The detection and analysis of hepatitis B virus genome variation and its use in clinical studies

by

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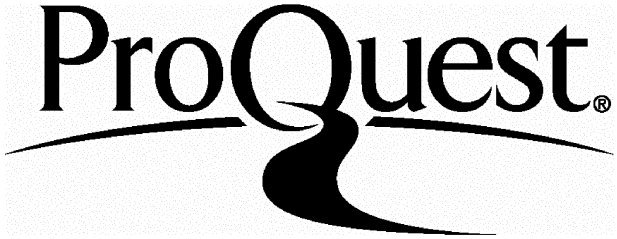

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Abstract

The detection and analysis of hepatitis B virus genome variation and its use in clinical studies.

This thesis describes the development of molecular techniques for the analysis of the hepatitis B virus (HBV) and their use in clinical studies.

The methods for detecting HBV in serum included a DNA dot blot assay; a DNA polymerase assay and a polymerase chain reaction (PCR) assay in a nested and a double nested format. Amplified HBV DNA was also analysed by direct sequencing and by a point mutation assay.

In a cohort of homosexual HBV carriers, concomitant HIV infection potentiated HBV replication and delayed HBeAg to anti-HBe seroconversion but did not appear to alter progression to AIDS. Fourteen patients in this cohort who received zidovudine were studied. The drug had no effect on HBV replication.

HBV DNA was amplified from the majority of our HBeAg negative carriers, indicating that anti-HBe positive carriers may remain infectious. Hepatitis B virus with precore mutations was identified in two patients in the cohort, both were anti-HBe positive. The mutant strain was not a prerequisite for HBeAg to anti-HBe seroconversion and was not common in asymptomatic male homosexual HBV carriers in London.

Reactivation of an HBV infection occurred in one of three HIV positive homosexual patients in whom an HBV infection recurred. Reinfection was implicated in the other two patients.

Precore mutant virus was sexually transmissible. Greek patients and their sexual partners had common viral nucleotide sequences which included mutations in the precore region. The mutant virus was detected equally in patients with a chronic, acute uncomplicated or fulminant hepatitis and therefore was not always associated with severe HBV disease.

Precore mutant virus was transmitted perinatally. Transmission of both precore mutant and the wild type virus from anti-HBe positive mothers resulted in a fulminant hepatitis.

A mutation at codon 145 in the HBsAg A determinant, which has been associated with immune escape, was detected as the major type in one of three liver transplant patients receiving HBIg prophylaxis.

Finally we identified a cluster of HBV infections from a group of control patients, illustrating the utility of this methodology in the study of HBV transmission events.

Note with regard to the presentation of this thesis

On the advice of my supervisor protocols relating to routine methods which have been used but not modified or adapted in any way as part this thesis are presented in Appendix 2. Results of individual studies are summarised in the text where possible, these results are also presented in tabulated form in Appendix 6. Full nucleotide sequence analyses are available for the studies described in Appendix 7.

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Chapter 1 Introduction

1.1 Viral causes of hepatitis

Thus far five hepatotropic viruses which cause hepatitis in humans have been identified and well characterised. A short description of the characteristics of these, other than hepatitis B, follows. Hepatitis B is discussed in sections 1.2 to 1.10

1.1.1 The hepatitis A virus

The identification of an epidemic type of infectious hepatitis (IH) was confirmed by Krugman *et al*, in 1967. Two types of hepatitis with different clinical, epidemiological and immunological features were identified. This offered an explanation for the recurrence of hepatitis in previously infected patients. The patients with IH were immune to that type whilst remaining susceptible to serum hepatitis (hepatitis B). The IH virus was found to be the hepatitis A virus (HAV).

HAV is a non-enveloped, 7.8 kb RNA virus that was first identified, by immune electron microscopy, in the faeces of infected individuals by Feinstone *et al.*,(1973). The size (27 nm) and morphology of the virus led to its classification within the picornavirus group (Siegle and Frösner,1978).

The mode of transmission of HAV is generally faecal-oral, either person to person or food borne. Viral titres of up to 10^8 infectious virions/ml have been measured in the faeces of infected individuals by electron microscopy (Mast and Alter, 1993).

HAV generally causes a self limiting infection, less than 5% of cases world wide are clinically recognised (Lemon *et al.*,1985). Infection with hepatitis A is indicated by the presence of IgM and IgG antibodies in the serum (Feinstone *et al.*,1973).

The culture of HAV in 1979 by Provost and Hilleman led to the development of HAV vaccines. Immunisation has been achieved with inactivated vaccines, one of which became licensed for use in some European countries in 1992 (Gust,1990, Andre *et al.*,1990). Pre- and post-exposure passive immunisation has been used successfully to prevent hepatitis A (Gust and Feinstone,1988).

1.1.2 The hepatitis C virus

Despite the identification of HBV in the 1960s and HAV in the 1970s there was still a relatively high proportion of patients who had undergone transfusion or used intravenous drugs who developed a non-A non-B hepatitis (NANB hepatitis) (Feinstone *et al.*,1975, Dienstag,1983). This form of hepatitis was related to a transmissible agent present in blood.

Infection with this virus was diagnosed only when other known causes of hepatitis had been excluded (Lau *et al.*,1991). Some of these NANB infected individuals developed a chronic infection. Choo *et al.*,(1989) first identified a viral, blood-borne infectious agent using molecular cloning techniques. This was homologous to a similar clone isolated from infected patients serum at the same time in Japan (Arima *et al.*,1989). This cloned virus was called the hepatitis C virus (HCV). HCV is a single stranded, 10 Kb, RNA virus which shows amino acid sequence homology to the flaviviruses (Miller and Purcell.,1990).

A serological assay for the detection of anti-HCV was quickly developed (Kuo *et al.*,1989) which showed that HCV infection occurred in essentially the same risk groups as HBV, patients receiving blood or blood products, homosexual men and sexual contacts of infected individuals, intravenous drug users and children born to infected mothers. The distribution of infection within these groups was different for HBV and HCV.

Coinfection with HBV and HCV is possible in some patients. A proportion of those identified by the early radioimmunoassays and enzyme immunoassays were shown be due to false reactivity of the assay. However, the general pattern of infection indicated by these early results was later confirmed by the application of the polymerase chain reaction (PCR) technique (Lau *et al.*,1991). Reverse transcription followed by PCR was an extremely sensitive and specific method for the direct detection of the viral genome in serum (Garson *et al.*,1990).

To date there is no method of immunisation, either active or passive, for the prevention of HCV infection. Passive immunisation is unlikely to be successful as many patients are chronic carriers of the virus. This suggests that the neutralising antibody response to the virus is poor and therefore unlikely to prevent infection.

1.1.3 The hepatitis D virus

Hepatitis D virus (HDV) (delta virus) was first detected as a new viral antigen associated with chronic HBV infection in 1977 by Rizzetto *et al.* and was later shown to be transmissible (Rizzetto *et al.*,1980).

HDV is a single stranded, 1.7 kb RNA virus that is generally thought to be reliant on a helper function of HBV for its replication (Rizzetto and Verme,1985). The HDV virus is a 36 nm particle. The HD viral envelope is HBV derived hepatitis B surface antigen, which HDV requires for hepatotropism and propagation.

HDV infection may coincide with acute HBV infection or be a super infection acquired later by HBV carriers. The documented routes of transmission are, close family contact in endemic areas and activities related to intravenous drug use (Rizzetto and Verme, 1985).

The acquisition of HDV as a super infection is associated with a decrease in markers of HBV replication but possibly the more rapid development of chronic active hepatitis and cirrhosis (Smedile *et al.*,1982). There is no method of immunisation for HDV infection. However, prevention of HBV infection may provide an indirect method of control.

1.1.4 The hepatitis E virus

Hepatitis E virus (HEV) is an enterically transmitted RNA virus. Initially outbreaks of hepatitis due to an enterically transmitted virus were all thought to be due to HAV. The development of assays for the specific detection of HEV showed that this was not so. Nine outbreaks in India that were investigated were caused by a faecal-oral transmitted non-A, non-B hepatitis which was named hepatitis E virus (Wong *et al.*,1980, Ramalingaswami and Purcell, 1988). In the majority of cases the disease was self limiting (Viewanath 1957).

The virus was identified as 23-27 nm particles by Balayan *et al.*,(1983), in faeces from an HEV infected individual. A suspension of the stool was then ingested by a volunteer who developed acute hepatitis and anti-bodies to HEV. HEV Ag was detected in the liver of experimentally infected macaques (Krawczynsky and Bradley, 1989). An enzyme immunoassay based on synthetic peptides, solid phase immune electron microscopy and

the polymerase chain reaction have all been used to identify HEV infection (Humphrey *et al.*,1990, Lau *et al.*,1991)

1.2 Hepatitis B

1.2.1 History

Although jaundice has been a recognised symptom of disease since the fifth century BC the relationship of this symptom with a specific etiologic agent was not recognised until the 19th century. The existence of a possible parenterally transmitted form of hepatitis was first reported by Lurman, (1885). This report noted that, in a group of shipyard workers who received smallpox vaccine prepared from human material, 191 of 1,289 workers developed jaundice 2-10 months after inoculation. The mode of transmission was further suspected after case reports of other hepatitis outbreaks associated with patients requiring parenteral injections, and individuals receiving tattoos.

The relationship between a virus that was transmissible through procedures involving the direct transfer of blood or biological material and jaundice was established in the 1930s. At which time outbreaks were associated with the administration of vaccines to large groups of individuals for example for yellow fever (Findlay and MacCallum, 1937). This outbreak was notable for the relatively long period of incubation before the disease became apparent of up to 2 to 7 months.

The terms hepatitis A and B were introduced by MacCallum, (1947). The distinction between infectious or epidemic hepatitis (hepatitis A) and serum hepatitis or homologous serum jaundice (hepatitis B) was initially based on epidemiologic evidence. A study at an institution for the mentally handicapped in the USA by Krugman *et al.*,(1967) showed that there were two types of hepatitis, hepatitis A which was characterised by a shorter incubation period and hepatitis B characterised by an incubation period of more than three months.

The antigen which was subsequently identified as the surface protein of the hepatitis B virus (the Australia antigen or HBsAg) was discovered as the result of a study carried out by Blumberg *et al.*,(1965). In this study a serum sample taken from an Aborigine contained an antigen that reacted with an antibody in the serum of an American haemophiliac patient. The prevalence of HBsAg varied among different populations. It

was rare among North Americans and Western Europeans but relatively common in some African and Asian populations.

Australia antigen was found in patients with Down's syndrome by Krugman *et al.*,(1967) and was initially thought to be associated with leukaemia (Blumberg *et al.*,1967). However, the Australia antigen was specifically detected in the serum of patients who had undergone transfusion and was demonstrated by Prince *et al.*,(1968) to be associated with the development of post-transfusion hepatitis B infection. The morphology of the virus was first identified by Dane *et al.*,(1970) using electron microscopy.

HBV infection may occur as a symptomatic, icteric or an asymptomatic acute infection. In a minority of patients who are infected as adults, and in the majority of those infected in early childhood or as infants, a chronic infection may develop and persist for years. In an acute infection viral replication generally ceases within 6 to 9 months, clinical recovery also occurs within that period.

The development of IgM antibodies to the core antigen (anti-HBc-IgM) in the presence of HBsAg is indicative of an early acute HBV infection. The presence of serum antibodies to HBsAg (anti-HBs) indicate a cessation of viral replication and the resolution of an acute HBV infection. Continued HBsAg production in the presence of anti-HBc, both IgM and IgG, for greater than six months is associated with chronic hepatitis B.

A non-structural protein, designated the hepatitis B e antigen (HBeAg) is present in the serum of both acutely and chronically infected individuals whilst active viral replication is ongoing. The development of the antibody to HBeAg (anti-HBe) generally indicates a cessation of viral replication. However, as will be discussed later, this is not always the case.

1.2.2 Epidemiology

Current estimates of the number of chronically infected HBsAg positive HBV infected individuals worldwide vary between 200 and 300 million (Zuckerman 1987, Maynard *et al.*,1988). The distribution of chronic carriage varies widely, with Northern Europe, USA and Canada having the lowest carrier rate ie less than 0.5% of the general population are carriers. The disease is endemic in Asia, South America and parts of Africa where the

carrier rate can be as high as 20%.

The majority of individuals in areas where HBV is endemic have acquired their infection perinatally or by familial, horizontal transmission (Brown *et al.*,1990). In areas where HBV is not endemic, infection is generally related to blood transfusion, intravenous drug abuse and sexual activities.

Original studies carried out by Blumberg *et al.*,(1972) showed that there were a higher number of males infected with HBV in areas where the virus was not endemic. Sexual transmission of HBV is covered in section 1.3.3.

The different groups of individuals most at risk of infection reflect the mode of transmission of HBV through the exchange of blood and other bodily secretions eg semen and saliva. The virus has been detected by the presence of HBsAg or DNA hybridisation in serum and saliva (Heathcote *et al.*,1974, Davison *et al.*,1987, Jenison *et al.*,1987, Karayiannis *et al.*,1985) and in menstrual fluid (Mosely 1975), urine (Davison *et al.* 1987), pleural fluid, tears, ascitic fluid, cerebrospinal fluid, breast milk and vaginal secretions (Capron *et al.*,1977, Dankert *et al.*,1975, Darani and Gerber, 1974). The highest viral concentrations are present in blood and in the liver, with lower concentrations in saliva and semen (Hollinger 1990).

The identification of HBV DNA sequences resembling replicative intermediates in the liver of patients with acute hepatitis indicated that this was a site of active viral replication.

The age of acquisition of an HBV infection and the serological markers of the infectious inoculum are important factors in determining the outcome of infection and disease. HBV acquired perinatally from an HBeAg positive mother is more likely to produce chronic infection than that acquired later in life through sexual contact or blood transfusion (Brown *et al.*,1990).

1.3 Transmission

1.3.1 Maternal/Perinatal

Most perinatal transmission occurs from HBeAg positive mothers (Okada *et al.*,1976). The age of infection, and by implication the route of infection, varies geographically (Botha *et al.*,1984). In Asia, babies are infected at birth (Stevens *et al.*,1975 and 1979). The majority (85%) of those born to HBeAg positive mothers became persistently infected (Beasley *et al.*,1977). In contrast 31% of the babies became carriers when the mother was HBeAg negative. The Beasley study also found a link between high HBsAg levels and transmission even in the absence of HBeAg.

The risk of perinatal transmission is greatest when the mother suffers an acute infection during the third trimester or the first two months post-partum (Merrill *et al.*,1972). Infants are usually HBsAg negative at birth but develop detectable HBsAg 1-3 months later. Although HBsAg has been detected in cord blood (Stevens *et al.*,1975) its presence does not correlate with the detection of HBsAg in venous blood in the infant at birth (Lee *et al.*,1978, Stevens *et al.*,1975). Stevens *et al.*,(1975) noted a window period during which the child remained HBsAg negative for up to six months before persistent antigenemia developed, even when HBsAg was detectable in the cord blood. This was probably because HBsAg in the cord blood represented contamination with maternal HBsAg and indicated that, in most cases, perinatal transmission probably occurred at delivery. Whilst *in utero* infection may be a rare event, the presence of relatively high levels of HBsAg in blood taken from the peripheral vein at birth was detected in 1.6% of infants examined in a study by Stevens *et al.*,(1988) which demonstrated that it can occur.

Mothers who are anti-HBe positive are much less likely to infect their babies. The number of reported infections is low and the result of transmission in this way has varied between a transient HBV infection and fatal, fulminant hepatitis (Sinatra *et al.*,1982).

In Africa, horizontal transmission is the more usual route of infection. Children are infected later in life (Botha *et al.*,1984) possibly by ingestion of breast milk and saliva. Transmission between familial contacts of HBsAg, HBeAg positive carriers is relatively common, particularly among the siblings of a carrier through close contact with blood or saliva (Szmuness *et al.*,1970).

1.3.2 Parenteral Transmission

This is still an important mode of transmission and a major cause of HBV infection, particularly in areas of the world where HBV is not endemic.

Screening of blood and blood products for HBV markers has markedly reduced the rate of post-transfusion hepatitis (Hollinger *et al.*,1990). However, some groups appear to remain at risk of infection. The stability of HBV and its retention of infectivity on mechanical surfaces, particularly those involved in haemodialysis (Bond *et al.*,1981) goes some way to explaining the high rate of infection that occurs in patients undergoing treatment in some haemodialysis units. This also explains, in part, the relatively high rate of transmission of HBV among drug abusers, by the sharing of contaminated syringes and needles. Healthcare workers are also at risk from HBV infection acquired from needlestick injuries and occupational exposure to blood products (Maynard *et al.*,1978).

The spread of infection from insect vectors has not been proven, even where HBsAg has been detected in insects (mosquitoes) (Berquist *et al.*,1976).

1.3.3 Sexual transmission

Evidence for the sexual transmission of HBV is provided by the strong epidemiological association between sexual behaviour in homosexual men and HBV infection.

Further evidence has been provided by studies of the prevalence of HBV serological markers in specific epidemiological groups of patients. HBsAg was detected in 15 of 658 men and 2 of 316 women attending the Department of Sexually Transmitted Diseases at the Middlesex Hospital (Fulford *et al.*,1973). Anti-HBs was detected in 36 men and 11 women in the same group. There was a significant correlation between the presence of serum-HBsAg and the country of origin of the patients, particularly those from Mediterranean and Middle Eastern countries. Similarly, a significant association between anti-HBs and a history of syphilis or gonorrhoea was found.

A significantly greater proportion of homosexuals and patients with three or more sexual contacts had serum anti-HBs. However, there was no correlation between serum HBsAg and these two parameters. This led to the suggestion that HBV was transmitted sexually and when acquired in this way, was unlikely to lead to a chronic infection. In a later

study, carried out in the same department, anti-HBc was detected in 48% of the homosexual men tested in a study by Gilson *et al.*,(1990) indicating a past or current HBV infection. In contrast 7.5% of the heterosexual tested men were anti-HBc positive. The detection of the virus in semen and in asymptomatic rectal mucosal lesions (Reiner *et al.*,1984) indicated a possible mechanism for the transmission of HBV.

The rate of chronic HBV carriage following infection via sexual contacts varies. Factors associated with higher rates of carriage include prior HIV infection ((Taylor *et al.*,1988) and an asymptomatic acute infection (Tassopoulos *et al.*,1987).

1.4 General Hepatitis B Virology

The hepatitis B virus is a member of the *Hepadnaviridae*. It is an enveloped 42 nm diameter particle enclosing a nucleocapsid containing a partially double stranded DNA molecule (Summers *et al.*,1975). Three envelope related proteins have been associated with HBV: the Australia antigen, displayed on the surface of the Dane particle or complete hepatitis B virion and renamed the surface antigen (HBsAg), (WHO.,1975). The pre-S2 antigen/antibody system (Alberti *et al.*,1984) and the pre-S1 antigen/antibody system (Heerman *et al.*,1984, Takahashi *et al.*,1986).

Detergent treatment of the Dane particle reveals the nucleocapsid which has a diameter of approximately 28 nm, (Almeida *et al.*,1971). The antigen associated with this structure is referred to as the core antigen and its presence is indicated by a specific antibody in convalescent serum (anti-HBc)(Almeida *et al.*,1970).

In contrast to HBsAg, HBcAg is not readily detected free in serum (Magnius and Espmark,1972) but has been identified in both the nuclei and cytoplasm of infected hepatocytes (Yamada and Nakane,1977). HBcAg is usually found as a complex with anti-HBc, specific treatments for the disruption of immune complexes are required to reveal HBcAg.

A second antigen/antibody system that was later shown to be associated with the pre-core/core open reading frame was also identified by Magnius and Espmark, (1972). This was the HBeAg/anti-HBe system. HBeAg was not detected in all HBsAg sera and was shown to occur most frequently in the sera of individuals with high numbers of circulating

Dane particles.

The viral core contains an endogenous, DNA dependent polymerase activity (Kaplan *et al.*,1973), a protein kinase activity (Albin *et al.*,1980) and a DNA linked protein (Gerlich *et al.*,1980).

Antibodies to HBV polymerase (anti-P) have also been identified in sera of infected individuals (Chang *et al.*,1989) and may provide a prognostic indicator for chronic infection (Kann *et al.*,1993).

Antibodies to the RNase H domain of the polymerase protein (anti-P/RH) have been associated with ongoing viral replication. Anti-P/RH is detected in serum early in the course of infection, just after the appearance of IgM anti-HBc. The presence of anti-P/RH correlated with serological markers of HBV replication and the level of anti-P/RH declined when HBeAg or HBV DNA were no longer detectable (Weimer *et al.*,1990). Antibodies to the X gene product have also been identified (Kay *et al.*,1985).

Hepatitis B virions may be present in serum at very high concentrations ($>10^9$ /ml) (Almeida, 1972). The minimum infectious dose of the virions was determined by the inoculation of diluted sera from HBV infected patients into chimpanzees. In this way sera from HBV infected individuals diluted by up to 10^{-7} remained infectious (Barker *et al.*,1975).

1.5 Hepatitis B proteins

1.5.1 Surface proteins

The coding region of the genome for the envelope proteins is divided into three sections, pre-S1, pre-S2, and S. These regions each have an in-frame start codon.

The surface glycoproteins of the HBV envelope are retained in a lipid bilayer. There are three proteins present in the Dane particle envelope, the major, the middle and the large proteins.

Initiation of translation at codon 1 in the pre-S1 region produces the large protein which

includes pre-S1, pre-S2 and S. The pre-S2 region is translated along with the surface gene to produce the middle protein. Translation from the start codon of the surface gene results in the major protein.

The major protein is 226 amino acids long and contains the immunodominant HBsAg *a* determinant and subtypic determinants *d/y* and *w/r* (Gerin *et al.*,1983, Bancroft *et al.*,1972, Courouce-Pauty *et al.*,1974, Le Bouvier,1971). This protein may be present in a non-glycosylated (P24) and also a glycosylated form (gP27).

The middle protein (Pre-S2, S) is larger and comprises 281 amino acids. Again, the protein may exist in a partially glycosylated form (P33) or a fully glycosylated form (gP36). The large protein (Pre-S1, Pre-S2, S) contains 389-400 amino acids and is detected as P39 or the glycosylated form,gP42.

HBsAg can be present in serum in two non-virion associated forms, 22 nm spheres or filaments dependent on the protein conformation. These empty viral envelope particles are present at much higher concentrations than complete Dane particles. The Dane particle envelope contains equimolar amounts of middle and large proteins and a ten-fold excess of the major protein (Heerman *et al.*,1984). The filamentous particles are of a similar composition. The spherical particles consist of mainly the major protein with no large protein and less than 1% middle protein during the non-replicative phase.

A receptor sequence for polymerised human serum albumin was detected within the pre-S2 region and may represent a binding site of HBV to hepatocytes (Machida *et al.*,1984). The pre-S1 region has also been implicated in the attachment of HBV to hepatocytes (Neurath *et al.*,1985).

The surface gene encodes an immunodominant group determinant region from codon 124 to 147 which is referred to as the *a* determinant. Two minor antigenic determinants, *d/y* and *w/r*, also located within the S gene, determine the subtype of the virus. There are two variants for each determinant which are generally mutually exclusive and conserved on transmission. Thus HBV falls into the following major subtype groups: *ayw*, ayw_2, ayw_3, *ayr*, adw_2,adw_4 and *adr* (Courouce-Pauty, 1976). Single amino acid changes have been associated with HBV sub-type variation. These have been determined as amino acid 122,

where a lysine to arginine change produces a *d/y* subtype alteration (Gerin *et al.*,1983) and a similar amino acid change at 160, where lysine is present in the *w* subtype and arginine present in the *r* subtype (Okamoto *et al.*,1987).

The geographical distribution of these subtypes has been determined. In Northern Europe, America and Australia *adw* predominates. However, the subtype *ay* has been associated with patients infected through intravenous drug associated activities in those regions. Subtype *ayw* occurs in north and west Africa, the eastern Mediterranean, eastern Europe, northern and central Asia and the Indian Subcontinent. *Adw* and *adr* are found in Malaysia, Thailand, Indonesia and Papua, New Guinea. *Adr* is predominant in Japan and the Pacific Islands. Individuals are usually infected with a single subtype. However, combinations of subtypes have been observed, *adwr, adyw* and *adyr* have all been reported (Courouce *et al.*,1976).

1.5.2 Nucleocapsid Proteins

The antigenic reactivity of the core protein is conserved within human HBV subtypes and to some extent across those of different species eg Woodchuck hepatitis Virus (WHV) and Ground squirrel hepatitis virus (GSHV) (Marion *et al.*,1980). The HBcAg is produced by translation of the core gene.

The predominant core protein is 183-185 amino acids in length and is 22,000 daltons (p22). It is found in purified virion cores and as particles in the liver (Hruska *et al.*,1977). Ferns and Tedder, (1986) used monoclonal anti-HBc to identify a single immunodominant HBcAg epitope.

An 87 nucleotide sequence with an in-frame translation initiation codon lies immediately upstream of the core gene. Translation from this upstream initiation codon and subsequent post translational modifications result in the soluble protein HBeAg. The treatment of HBV core particles with detergent and 2-mercaptoethanol removes core antigen reactivity and reveals HBeAg reactivity (Mackay *et al.*,1981).

HBeAg is smaller than HBcAg due to the cleavage of 34 carboxyl terminal amino acids (Pasek *et al.*,1979). Proteolytic cleavage of 19 amino acids at the amino terminal occurs in the endoplasmic reticulum. From that point HBeAg is either transported to the Golgi

apparatus and secreted from the cell or to the nucleus (Ou *et al.*,1986, Ou *et al.*,1989, Uy *et al.*,1986). HBeAg was shown by Schlitt *et al.*,(1987) to be a non-essential protein for viral formation.

The close relationship between HBeAg and HBcAg was further confirmed by studies of serological responses to HBcAg. The inoculation of chimpanzees with HBcAg elicited both an anti-HBc and an anti-HBe response (Iwarson *et al.*,1985a, Marion *et al.*,1987).

1.5.3 The polymerase protein

Hepatitis B virus also contains a polymerase protein (DNAp) which is DNA-dependent (Robinson & Greenman 1974). This is a large protein of up to 845 amino acids. The polymerase protein encodes a terminal protein and an RNase H activity as well as the reverse transcriptase polymerase function. The polymerase and reverse transcriptase are both essential for replication. The reverse transcriptase shares nucleotide sequence homology with the corresponding genes of retroviruses (Toh *et al.*,1983). The polymerase region amino terminus encodes the terminal protein which is required for synthesis of the negative strand (long, L-) HBV DNA(Bartenschlager & Schaller 1988, Bosch *et al.*,1988). The carboxyl-terminus encodes RNase-H which degrades the RNA template during viral replication (Radziwill *et al.*,1990)

1.5.4 The X protein

The hepatitis B X gene product (HBx) is expressed during viral infection (Rossner, 1992). Antibodies to a synthetic HBx protein have been detected in the sera of infected individuals (Kay *et al.*,1985, Vitvitski-Trepo *et al.*,1990).

HBx has been detected in HBV infected liver (Moriarty *et al.*,1985) but is not essential for the encapsulation of viral DNA (Koike *et al.*,1989). The HBx protein acts as a transcriptional transactivator (Spandau and Lee,1988) and may be involved in the development of human hepatocellular carcinoma (Beasley *et al.*,1981).

1.6 Natural History of Acute and Chronic HBV Infection

The natural history of HBV infection may follow essentially two courses. In the first, an acute infection, the virus is cleared from the infected individual, in the second a persistent or chronic infection is established which continues for many years. A variety of clinical

courses may be associated with either type of infection. The infection can range in severity from asymptomatic, subclinical to a severe symptomatic infection.

An incubation period of one to three months follows infection. The duration of the incubation period may be influenced by the infecting dose. A higher dose stimulating a more rapid immune response and producing levels of HBsAg earlier that are detectable in serum. As many as 75% of infected individuals will have a subclinical infection and their infection may only be discovered by the presence viral antibodies. The presence of anti-HBs alone in serum generally indicates a vaccine induced, protective immunity whilst anti-HBs with anti-HBc is indicative of a resolved, naturally acquired infection. Levels of anti-HBs generally fall more quickly than anti-HBc thus anti-HBc may be present in serum as the only marker of a past HBV infection.

1.6.1 Acute infection

HBsAg is generally the first serologic marker to appear. This is generally cleared within three to four months post-detection. HBV replication as indicated by the presence of HBeAg, HBV DNA by DNA hybridisation assay and HBV DNA polymerase is detectable shortly after HBsAg. The development of anti-HBe follows the appearance of HBeAg and may persist for years.

The infectivity of a patient is related to the HBeAg/anti-HBe status. The presence of HBeAg in the serum is generally regarded as being consistent with a high level of infectivity and correlates well with the presence of HBV DNA in patients' serum as detected by HBV DNA dot blot (Krugmann *et al.*,1974, Krugmann *et al.*,1979, Hoofnagle *et al.*,1981). The presence of anti-HBe in serum is generally taken as an indication of low infectivity. Lok *et al.*,(1984) and Bonino *et al.*,(1986) noted that in some individuals HBV DNA was detectable by DNA hybridisation assays in anti-HBe-positive patients. This will be discussed more fully in Chapters 3 and 4. As HBsAg levels decrease there may be a "window" period when only anti-HBc is detectable.

IgM anti-HBc is the first antibody to appear in serum. Tedder *et al.*,(1981) noted that levels of IgM anti-HBc remained higher for longer in patients with an acute resolving infection than in patients who had developed a chronic infection.

In some patients an acute HBV infection is associated with a fulminant hepatitis which involves a massive immune response to the infection and consequent severe liver damage which may prove fatal. The levels of virus found in such patients is often low.

1.6.2 Chronic infection

The term chronic carrier is applied when HBsAg remains detectable in the serum of an infected patient for greater than six months. Anti-HBc IgM may also remain detectable in carriers at low levels for many years. In the majority of carriers there are three phases of the infection (Bonino, 1992). The first involves high levels of viral replication and serological events are similar to those described for an acute infection. The presence of detectable HBcAg in the liver is taken as a marker of ongoing viral replication. This is carried out in-situ on biopsy material and is not used routinely for the monitoring of HBV infected individuals.

In the second phase the patient may seroconvert from HBeAg to anti-HBe positive. This happens in approximately 10% of carriers per year (Weller *et al.*,1986). This figure is lower for HIV infected individuals (5%) (Krogsgaard *et al.*,1987). At that stage, in immunocompetent patients, ALT levels may become raised as the patient loses immune tolerance toward the virus but decrease following seroconversion (Seeger *et al.*,1986). Histological manifestations of this may take the form of chronic active hepatitis.

In the third phase HBsAg remains detectable but there is no serological or histological evidence of HBV replication. Consequently there is also very little evidence of hepatitis. The rate of seroconversion in carriers from HBsAg to anti-HBs is 0.5-1% per year (Hoofnagle *et al.*,1978).

The patterns of infection described above are extremely generalised. The characterisation of a hepatitis B virus that was unable to produce HBeAg (Carman *et al.*,1989) provided a mechanism for the presence of HBV DNA in sera that lacked HBeAg (Lok *et al* 1984). Other serological patterns that do not follow the above schemes will be discussed in Chapters 3 and 4.

To date there is no confirmed evidence of a directly cytopathic role for HBV. The variability in clinical presentations and the length of time which an infected individual

may carry the virus asymptomatically help to confirm this. The inference is that the immune response to HBV is causing HBV associated liver disease (Dudley *et al.*,1972).

The target of cytotoxic T cells was shown to be the core antigen expressed on the surface of infected cells. Mondelli *et al.*,(1982) showed that the cytotoxic response to hepatocytes expressing HBcAg could be blocked by the addition of monoclonal and polyclonal anti-HBc . Naumov *et al.*,(1984) confirmed this and also showed that hepatocytes expressing surface antigen were generally not destroyed.

In some cases this process results in the clearance of hepatocytes containing replicating HBV. In other cases prolonged destruction of the hepatocytes may result in severe hepatic damage (Thomas and Novick, 1985) including severe chronic lobular hepatitis, chronic persistent hepatitis, and also extrahepatic diseases (Thomas *et al.*,1984)

Infection with HBV has also been associated epidemiologically with hepatocellular carcinoma (HCC)(Szmuness, 1978). The HBV genome has been found to be integrated with the chromosomal DNA of HCC tumour cells (Schafritz *et al.*,1981).

The outcome of HBV infection may be related to the immunocompetence of the infected individual. The likelihood of developing a chronic infection is also increased in previously HIV infected individuals (Taylor *et al.*,1988). Increased rates of longterm carriage amongst individuals with an asymptomatic acute infection may similarly reflect a depressed immune function.

1.7 Prevention of hepatitis B infection

1.7.1 Active immunisation

The development of a naturally acquired immunity to HBV was demonstrated by Lander *et al.*,(1971). This group showed that the development of an anti-HBs response in HBV infected individuals prevented reinfection. This indicated that efforts in the development of successful vaccines could be directed towards the promotion of a high, sustained anti-HBs response. Other antibody systems (anti-HBc and anti-HBe) have not been shown to have virus neutralising activity.

The first HBV experiments used human plasma (Krugmann *et al.*,1971 & 1973). Plasma vaccines containing the non-infectious 22 nm HBsAg particles (subvirion vaccines) were then developed (Purcell *et al.*,1975).

The efficacy of the subunit vaccine was demonstrated in a study carried out among staff and patients of a renal dialysis unit (Maupas *et al.*,1978). This study also confirmed the increased efficacy of an alum precipitated adjuvant form of vaccine when compared with an aqueous form. Protocols for the safe production of 22 nm particles which were free from infectious HBV were designed, all involved either heat and formalin treatment or formalin treatment alone (Reviewed by Thomas and Novick,1985).

The first commercially licensed vaccine was produced by Merck, Sharp & Dohme (Hilleman, 1979). The vaccine prevented HBV infection in all six chimpanzees that were challenged with HBV subtype *adw*. Szmuness *et al.*,(1980) also demonstrated the efficacy of the plasma derived, inactivated vaccine in a randomised double blind, placebo controlled trial in a high risk population of 1083 homosexual men.

The identification of HIV, which had been transmitted via blood products highlighted the requirement for a non-plasma derived vaccine. The production of HBsAg in transformed cell lines was unsuitable because of the risk of neoplasia (Moriarty *et al.*,1981). The low level of expression of cloned HBsAg in bacteria mitigated against this as an alternative source of antigen (Valenzuela *et al.*,1980). Oligopeptides carrying the antigenic determinants of HBsAg were of low potency and therefore not suitable (Dreesman *et al.*,1982).

Thus the second generation of vaccines involved yeast-derived recombinant vaccines and were first licensed in 1986. The recombinant vaccine contains a surface gene cloned into *E coli* and expressed in yeast cells (Saccharamyces cervisiae). The efficacy of the purified HBsAg was demonstrated in chimpanzees by McAleer *et al*,(1984).

The plasma derived and recombinant vaccines have been widely administered to high risk groups including the babies of HBsAg positive mothers (Zuckerman 1990, Stevens *et al.*,1984). Initially vaccination was suggested only for babies born to HBeAg positive mothers (Wheeley *et al.*,1990), this has now been modified to include babies born to anti-HBe positive mothers (Sinatra *et al.*,1982, Deinhardt *et al.*,1985).

1.7.2 Passive Immunisation

The administration of hepatitis B immunoglobulin (HBIg) both pre- and post-exposure is effective in the prevention and neutralisation of HBV infection. Early studies of pre-exposure prophylaxis were hampered by the lack of consistent levels of anti-HBs in preparations and by the possibility of the inclusion of HBsAg which would produce a compound active, passive immunisation.

HBIg was licensed for use in 1977 for the treatment of patients with accidental percutaneous exposure to infected blood and for the treatment of babies born to mothers who had an acute HBV infection during the third trimester (McAuliffe *et al*,.(1980). Beasley *et al.*,(1981) demonstrated the efficacy of HBIg in a study of HBeAg positive mothers in Taiwan.

HBIg is rarely used for pre-exposure prophylaxis but may be appropriate for use in the severely immunocompromised. HBIg is still used for post-exposure prophylaxis in the following situations: sexual contacts of an acutely infected patient, perinatal exposure from HBV infected mothers and percutaneous exposure to HBV. The consensus of studies carried out in the efficacy of HBIg is that it is effective in preventing hepatitis B and that the HBIg should be administered as soon as possible after exposure.

1.8 Treatment of Hepatitis B

An important aspect of the development of any therapy against HBV has been the choice of monitoring methods and the determination of appropriate end points. Antiviral agents

have been assessed with the aim of inhibiting active viral replication, mainly in chronic carriers. However, the removal of integrated virus or the prevention of integration of the virus into the host genome has not been achieved (Rizzetto, 1988). Treatment has largely been directed at those patients with evidence of active viral replication and inflammatory liver disease ie HBeAg positive with HBV DNA or HBV DNA polymerase activity detectable in serum or HBcAg detectable in the liver.

Trials of antiviral drugs have also been carried out in anti-HBe positive patients in whom viral DNA is detectable in serum Bonino *et al.*,1986). To date clearance of circulating virus has been achieved with interferon therapies where progression to the integrated phase of infection as indicated by HBeAg to anti-HBe seroconversion has been promoted. To obtain a true measure of the efficacy of any treatment the rate of HBeAg/anti-HBe seroconversion while on therapy must be compared with the natural rates of conversion which have been shown to vary between 3% and 21% (Weller *et al.*,1986). Alpha interferon has been licensed for the treatment of chronic hepatitis B and more recently 3'-dideoxy-3'-thiacytidine (3TC) has been shown to inhibit HBV replication *in vivo*.

1.9 Genetic Organisation of HBV

The structure of the HBV genome in the virion is unusual, consisting of a circular 3.2 kb, partially double stranded DNA molecule. The existence of a shorter, positively orientated strand was first demonstrated by restriction analysis (Summers *et al.*,1975). Kaplan *et al.*,(1973) had previously shown that the virus contained an endogenous DNA-dependent polymerase. HBV DNA polymerase activity could be used to incorporate radiolabelled nucleotides into the HBV genome. This function was important as it provided a screening assay, allowed for the discovery of Hepadna viruses in other animals and also gave an indication of the genetic arrangement of the virus.

The virus has a short positive strand (S+) and a long negative (L-) strand. The L- strand contains information for coding and is complementary to viral mRNA. The S+ strand has a fixed position at the 5' end but the length of the gap is variable (Delius *et al.*,1983).

The HBV genome is maintained in a circular configuration by the presence of a complementary 224 base 5' terminus between the long and short strands. Sattler and Robinson, (1979) showed that the denaturation of this region provided linear molecules

which could be returned to the circular form under appropriate conditions and which could be prevented from recircularising with S1 nuclease.

The long strand has a polypeptide covalently attached to the 5' end (Gerlich & Robinson 1980). There is also a capped oligoribonucleotide attached at the same point to the short strand which may function as a primer for short strand synthesis (Tiollais *et al.*,1985).

The genome is divided into four major open reading frames (figure 1(i)). These encode the HBV nucleocapsid core gene, the polymerase gene, the envelope (HBsAg) region and a transactivational X gene. There are also two direct repeat sequences, DR1 and DR2, located at the overlapping 5' end of both strands which help to maintain the circular format of the genome. There are two additional potential open reading frames for which no translation products have been identified (Miller *et al.*,1989).

1.9.1 Enhancers

The genome also contains enhancer regions. One enhancer region (En I) has been mapped by Shaul *et al.*,(1985) to a 200 nucleotide region, 600 base pairs upstream from the genomic RNA start site.

En I lies 450 bp upstream from the HBcAg gene promoter within the polymerase coding region and is close to the X gene promoter. It was identified by Hu and Siddiqui,(1991) as an up-regulator of all four of the viral promoters that have been identified in recircularised genomic DNA. Shaul *et al.*,(1985) and later Antonucci *et al.*,(1989) suggested that En I was only active in a tissue dependant manner. Further work has shown that the En I and core promoter are much more active in hepatocytes (Honigwachs *et al.*,1989) and in particular some hepatoma cells but are not silent in other cell types (for review see Yen, 1993).

A second enhancer (En II), which is active on the S promoter has also been identified (Yee 1989). This enhancer is apparently tissue specific and its activity is restricted to hepatoma cells.

1.9.2 Promoters

The core promoter gives rise to two transcripts, which are translated respectively to give the HBc protein and HBeAg. These proteins were shown by Hu and Siddique, (1991) to be differentially activated by En I when the viral genome was circularised.

There are two surface gene promoters. The pre-S1 promoter, which is highly tissue specific, has been shown to be crucially related to the binding of a liver enriched factor (Chung *et al.*,1989) and also a non-specific factor (Zhou & Yen 1991).

The S promoter consists of two initiation elements which may act independently (Zhou & Yen 1991), the upstream element producing the middle surface protein coding transcript and the downstream initiator producing the small surface protein (Yen 1993).

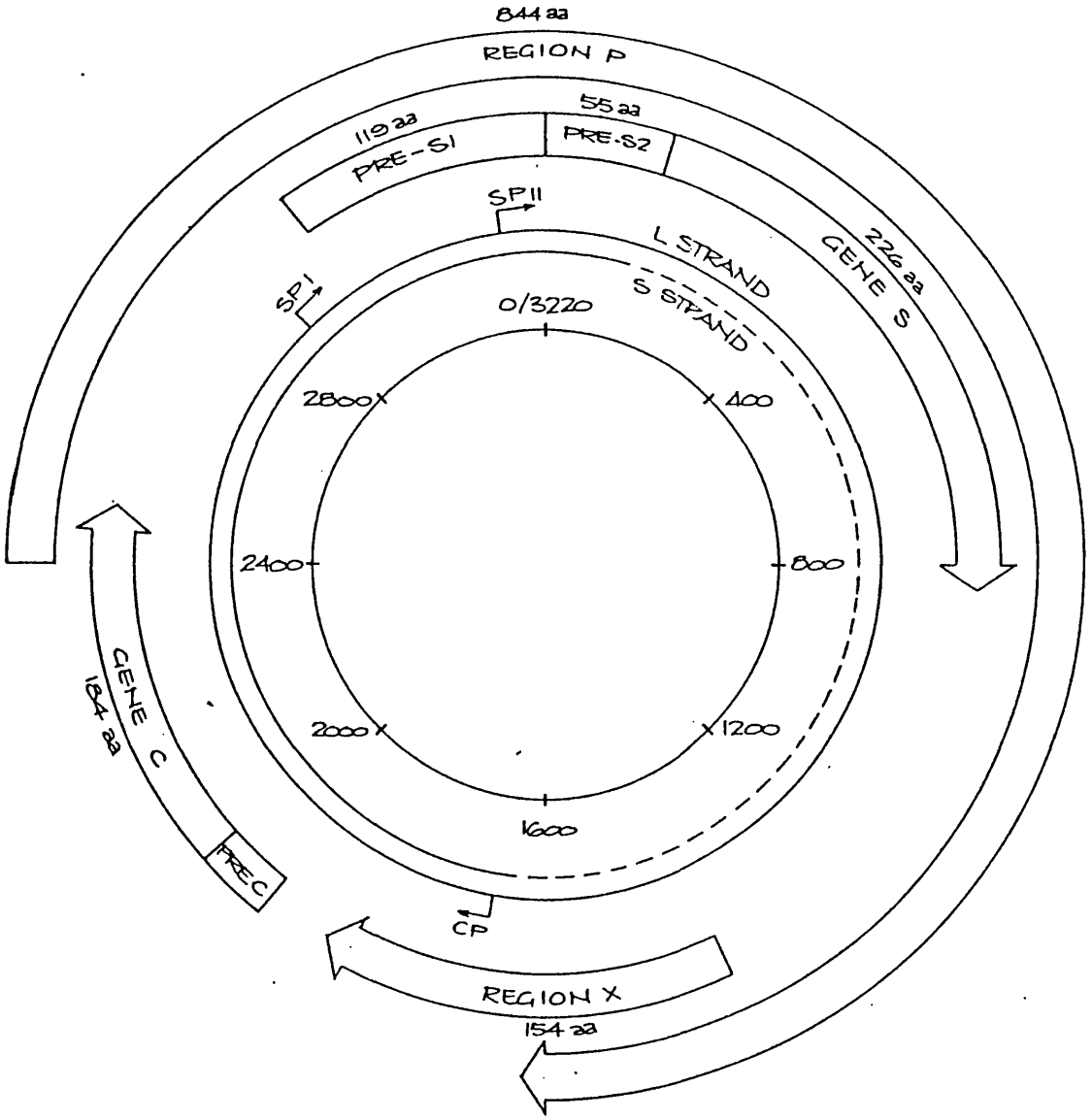
The X protein has been identified as a regulatory protein. Miller and Robinson (1986) first suggested that the X protein may be an expression regulator by comparing its sequence with that of known retroviral regulatory proteins. This function has been confirmed and the X protein has been shown to be a ubiquitous regulator.

The HBV X protein has been implicated in HBV-associated carcinogenesis but its role remains unclear. HBx has been shown to transform cells in culture (Hohne *et al.*,1990) and is expressed at high levels in transgenic mice which develop hepatocellular carcinoma. Both properties indicate that HBx may be involved in HCC but as yet no mechanism has been proven.

The existence of factors which down-regulate transcription was reviewed by Yen,(1993). A core gene product, which regulates the viral X gene promoter, has been identified.

A glucocorticoid response element within the S open reading frame has been identified by Tur-kaspa *et al.*,(1986). This element acts with En1 to up-regulate the SV46 promoter. This response has been seen *in vivo* in transgenic mice where gene expression is increased in response to glucocorticoids. A similar effect has been noted with androgen mediated cis-activation.

Figure 1(i) Hepatitis B Virus genome organisation



Notes

Gene S, Surface gene. Gene P, Polymerase gene. Gene C, Nuclear capsid gene. Gene X, X region. CP, Core promoter. SP 1, Surface promoter 1. Sp 11, Surface promoter 2

1.10 Viral Replication

Replication of the hepadnaviridae takes place by reverse transcription of a RNA intermediate. This was first determined by Summers and Mason,(1982) who studied the duck hepatitis virus (DHBV). Subviral particles were shown to have a DNA polymerase activity that completed the positive strand, closing the nick in that strand and allowing replication. The DNA minus strand is synthesised from an RNA template that is degraded concomitantly with growth of the DNA strand by the polymerase encoded RNase H. The plus strand is then synthesised using that completed strand as a template. Summers and Mason,(1982) designated the RNA replicative intermediate, the pregene, allowing it to be distinguished from mRNA transcripts.

Fowler *et al.*,(1984) showed that replication of HBV was asymmetric and that a reverse transcription replication strategy, similar to that described for DHBV, was followed by HBV. The detection of RNA replicative intermediates was similar to that noted in DHBV by Mason *et al.*,(1982). Single stranded DNA copies of the viral DHBV genome were detected by northern blotting and shown to be predominantly minus strands.

The scheme proposed by Summers and Mason (1982) has been largely confirmed. On entering the hepatocyte the viral envelope is stripped and the core, containing the partially double stranded DNA, transported to the nucleus. At this stage the HBV DNA is in the non-replicative, relaxed circular form.

The short positive strand (S+) is completed by the HBV DNA polymerase and the completed DNA converted to the covalently closed circular form, which is a suitable template for transcription.

Transcription of the minus strand produces several RNAs which vary in length. A 3.4 kb RNA is produced which codes for the major structural core protein (p22), the reverse transcriptase protein and the polypeptide primer required for minus strand synthesis. There are also a 2.4 kb RNA and an approximately 2.1 kb RNA which have heterogenous 5' ends. All of these RNAs have fixed 3' polyadenylated ends that are mapped to the start of the core gene. The 2.1 kb RNA codes for the major and middle surface protein and the 2.4 kb mRNA for the large surface protein. The 3.4 kb RNA is transcribed and serves as the template for HBV replication.

Junker-Niepman *et al.*,(1990) identified a short sequence at the 5' end of the RNA pregene which is the signal for encapsidation. Bartenschlager *et al.*(1990)determined that a fully functional polymerase gene product is required for encapsidation. Once encapsidated, reverse transcription of the pregene begins at the direct repeat 1 sequence (DR1) and continues until a small fragment of RNA pregene remains. The RNase H activity degrades the pregene as the reverse transcription proceeds.

The polynucleotide containing the second DR1 sequence is cleaved and translocated to DR2 on the minus strand DNA. Staprans *et al.*,(1991) constructed DHBV mutants in which nucleotides surrounding DR1 were altered. Mutations were found which prevented translocation and did not allow circularisation of DNA as in the normal replication cycle. Other mutations in this region prevented either primer cleavage or translocation and showed that these processes can be dissociated. Once plus strand synthesis has started the mature core particles are packaged into the virus envelope and the virus exported.

1.11 Interactions between HIV-1 and HBV

Similarities exist between HIV-1 and HBV in several ways. Both have an RNA stage during the replication cycles and both have an endogenous reverse transcriptase function coded for by regions with a high degree of homology. There are also repeated sequences which are involved in viral replication. The major cellular targets for infection are different, HBV being hepatotropic and HIV lymphotropic. However, Laure *et al.*,(1985) also detected HBV DNA in lymphoid cells from patients with HIV. A potential direct action of HBV on HIV whereby the LTR enhancer region of HIV is activated by the HBV transactivator protein was shown by (Siddiqui *et al.*,1989).

Co-infection with HIV in HBV carriers is associated with a decrease in inflammatory liver disease but a rise in circulating HBV (Krogsgaard *et al.*,1987). The annual rate of spontaneous loss of HBeAg is lower in seropositive patients than in seronegative patients (Weller *et al.*,1986).

1.12 The development of the thesis

Sections 3.1 to 3.4 describe the development of methods for the analysis of HBV DNA in various clinical settings; section 3.1 describes the development of a specific, sensitive assay for the detection and quantification of HBV DNA.

This method was applied to a study comprising the analysis of samples from a cohort of homosexual men attending the Middlesex Hospital hepatitis clinic (section 3.5). The aim was to determine the effect of coinfection with HIV-1 on the natural history of chronic hepatitis B. Both diseases are sexually transmitted and are prevalent in similar patient groups. Thus the number of dually infected individuals was predicted to be sufficient for a study of this population.

The effect of coinfection on the level of HBV replication was examined directly by the assay of HBV DNA and HBV DNA polymerase and indirectly by the analysis of serological markers.

A number of the patients within this group were receiving Zidovudine (section 3.6). This is a reverse transcriptase (RT) inhibitor used in the treatment of HIV-1 infection. The mechanism of this drug is such that it may exert an effect on any virally encoded RT. The HBV polymerase protein has sequence similarity with the RT gene. Reverse transcription is also part of the HBV replication cycle. Therefore the possibility that Zidovudine treatment may have an effect on HBV replication was investigated.

Sections 3.2, 3.3 and 3.4 describe the development of an *in vitro* amplification system (polymerase chain reaction, PCR) for HBV DNA, the development of methods for nucleotide sequencing amplified HBV DNA, and the application of a point mutation assay for the detection of specific single nucleotide mutations.

Within the clinic it was noted that, a small number of anti-HIV positive patients, who had an acute hepatitis B (section 3.7), failed to develop anti-HBs. HBsAg became detectable in the serum again several years later, indicating either reinfection or reactivation of an existing chronic HBV infection. HBV DNA amplification and sequence analysis was used to determine if these patients had become reinfected or had reactivated an existing infection (section 3.7).

The possibility that variation in the HBV genome may be related to disease severity was investigated using methods described in sections 3.2 to 3.4. The pre-core and 5' core regions of the genome, which are known to be highly conserved between subtypes and are immunogenic, were examined. The immune response to the translation product of these regions has been associated with immune mediated hepatocellular damage. Thus, any variation in these regions may be significant for the clinical outcome. HBV sequence variation in clinic attenders was studied (section 3.8).

Carman *et al.*,(1989) linked the presence of a nonsense mutation of the pre-core region with seroconversion from HBeAg to anti-HBe and a continuing high level HBV replication. This pattern of infection is common in the Mediterranean and areas where HBV infection is endemic. In collaboration with colleagues from Greece we examined the sexual transmission of pre-core mutant (HBeAg-minus) HBV by PCR amplification, sequence analysis and point mutation analysis (PMA) of amplified HBV DNA (section 3.9). In the same group of patients the effect of pre-core mutants on severity of disease in acute hepatitis was examined.

Cases of perinatal transmission from anti-HBe positive mothers were examined to study the transmission of pre-core mutants in this setting using the PMA (section 3.10).

In addition the role of pre-core mutants in the reactivation of HBV infection following liver transplantation and immune-globulin prophylaxis was examined. Amino acid substitutions in the a determinant of the HBV surface protein have been noted in vaccine recipients who have become HBV infected, despite achieving apparently protective levels of immunity. Thus, nucleotide sequence variation in this region may be associated with immune escape, and was therefore studied in these patients.

Nucleotide sequence analysis may be used in the investigation of transmission events. We have identified two regions of the HBV genome which may be useful for the confirmation of clustered out-breaks of HBV infection and the identification of the source of such out-breaks (section 3.12).

Chapter 2 Materials and Methods

2.0 Introduction

The following section describes the methods applied throughout this work. The major processes described are; the HBV DNA dot blot assay; the HBV DNA polymerase assay: the polymerase chain reaction (PCR); sequencing of HBV DNA amplified by the polymerase chain reaction; the point mutation assay for the detection of single nucleotide mutations in the PCR product.

2.0.1 An overview

The hepatitis B virus (HBV) was detected in serum samples by the analysis of HBV DNA polymerase (HBV DNAp) activity and by the isotopic detection of HBV DNA with a cDNA HBV probe. HBV DNAp was assayed according to a method derived from Weller *et al.*,(1985) and Marion *et al.*,(1980). A modification of this method allowed the detection of HBV DNA and HBV DNAp in the same serum sample (Weller *et al.*,1982 (1), Weller *et al.*,1982 (2)). Both the sample preparation time and the sample volume required were reduced in a modification of the method described by Hsiang *et al.*,(1987). These methods were used in all analyses of HBV by HBV DNA dot blot and HBV DNAp.

Two cDNA probes were used to detect HBV DNA immobilized on nitrocellulose filters. The probes used for HBV DNA detection were donated by Professor K Murray (University of Edinburgh). They consisted of the entire HBV genome cloned into the plasmid pBR322 (pHBV 130) (Gough and Murray.,1982) and the HBV genome ligated into a phage vector (Lambda 1150).

The plasmid or phage DNA supplied was used to transfect *E Coli* strains HB101 and QR48 respectively and to produce further stocks by culture of the cells and the purification of either phage or plasmid DNA from those cultures. Transfected cells, stored as stab cultures, were grown and harvested to produce DNA. Purified DNA was stored at -20 °C and the stab cultures at room temperature.

Two methods of labelling were assessed, nick translation and random primed oligolabelling (Feinberg and Vogelstein.,1983). The latter method was found to produce

probes with a greater level of radioisotope incorporation (specific activity).

When the entire phage construct was used as the HBV DNA probe it gave relatively high levels of non-specific binding to the nitrocellulose filters used for the sample DNA. This problem also occurred with the pHBV130 construct (pHBV 130). Purer HBV DNA was separated from the vector using Bst EII, the insert was isotopically labelled and used as the probe in subsequent DNA analyses. This form of the probe resulted in much lower non-specific background hybridization to the filter. Hybridization and removal of excess probe were carried out under stringent conditions.

The stringency of the reaction was determined according to the method described by Bolton and McCarthy,(1962). The resultant autoradiographs were analyzed both by scanning densitometry and by eye.

The absolute sensitivity of this method was established by the detection of serial dilutions of cloned HBV DNA. The sensitivity and specificity of the system for native HBV DNA was evaluated in a cross sectional study of serotypically confirmed HBV carriers.

2.0.2 HBV DNA detection by the Polymerase Chain Reaction

This method was applied to HBV DNA purified from serum as described for the HBV DNAp assay.

The concentration of virus by centrifugation was found to be unnecessary for samples with a viral titre of $>10^8$ copies /ml as HBV DNA could be detected directly from 1 µl of serum in a 25 cycle PCR reaction. The PCR product was analysed by agarose gel electrophoresis and ethidium bromide staining. The specificity of the PCR reaction was confirmed by Southern blot transfer of the product and subsequent detection with an isotopically labelled HBV specific probe.

HBV DNA was extracted from serum in samples with viral titres of $<10^8$ copies /ml. Several extraction procedures were compared. The most sensitive was found to be a modification of the method described by Ljunggren and Kidd (1991).

The sensitivity of the assay was further increased by the inclusion of a second (nested)

round of amplification (Saiki *et al.*,1988). This increased the sensitivity to between 10 and 1 copy/ volume of inoculum. However, the amplification reaction was shown to be less sensitive than this for first round products of >1.5 kb. A reaction in which 2.7 kb of HBV DNA were amplified was used to obtain the x, precore and core regions in samples where there was a low viral titre and insufficient serum for repeated separate amplification reactions. By using a third ('double nested') round of amplification ethidium bromide detectable quantities of DNA could then be obtained, even from a PCR where the product of the first round was very long.

A method of sample preparation was developed in which a 20 µl aliquot of sample was boiled with water, the denatured serum proteins were pelleted by centrifugation and the supernatant used as the inoculum for the PCR. This procedure was both rapid and extremely sensitive (1 copy/volume of inoculum), which was comparable to that achieved after conventional sample extraction from 100 ul of serum. This method was therefore used for screening all samples in subsequent studies.

In some HBsAg-anti-HBe-positive samples HBV DNA was not detected by PCR amplification after these standard procedures. Pretreatment of these samples with RF-Absorbent and subsequent purification of HBV DNA by phenol chloroform extraction restored the sensitivity of the assay.

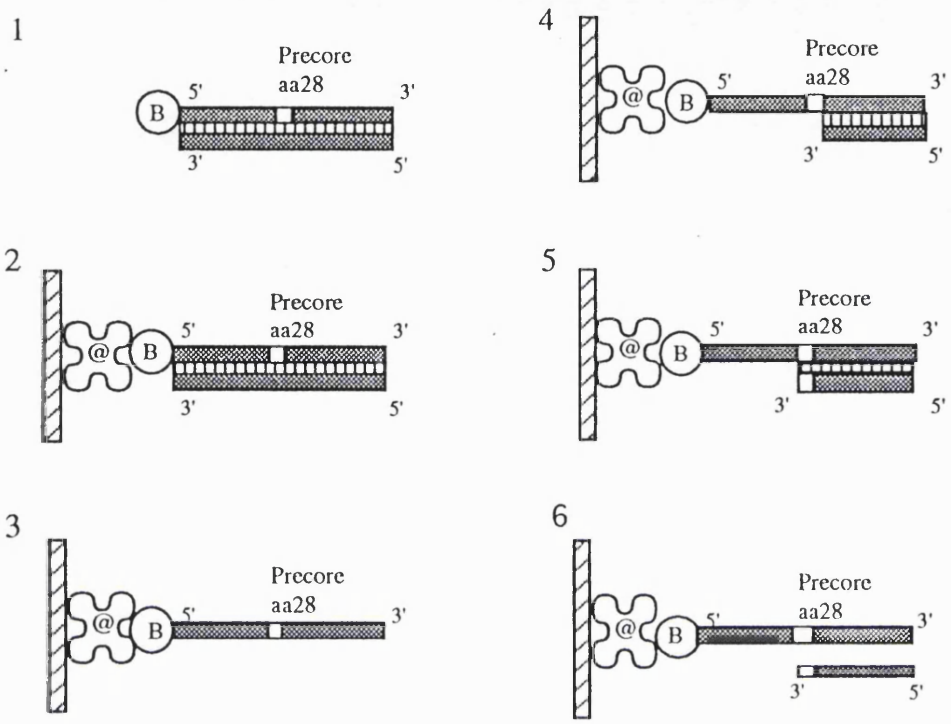
The polymerase chain reaction generated sufficient product for analysis by direct sequencing; both double stranded and single stranded sequencing methods were compared. For both methods PCR product had to be purified away from unreacted nucleotides primers. In the case of double stranded sequencing this was carried out by agarose gel electrophoresis followed by band excision and Mermaid™ kit purification. For single stranded sequencing the PCR product was generated using a biotinylated primer and captured onto streptavidin coated M-280 Dynabeads™. Unreacted nucleotides and non-biotinylated primers were removed and the product was denatured. The absence of the complementary strand and the consequent lack of secondary structure of the template meant that single stranded sequencing gave clearer, more reproducible results and hence was used for most studies.

Direct sequencing of PCR products was not possible for viral subpopulations of less than

15-20% abundance. The relative concentrations of such mixed populations were also difficult to estimate using this method. The application of the Point Mutation Assay (figure 2.0(i) (Kaye *et al.*,1992) allowed the quantitative and qualitative analysis of mixed samples and was shown to be sufficiently sensitive to detect subpopulations of between 2% and 3%.

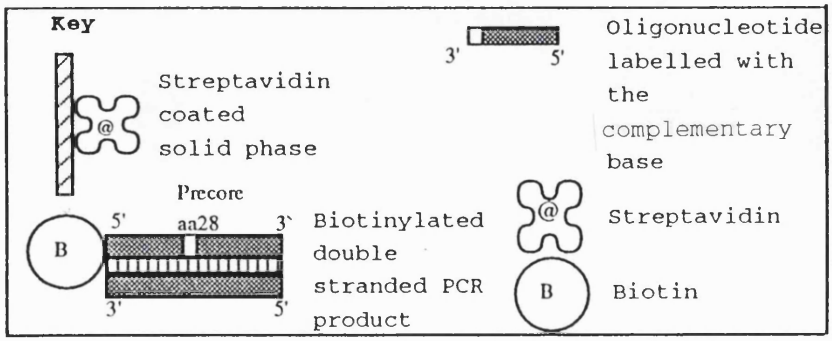
Point mutations at codon 1 and codon 28 of the precore region and amino acid 145 of the A determinant of the surface gene were assayed.

Figure 2.0(i) Principle of Point Mutation Assay (Kaye *et al.*,1992)



Notes

1. Double stranded, biotinylated PCR product. 2. The product is captured onto a streptavidin coated Microtiter well. 3. The product is denatured to a single strand. 4. A probe with its 3' end one base upstream of the point mutation under analysis (Amino acid 28 ofthe precore region) is annealed to the template. 5. A single labelled dNTP is used to extend the probe by a single complementary nucleotide. 6. The extended probe is denatured away from the target and added to a scintillation cocktail for counting.



2.1 Methods in detail

2.1.1 Serological assays

Hepatitis B surface antigen (HBsAg) (Cameron *et al.*,1978), antibody to HBV core (anti-HBc, both total and IgM fraction) (Tedder *et al.*,1980, Tedder *et al.*,1981), and HBeAg/anti-HBe, (Ferns and Tedder.,1985) were assayed by colleagues in the diagnostic service of the UCLMS Virology Division . These are standard protocols, which were not developed as part of this thesis and are described in Appendix 2.

Assays for the detection of HBsAg

I. A commercially available, amplified enzyme immunoassay (Wellcozyme, Murex diagnostics Ltd)(Appendix 2, page 232).

II. Reverse passive haemagglutination assay (Hepatitis B surface antigen HA screening kit. Murex diagnostics Ltd)(Appendix 2, page 232).

III. HBsAg subtype analysis (Appendix 2, page 232).

IV. HBsAg neutralisation (Appendix 2, page 232).

A radioimmunoassay for the detection of anti-HBs (Appendix 2, page 232).

Hepatitis B core antigen detection (Neurath *et al.*,(1982) (Appendix 2, page 232).

Assays for the detection of anti-HBc

I. A Passive Haemagglutination test kit for the detection of anti-HBc. Modified from Corecell™ (The Green Cross Corporation)(Appendix 2, page 233).

II. Radioimmunoassay for anti-HBc IgG fraction (Appendix 2, page 233).

III. Radioimmunoassay for anti-HBc IgM fraction (Appendix 2, page 233).

A radioimmunoassay for the simultaneous detection of HBeAg and anti-HBe (Appendix 2, page 234).

Commercial assays for the detection of delta and hepatitis A serological markers (Murex Diagnostics Ltd)(Appendix 2, page 234).

2.2 HBV DNA detection

2.2.1 The detection of hepatitis B virus (HBV) DNA polymerase in serum (Modification of Weller *et al.*,1985, Marion *et al.*,1980).

One hundred microlitres of serum was layered onto 600 μl of a 30% sucrose cushion (Appendix 1) in a sterile polycarbonate 1.5 ml ultra-centrifuge tube. The virus was pelleted by centrifugation for 4 1/4 hours at 25,000 r.p.m. in a fixed angle, type 25 Beckman rotor. The supernatant was removed and the pellet resuspended (by vortex) in 12.5 μl of nucleotide solution (mix E, Appendix 1) containing 25 μCi of tritiated thymidine. Twenty-five microlitres of TN/NP40/2 ME buffer (Appendix 1) were added and, after further mixing, the sample was sealed with Nescofilm. The samples were incubated for 3 hours at 37°C. The sample was denatured with 135 μl of 0.667M NaOH/0.667M NaCl and neutralized with an equal volume of 0.1M Tris HCl/1.0M NaCl. The sample was added to a nitrocellulose filter via a hybridot apparatus (BRL Manifold model number 1050 M).

The filter was washed in 100 ml of 6 x SSC (Appendix 1) on an orbital shaker for 20 minutes after removal from the Hybridot apparatus. The sample DNA was fixed to the filter by baking under vacuum for 2 hours at 80°C.

Excess unincorporated nucleotide was removed by the following washing procedure:

1) 2.5 ml/cm^2 cold 5% TCA 0.1M sodium pyrophsphate for 60 minutes. This step was repeated twice.
2) 2.5 ml/cm^2 cold 5% TCA for 60 minutes.

The samples were air dried and removed from the sheet using a 4 mm cork borer. Samples were placed individually into 5 ml of Betafluor scintillation liquid for 30 minutes before analysis each sample was counted for 10 minutes in a Packard 1500 Tri-Carb liquid scintillation analyzer.

2.3 The preparation of samples for direct detection of HBV DNA in serum by dot blotting (Hsiang Ju-Lin *et al.*,1987, Harrison *et al.*,1985).

Twenty five microlitres of each serum sample was placed in a sterile microfuge tube. Twenty microlitres of TE buffer pH 7.5 (Appendix 1) containing 5% NP40, 1.5% 2ME were added and the sample vortexed for 30 seconds. The sample was incubated at room

temperature for 10 minutes in the above buffer, denatured with 135 μl of 0.67M NaOH/0.67M NaCl and incubated at +4°C overnight. The sample was applied to the nitrocellulose filter using a hybridot apparatus as previously described. The filter was rinsed and the sample neutralised by adding 200 μl of 0.1M Tris pH 7.4/1.0M NaCl per well. The filter was then removed from the hybridot apparatus and rinsed in 6 x SSC. The samples were fixed by baking the filter at 80°C for 2 hours under vacuum. The filter was stored in a dry container at room temperature.

2.4 HBV cDNA Probe preparation

The protocols used for lambda phage preparation; transfection of *E.Coli* (HB 101); plasmid preparation and transformation of NEM 259 cells were provided by Professor K Murray in Edinburgh University.

Initially Professor Murray provided purified phage and plasmid DNA. These supplies were replaced with stocks of phage and plasmid produced in this laboratory. Cells containing either phage HBV 1150 or plasmid pHBV 130 were also supplied and cultured to provide material for use as an HBV DNA probe. The standard protocols listed below were not developed as part of this thesis, and are described in Appendix 2.

Lambda HBV 1150 Phage Preparation (Appendix 2, page 234).

Transformation of *E. Coli* (HB 101) with lambda HBV 1150 DNA (Lederberg and Cohen.,1974)(Appendix 2, page 235).

Plasmid preparation (pHBV 130)(Appendix 2, page 236).

Transformation of NEM 259 cells by pHBV 130 DNA (Lederberg and cohen 1974)(Appendix 2, page 236).

Restriction endonuclease digestion of DNA (Appendix 2, page 237).

Phenol/Chloroform extraction of DNA (Appendix 2, page 238)

Concentration of DNA by ethanol precipitation (Appendix 2, page 238)

2.4.1 Isotopic labelling of HBV DNA

2.4.1.1 Labelling of HBV/Lambda DNA probes with ^{32}PdCTP by nick-translation (Rigby *et al.*,1977).

The DNA to be labelled consisted of the complete HBV genome with two terminal repeats (in the modified lambda phage vector). 350 ng of HBV DNA probe were labelled with 50 μCi ^{32}P by nick translation using a commercial nick translation kit (Amersham International). Unincorporated nucleotides were separated from the probe DNA by fractionation through a 1 ml Sephadex G50 column.

2.4.1.2 Random primed labelling of HBV DNA probes (Feinberg and Vogelstein, 1983)

HBV DNA, lambda DNA and gel purified digested HBV DNA fragments were labelled by this method.

2.4.1.3 Labelling of Lambda DNA

Three hundred and forty nanograms of total lambda probe were labelled, which included 20.4 ng of specific HBV DNA in the vector. The DNA was labelled with 1x oligolabelling buffer (Appendix 1); 2 μl of 10 mg/ml bovine serum albumin; 31 μl of sterile demineralized water; 5 units of DNA polymerase Klenow fragment and 25 μCi of ^{32}P dCTP.

2.4.1.4 ^{32}P Labelling of HBV DNA in low melting point agarose

Separation of the insert and vector DNA prior to oligolabelling was carried out in a low melting point agarose gel by electrophoresis in Tris-acetate buffer (Appendix 1). Following excision of the gel slice the DNA was boiled for 7 minutes with 50 μl of sterile demineralised water and stored at -20°C. Before each labelling reaction the DNA in the gel was denatured by re-boiling. The labelling reaction was found to be successful over a range of concentrations of agarose (2% - 0.8%). The labelling constituents were used in the same way as for purified lambda DNA.

For both types of DNA used the labelling reaction was carried out over 4 hours. For purified DNA the reaction was carried out at room temperature, for DNA in agarose the temperature was raised to 37°C. Removal of unincorporated nucleotides was performed using Sephadex G50 columns.

2.4.2 Detection of HBV DNA using a ^{32}P labelled probe (modified from Anderson *et al.*,1985)

This method was used with all of the probes that were prepared. Samples were treated as described in section 2.2.1 stored on nitrocellulose filters and analyzed using a ^{32}P labelled HBV DNA probe. Prehybridisation was carried for 4 hours at 65°C in prehybridisation buffer (Appendix 1).

2.4.2.1 Hybridisation

Hybridisation was carried out in hybridising buffer (Appendix 1) at 65°C overnight in a shaking water bath. The volume of hybridising solution used was 100 μl/cm^2. The probe was denatured by boiling, spun briefly and then added to a final concentration of 3.4 ng/cm^2 of filter. This was equivalent to 0.204 ng/cm^2 of HBV (insert) specific DNA.

2.4.2.2 Post hybridisation sample washing

The washing stages were carried out at 65°C in a shaking water bath. The washing conditions were as follows (for buffer constituents see Appendix 1). Washing buffer 1, for 20 minutes (2.5 ml/cm^2) twice; Washing buffer 2, for 20 minutes (2.5 ml/cm^2) twice; Washing buffer 3, for 10 minutes (2.0 ml/cm^2) once

2.5 Autoradiography of samples

After washing the filter was air dried at room temperature for 10 minutes and sealed into a plastic bag. The filter and an autoradiograph film were then placed in a cassette with intensifying screens (Dupont, Cronex lightning plus). The film used was X-omat S,(Kodak) and the samples were left at -70°C for 24 and 72 hours. The film was then developed (Kodak D90) and fixed (Kodak liquifix).

The films were dried and analyzed both by scanning densitometry and by eye. The area under the curve, as measured by the densitometer, was recorded as was the height. These measurements allowed samples to be compared more accurately than by eye.

2.6 Introduction to the Polymerase Chain Reaction

The current methodology for the *in-vitro* amplification of DNA sequences derives from experiments first carried out by Saiki *et al.*, (1985) on human β globin sequences.

The Klenow fragment of the DNA polymerase which was originally used for amplification of the target DNA by Saiki *et al.*, (1985) was deactivated by the high temperatures required for sample denaturation. The enzyme was therefore replenished manually after every cycle.

A major advance was made by the use of a previously isolated thermostable deoxyribonucleic acid polymerase from the extreme thermophile *Thermus Aquaticus* (*Taq* polymerase)(Chien *et al.*,1976, Saiki *et al.*,1988), which could withstand the high temperatures used in the denaturation step. *Taq* polymerase was added at the beginning of the first cycle and remained active throughout the process. The thermal stability of *Taq* polymerase also permitted the use of higher annealing temperatures. This increased the specificity of the reaction and, in some cases, the sensitivity of the assay (Larzul *et al.*,1987).

The procedure was based on the specific amplification of DNA sequences by a DNA polymerase. Two oligonucleotide primers, flanking the region to be amplified, were used to initiate elongation, one primer complementary to the (+) strand, the other to the (-) strand. These primers annealed to the heat denatured (+) and (-) strands. DNA polymerase was then used for primer extension with deoxynucleotide triphosphates, resulting in the synthesis of 1(-) strand containing the target sequence and, at the same time, 1(+) strand.

The polymerase chain reaction (PCR) has been used to detect β globin sequences with modified primers that allowed the cloning of the amplified DNA (Scharf *et al.*,1986). Sickle cell anaemia has also been diagnosed prenatally using *in vitro* amplification (Embury *et al.*,1985), the products of the amplification were digested with restriction endonucleases. The digestion indicated the presence or absence of the point mutation in the β globin gene associated with the disease. Restriction endonuclease digestion can also be used to verify the product of the reaction when the sequence of the PCR template is known. HIV-1 proviral DNA was detected by this method in children and new-born

infants from infected mothers (Wolinsky *et al.*,1988). RNA viruses can be detected using an initial reverse transcriptase step (cDNA synthesis) (Kwok *et al* (1987), Ou *et al.*,(1988)).

The PCR method was first used for the detection of hepatitis B virus by Larzul *et al.*,(1988) and greatly increased the sensitivity of viral detection. In that study the template concentration was estimated to have increased by a factor of 2 x 10^5, after 20 cycles. The primers used were designed from an alignment of sequences from subtypes *adw, adr, ayw* and *ayr*, and were targeted to the surface gene. For these experiments the Klenow fragment of DNA polymerase I was used for primer elongation and therefore had to be replenished after each cycle. Twenty four cycles produced a high yield of a specific product. The specificity was reduced when the number of cycles was >32.

Thiers *et al.*,(1988) also amplified HBV DNA from serum but used *Taq* polymerase in place of Klenow In these experiments HBV DNA was detected by PCR in three patients negative for all serological markers. Previous methods of HBV DNA detection by dot blot and liquid hybridisation assays were relatively insensitive, with limits of 100 times above the level required for infectivity. A concentration of 100 virus particles/ml was the dose required to establish infection in chimpanzees (Hoofnagle, 1981).

Nested or double PCR (Simmonds *et al.*,1990) was first described for hepatitis B by Carman *et al.*,1991. In this process DNA fragments were sequentially amplified with two pairs of primers. An outer pair of primers was used in the first amplification reaction, carried out over 40 cycles. A 1 μl aliquot of the completed first PCR reaction was then transferred to a fresh volume of PCR solution containing the second (inner) pair of primers and amplified for a further 20 cycles. This second round of amplification increased both the specificity and the sensitivity of the reaction and did not lead to the formation of non-specific products as predicted by Larzul *et al.*,(1988).

The number of rounds of amplification carried out on HIV proviral DNA was increased to three in the double nested PCR (Simmonds *et al.*,1990). This further increased the yield of the assay and generated sufficient PCR product for direct sequencing (Simmonds *et al.*,1990, Winship, 1989). The inclusion of a 5' biotinylated primer in the final round of amplification enabled the product to be captured onto a streptavidin coated solid phase

(Stahl *et al.*,1988, Hultman *et al.*,1989).

The detection of specific point mutations within an amplified sequence, which may have a profound effect on the expression of that sequence, was first described by Syvanen *et al.*, (1990). This method was adapted by Kaye *et al.*,(1992) for the detection of point mutations associated with Zidovudine resistance in Human Immunodeficiency Virus Type-1. As part of this thesis the method was modified for the detection of mutations in the pre-core and surface genes of HBV and then applied in clinical studies.

2.6.1 Primer design and purification.

Primers were designed following the suggested guidelines of Saiki, PCR technology,(1989). Primers were selected to amplify well conserved regions of the HBV genome, as assessed by comparing published HBV subtypes using the Microgenie sequence analysis database (Genbank Issue 59, Beckman instruments Inc, Palo Alto, Ca 94304).

Primers were selected to have as near as possible a 50% G/C content and to have no self complementarity, particularly at the 3' end. Primers were designed to be between 18 and 25 nucleotides in length. This primer length allowed a short denaturation step and increased the specificity of the reaction. Where some sequence heterogeneity between HBV subtypes was present in the area chosen for annealing, the primers were made so that variable nucleotides were close to the 5' end of the primer. Sequence variation at the 3' end of the primer was avoided. Up to three potentially mismatched bases were allowed in the design of each primer. Primers were designed to have similar temperatures of dissociation (T_d), in the range of 50-60 °C.

Primers were produced by Oswel (University of Edinburgh). The primer positions were as follows: core primers C188. 1944-1964, C189. 1473-1444, C190. 3182-3212, C666. 531-516, C664. 1-119. Precore primers PC439. 2951-2970, PC441. 54-35, PC440.77-55, PC373. 45-27, PC459.2961-2979. Surface primers S376. 1740-1765, S377. 1991-1965, S374. 1767-1796, S375. 1966-1935, S185. 914-933, S186. 2157-2136, S187. 2063-2051, S163. 2073-2054. Nuclotide 1 is counted as the A of the core start codon, AUG. The primer identification numbers, sequences, the calculated temperatures of dissociation and the annealing temperatures used for each primer PCR of amplifications

are shown in detail in Appendix 6, tables 2.6(i) (page 244) and 2.6(ii) (page 246). The position of the primers in relation to the HBV genome are shown in Appendix 6, figure 2.6(i) (page 248). The combinations of primers used for the PCR are shown in Appendix 6, tables 2.6(iii) (page 249), 2.6(iv) (page 250) and 2.6(v) (page 251).

Four methods of serum preparation were used, the volumes used in each of these methods were determined by the level of sample purification. The presence of *Taq* polymerase inhibitors in serum restricted the volume that could be added directly to the reaction mixture, thus a small volume, 1 μl was used in this method. The addition of 10 μl of eluate from 20 μl of heat treated serum allowed larger volumes of serum to be analysed without compromising the sensitivity of the assay. The volume of serum incubated with RF absorbent was limited in practice because of the large volume of absorbent required for each sample but was applicable to larger sample volumes. The phenol/chloroform extraction method is applicable to any volume of serum and was limited only by the practical considerations of sample handling and availability.

2.6.2 Direct serum addition to the PCR reaction mix

One microlitre of serum, that had not been heat treated, was added directly to 49 μl of PCR mix. The samples were then amplified as described below.

2.6.3 Sample preparation by heat treatment of serum

Twenty microlitres of serum were aliquoted into a sterile 1.5 ml eppendorf using a positive displacement pipette (Gilson). Twenty microlitres of sterile water were added to each sample and mixed (by vortex). The sample was placed in a boiling water bath and incubated for 10 minutes to denature serum components and lyse Dane particles. The sample was then pelleted by centrifugation at 15000 RPM for 5 minutes. Ten microlitres of the supernatant were transferred to a 0.5 ml Sarstedt tube containing 40 μl of PCR reaction mix.

2.6.4 Removal of non-specific IgG and IgM rheumatoid factors with RF-absorbent™

Fifty microlitres of serum were incubated for 15 minutes at room temperature with 250

μl of RF-absorbent in a sterile 1.5 ml Eppendorf. The samples were pelleted by centrifugation at 2000 RPM for 10 minutes. The supernatant was removed to a sterile Eppendorftube for further processing.

2.6.5 HBV DNA extraction (Modification of Ljungren and Kidd.,(1991).

A 100 μl aliquot of a serum sample or 250 μl of supernatant from samples pretreated with RF absorbent were incubated with 130 μl or 260 μl of HBV DNA extraction buffer respectively (Appendix 1) at 57°C for 2 hours. This was followed by a proteinase K inactivation step of 10 minutes incubation at 95°C. Sample that had evaporated during heat inactivation was collected by centrifugation for 30 seconds at 15000 RPM before the tubes were opened. Denatured salmon sperm DNA was added to the sample at a final concentration of 10 ug/ml.

HBV DNA was purified by Phenol/Chloroform extraction (Appendix 2, page 238) and precipitated with 2 vols of 100% ethanol, 0.1 vols of 3 M sodium acetate and 20 μg of glycogen. The HBV DNA was resuspended in 20 μl of sterile water by gentle shaking at room temperature for 1 hour. 5 μl of the HBV DNA were added to a 45 μl PCR reaction mixture.

2.6.6 PCR reaction mix preparation

All components, except water, were stored at -20 °C. All solutions, except the *Taq* polymerase, were brought to room temperature before use. The concentrations of the components for the first round of amplification were as follows:

Sense primer	2.4 μg /ml
Anti sense primer	2.4 μg /ml
10 x Reaction buffer (RB)*	100 μl/ ml
Nucleotide mixture (1.25 mM)*	160 μl/ ml
Magnesium chloride (25 mM)	80 μl/ ml
Taq polymerase (5 units /ul)	10 μl/ ml

* (Appendix 1)

The solutions were mixed by vortex and aliquoted to sterile 0.5 ml Sarstedt™ tubes. Each aliquot was overlayed with 50 μl of Light white mineral oil and the tubes closed before any of the samples were added. The tubes were opened and closed sequentially to minimise contamination which could arise from aerosols during sample manipulation.

The Sarstedt tubes containing the PCR mixtures were placed in a Techne PHC-1 thermal cycler and run with the appropriate cycling program. Cycling times and conditions are summarised in Appendix 6 tables 2.6(iii)(page 249), 2.6(iv) (page 259) and 2.6(v) (page 251).

2.6.7 Nested PCR

The second (nested) round of amplification was carried out in 25 μl volumes. The reaction mixture was prepared as for the first round with the exception that the concentration of the second (inner) pair of primers was reduced by 50% to 1.2 μg/ml and the concentration of *Taq* polymerase was also reduced, to 25 units/ml. One microlitre of the completed first round reaction mixture was transferred to the second reaction mix. This was then amplified by a second cycling program (Appendix 6, tables 2.6(iii)(page 249), 2.6(iv)(page 250) and 2.6(v) (page 251).

After the second round was completed 10 μl of the sample was removed and electrophoretically separated on a 2.0% agarose gel. The 7 cm gel was run at 50 V for 2 hours in 1 x TBE buffer (Appendix 1) and photographed under U.V. illumination (306nm). A 10 μl aliquot from the completed 1st round reaction mixture was also run on the gel, and compared to the 2nd round product.

2.6.8 Double nested PCR

A third pair of primers were used for this round. The PCR reaction components for the third round of amplification were as described for the second round. One microlitre of the second round PCR product was added to 24 μl of reaction mixture and thermal cycling performed as previously described. The PCR product was analyzed in the same way as described above for the second round product.

2.7 Biotinylation of PCR products

The inclusion of a biotinylated version of one of the primers in the final round of the PCR

did not affect the sensitivity of the reaction. The biotinylated DNA produced by this method was suitable for analysis by direct sequencing or PMA.

2.8 Southern transfer of DNA (Southern E,1975)

This standard protocol was not developed or modified as part of the thesis and is therefore described in Appendix 2, (page 237).

2.9 Double stranded HBV DNA sequencing

2.9.1 PCR product purification

Twenty microlitres of PCR product was separated from un-reacted primers and dNTPs by electrophoresis through a 2.5% low melting point agarose gel in TAE buffer. The PCR product was excised from the gel and the agarose removed by processing the gel with the Mermaid Kit (United States Biochemicals USB) according to the manufacturers instructions.

2.9.2 Quantification of PCR products for sequencing

The purified product was quantified by comparison with a DNA marker (Hind III lambda digest) of known concentration, approximately 200 ng of product was used per sequencing reaction.

2.9.3 Sequencing

Sequencing of the product was carried out using an in-house version of the Sequenase™ 2 kit (United States Biochemicals, Winship, 1989).

All sequencing samples were stored at -20°C and allowed to warm to room to temperature before use. 2.5 μl of each termination mix , mixed with 0.25 μl of DMSO, were aliquoted into the wells of a 96 well V bottom microtitre plate (Dynatech). The plate was then sealed (Costar plate seals) until use. Primers were diluted to a concentration of 100 ng/μl, the labelling dNTP mix was diluted 1/30 with sterile demineralised water and stored on ice. The annealing and labelling mixtures were prepared in advance and kept on ice until required.

Anneal mix

Primer	1 μl (50 ng/sample)
5 x Sequenase buffer*	2 μl/sample
DMSO	1 μl/sample
H_2O	6 μl/sample

Labelling mix

0.1M Dithiothreitol	1 μl/sample
1/30 Labelling dNTP mix*	2 μl/sample
^{35}SdATP	0.5 μl/sample (5 uCi)
Sequenase™	0.16 μl/sample
H_2O	0.84 μl/sample

* (Appendix 1)

One hundred nanograms of sample was placed in a 1.5 ml Eppendorf tube and annealing mix added to give a final volume of 10 μl. The samples were incubated at 100 °C for 5 minutes, immediately placed on ice for 2 minutes and the sample spun briefly.

The Sequenase enzyme was added to the labelling mix immediately before use. 4.5 μl of labelling mix was added to each sample and the sample incubated at room temperature for 5 minutes. The termination mixes were preheated to 37 °C for 2 minutes on a heating block. Three microlitres of sample were added to each of the four termination mixes and the reaction incubated for a further 5 minutes. The reaction was then stopped by the addition of 4 μl of stop solution (Appendix 1).

2.9.4 Sequence electrophoresis

The sequences were analysed on an 8% polacrylamide gel. The glass plates were carefully cleaned by sequential washing with distilled water, 70% ethanol and acetone. The sequences were analysed on 0.4 mm wedge gels using Sequagel™ reagents made according to the manufacturers instructions.

The gel was run at a constant power of 70 W for 2 hours, sodium acetate was then added to the cathode chamber to a final concentration of 1.0 M and the run continued for a further 1 hour (Ausubel, 1988).

The gel was removed from the plates and fixed on 3 MM filter paper (Whatman) in 10% methanol, 8% Acetic acid . The gel was incubated twice at room temperature for 15 minutes in fixing solution. The gel was dried under vacuum for 90 minutes using a vacuum gel drying apparatus (Biorad).

2.9.5 Autoradiography of sequences

The gel was placed in an autoradiograph cassette (Dupont) and exposed to X ray film at room temperature for 24 hours. The autoradiograph was developed in a automatic developer in accordance with the manufacturers instructions (X-Ograph).

2.10 Single stranded sequencing

2.10.1 Purification of biotinylated PCR products (modification of Hultman *et al.*,(1989)

The incorporation of a biotinylated primer in the final round of amplification allowed the PCR product to be captured onto a streptavidin coated solid phase. The volume of the final PCR reaction was increased to 75 μl, 10 μl of this was visualised by agarose gel electrophoresis and ethidium bromide staining. 60 μl of the sample was carefully removed to a 1.5 ml Eppendorf tube, 20 μl of streptavidin coated magnetic particles (Dynanbeads™), prepared according to the manufacturers instructions, and 60 μl of '2 x Bind and Wash solution' (Appendix 1) were then added. The sample was incubated with the Dynabeads™ at room temperature with gentle agitation for 15 minutes. The sample was then pelleted by a magnetic field in a specifically designed rack (Dynal MP-C). The unreacted nucleotides and the non-biotinylated primer in the PCR reaction mixture were removed and the pellet washed a second time in 100 μl of 2 x Bind and Wash solution.

The sample was denatured by incubating for 5 minutes at room temperature with 8 μl of 0.1 M NaOH. The eluted strand was placed in an Eppendorf tube and stored at - 20°C until use. The sample was then washed sequentially in 50 μl of 0.1 M NaOH, 40 μl of 2 x Bind and Wash solution and finally in 50 μl of TE buffer (PH 7.4). The TE buffer was discarded and the sample finally resuspended in water and stored at -20°C.

The eluted, non-biotinylated, strand was neutralised by the addition of 4 μl of carefully titrated 0.2 M HCl and 4 μl of TE ph 7.4 before sequencing.

2.10.2 Sequencing of single stranded biotinylated PCR product

The reagents used for sequencing double stranded DNA may also be used for single stranded sequencing. The single strand of biotinylated HBV DNA PCR product attached to the magnetic particles was resuspended by vortex, 5 μl of the sample transferred to a 0.5 ml Sarstedt™ tube and 5 μl of annealing mix added. After mixing the samples were placed in a thermal cycler, incubated at 70°C for 5 minutes and cooled to 25°C over 30 minutes. The samples were then held at +4°C until required. Sample that had evaporated onto the lid during incubation was collected by centrifugation of the samples at 6000 RPM for 3 seconds before the labelling reaction. 4.5 μl of labelling were mixed with each sample by gentle pipetting and the samples incubated at room temperature for 5 minutes.

Termination mixes (10% DMSO) were aliquoted into a 96 well V bottom microtitre plate and preheated to 50°C on a heating block for 2 minutes. Three microlitres of each sample were added to each termination mix and the reaction incubated at 50°C for 5 minutes. The reaction was stopped by the addition of 4 μl of Stop solution (Appendix 1). The sequence was analysed on an 8% polyacrylamide gel as described for double stranded sequencing.

2.11 The microtitre point mutation assay

2.11.1 Streptavidin coating of Microtitre wells

Strepatavidin was prediluted in water to a concentration of 1 mg/ml and stored as a stock solution at -20°C. The stock dilution was diluted in 100 mM Tris/HCl pH 7.6, containing 0.1% sodium azide to a final concentration of 25 μg/ml.

A 25 μl aliquot of streptavidin solution was added to each well of a 96 U-well microtitre plate (Nunc Maxisorp U-Wells). The plate was incubated overnight at room temperature

in a moist environment. The plate was washed twice with PBS (Dulbeco A) using a Skatron hand washer. The plate was turned between washes to ensure even washing.

A 350 μl aliquot of blocking solution, containing 1% BSA (Appendix 1) was added to each well. The plate was then incubated at room temperature for a minimum of 1 hour. The plate was aspirated and 100 μl of fresh blocking solution added to each well. The plate was stored in a moist container at 4°C.

2.11.2 Point mutation assay

Forty microlitres of the biotinylated product from a nested or double nested PCR was analysed. The storage buffer was aspirated from the Streptavidin coated plate and the plate washed twice in TTA buffer (Appendix 1). Four wells were used for each sample, positive and negative controls were included on each plate. A maximum of 20 samples could be analysed in each assay. Fifteen microlitres of 1 x TTA buffer was added to each well using a multichannel pipette. After the final round of amplification 10 μl of each sample was added to each of four wells of a microtitre plate, fifteen microlitres of PMA diluent were then added to each sample. The sample was mixed with the PMA diluent and incubated at room temperature for 15 minutes. The unbound sample was aspirated away and the plate washed three times in TTA buffer. The plate was turned through $180^{°C}$ after two washes to ensure even washing.

A 40 μl aliquot of 0.15 M NaOH was added to each well of the plate, which was then incubated at room temperature for 5 minutes. The NaOH was aspirated away and the plate washed four times in TTA buffer.

Twenty five microlitres of anneal mix (Appendix 1) containing 10 ng of the appropriate probe-primer were added to each well and the plate sealed. A metal block was preheated to 63°C in a water bath. The sealed plate was incubated on this block for 3 minutes. The plate and the block were then removed from the water bath and allowed to cool to room temperature over 30 minutes.

A 10 μl aliquot of labelling mix (Appendix 1) containing either ^{35}S labelled dATP, dGTP, dCTP or dGTP was added to each of the sample wells and incubated at room temperature for 2 minutes.

The anneal and labelling mixture was then aspirated and the plate washed six times in TTA. A 1 minute incubation in TTA was included between the fourth and fifth washes to ensure that all of the non-incorporated labelled nucleotide was removed.
The labelled probe was removed from the template by the addition of 40 μl of 0.15 M NaOH to each well and incubation at room temperature for 5 minutes. The NaOH, containing the eluted probe, was removed to a white microtitre plate which contained 100 μl of scintillation cocktail (Microscint^{TM}) per well. The plate was sealed in a Beckton Dickenson heat sealing device. The samples were analysed in a (Top count analyser, Tricarb) for 1 minute.

The probes used to assay each position were:

PMA 1,	3149-3130	GCAGAGGTGAAAAAGTTGCA,
PMA 28A,	17-3212	TAAGGGTCAATGTCCATGCCC,
PMA 28B	3191-3210	GCTGTGCCTTGGGTGGCTTT,
PMA 145A	1922-1903	GGGAATACAGGTGCAGTTTC,
PMA 145B	1922-1903	GGAATACAGGTGCAATTTC

The nucleotide positions are numbered according to Pugh *et al.*,(1986) where nucleotide 1 is A of the core region start codon. Point Mutation Assay probe-primers 1 (PMA 1) and PMA 28a are used with biotinylated primer PC439. Probe PMA 28b is used with biotinylated primer PC441 and probes PMA 145a and 145b are used with biotinylated primer S374.

The results were calculated as follows:

	cpm dATP	cpm dCTP	cpm dTTP	cpm dGTP
Negative control	W	X	Y	Z
Sample	1	2	3	4

Corrected cpm dATP = 1 - W

Corrected cpm dCTP = 2 - X

Corrected cpm dTTP = 3 - Y

Corrected cpm dGTP = 4 - Z

% of Nucleotide A in the sequence at that point = N

This value is calculated for each nucleotide at that point.

$$N = \frac{\text{Corrected cpm dATP}}{[(\text{Corrected cpm dATP})+(\text{Corrected cpm dCTP})+(\text{Corrected cpm dTTP})+(\text{Corrected cpm dGTP})]} \times 100$$

Chapter 3 Results

3.1 Optimisation of the HBV DNA dot blot assay

3.1.0 Objectives

- To develop a specific, sensitive assay for the detection of HBV DNA

3.1.1 Introduction

The analysis of serologic responses to HBV in patients may be used to identify or confirm a past or current HBV infection. The pattern of their response may also be used to follow the natural history of the infection.

Gudat *et al.*,(1979) also used electron microscopy to provide early evidence of HBV replication in the presence of serum anti-HBe. Whilst it was generally true that the presence of anti-HBe in serum indicated an absence of Dane particle formation and by implication HBV replication, 11% of the samples tested by Gudat *et al.*, had anti-HBe in the presence of circulating Dane particles.

This finding has since been confirmed in many studies. However, the frequency of this serological pattern varies geographically, the highest frequency occurring in areas where HBV is endemic. Thus the detection of serologic HBV markers alone may not provide sufficient information for determining potential levels of infectivity in patients.

The method of virus particle detection, in the form of the HBV genome, was simplified considerably by the use of DNA hybridisation techniques. The cloning of the virus into *E. coli* by Burrell *et al.*,(1982) and Sninsky *et al.*,(1979) provided suitable material for radiolabelling and direct HBV DNA detection.

Brechot *et al.*,(1990) and Weller *et al.*,(1982) described the detection of HBV DNA in plasma and serum. The HBV DNA dot blot assay may be used to monitor the efficacy of new treatments of HBV infection throughout the treatment course. HBsAg to anti-HBs seroconversion rarely occurs in response to therapy and HBeAg to anti-HBe seroconversion may similarly only occur at the end of treatment.

In the following section the development of a simplified method of sample preparation

and the optimisation of an isotopic HBV DNA detection system is described.

3.1.2 A comparison between random primed labelling and nick translation with ^{32}P dCTP of HBV DNA for use as a nucleic acid probe

When measured by the relative level of ^{32}P dCTP incorporation, the random primed method of labelling the HBV cDNA (rOLB labelling) produced a probe of higher specific activity (35%), than nick translation (15%). The levels of incorporation are shown in Appendix 6, table 3.1(i) (page 252).

The sensitivity of the probe was determined by hybridising the labelled HBV DNA probe to homologous unlabelled DNA. The limit of sensitivity of the random primed labelled probe against unlabelled DNA was 0.1 pg, the corresponding value for the nick translated probe was 1 pg.

3.1.3 A comparison of probes synthesised from Lambda HBV 1150, pHBV 130 and HBV DNA purified from pHBV 130 by restriction endonuclease digestion.

HBV DNA was obtained by excision from the vector after digestion of pHBV130 with Bst EII. The HBV DNA was separated from the vector DNA by electrophoresis through a low melting point agarose gel. The agarose slice containing the HBV DNA was removed from the gel, placed in a sterile 1.5 ml Eppendorf tube and water added to give a final concentration of 10 ng HBV/ml.

The DNA was labelled directly in agarose (Feinberg and Vogelstein, 1983). An equivalent amount of specific HBV DNA was labelled as either a 20 ng aliquot of gel purified HBV DNA, 52 ng of pHBV 130 or 340 ng of Lambda 1150 DNA. The percentage incorporation of the probes were 40%, 47% and 32% respectively.
The probes were all equally sensitive (0.1 pg of HBV DNA). The level of non-specific hybridisation was lower with the purified fragment, hence the Bst EII digested HBV DNA was used for all subsequent analyses.

3.1.4 Results of treatment of serum with NP40/2 ME

Serum was prepared and transferred to the filter as described in section 2.3. The filter was prehybridised for 4 hours in prehybridisation buffer (Appendix 1). The probe was prepared with 25 μCi of ^{32}P dCTP/25 ng of HBV DNA, (see above). The filter was

washed as previously described. The resultant autoradiograph showed the positive samples as clearly defined, uniform dark dots and the negative samples as clear areas with no non-specific hybridisation, as evidenced by the presence of 'hot-spots' either in the samples or around them. The overall level of background signal was low.

The resultant autoradiographs were analysed densitometrically. The height of the peak and the area under the curve were measured, the area measurement correlated most closely with the HBV DNA polymerase values ($r=0.858$, $p<0.001$), and so was used in all subsequent analyses (Appendix 6, table 3.1(ii)(page 253).

A comparison of mean values of duplicate samples after a 24 hour exposure of the filter to the X-ray film at -70°C and a 72 hour exposure of the same filter under the same conditions was carried out. This was done both to ascertain that the shorter time of exposure would provide an autoradiograph suitable for densitometric analysis and to ensure that the longer time exposure did not disproportionately represent any background signal, either on the negative control samples or the filter as a whole. The additional 48 hours of exposure resulted in an increased signal of an average 70367 units per sample, but a decreased positive:negative ratio; from 55/1 after 24 hours to 39/1 after 72 hours (Appendix 6, table 3.1(iii), page 254).

On the basis of these results, 24 hour autoradiographs were used for subsequent analyses. The experiment was repeated to assess inter-assay variation (table 3.1(iv), page 255). Analysis of the correlation coefficient between the initial and confirmatory tests indicated that there was a highly significant correlation coefficient between these experiments ($r= 0.9448$, $p<0.001$). Some non-specific background hybridisation of the filter occurred in both of the above experiments, but was only a problem where a non-specific 'hot spot' coincided with the sample dot.

3.1.5 Evaluation of NP40/2 ME treatment of 36 HBsAg negative samples and 57 samples of unknown HBV serological status by hybridisation to an HBV DNA probe.

In order to investigate the extent of non-specific binding of probe to the filter and to serum sample constituents, 36 samples, known to be HBsAg negative, were assayed for the presence of HBV DNA.

Prehybridisation was carried out overnight at 65°C. The probe used in this experiment was as described in previous experiments as were hybridisation conditions and post hybridisation washes. Fifty seven sera that had not previously been tested for HBV serological markers were also analysed.

None of the samples in either group were positive in this assay. The positive serum control was always positive and the sensitivity of the assay was 0.1 pg. From these results it was concluded that the assay should be further evaluated in a cross sectional study of HBV carriers. These samples were also analysed with the HBV DNA polymerase assay (section 3.5).

3.1.6 Discussion

In our study the rOLB method of probe labelling gave a higher percentage of isotope incorporation than nick translation and was used for all analyses described in this thesis. Greatest specificity was achieved with the Bst EII digested highly purified probe.

The use of biotinylated probes for the detection of HBV DNA has been described (Saldanha *et al.*,1987). The level of sensitivity in most of these cases has been approximately 10 fold lower than for radiolabeled probes. In addition specificity has been shown to be lower in some cases (Kejian and Bowden,1991).

Using the radiolabeled probes in the assay developed in this thesis we did not have any false positive results. The contamination of probes with bacterial or plasmid sequences may lead to spurious hybridisation. In our study we found that purification of the HBV sequence away from the plasmid improved the specificity of the assay.

Lin *et al.*,(1987) suggested that oligonucleotide probes may also be used to improve specificity. However, sequence variation in the region of the probe may reduce sensitivity of the assay compared with assays which use full length probes.

The reduction of sample preparation to exclude phenol/ chloroform purification did not adversely effect either the specificity or the sensitivity (0.1-1 pg) of the assay. The results were analysed by scanning densitometry of autoradiographs. The area under the curve demonstrated a positive correlation with HBV DNA polymerase values and was used for

all subsequent analyses.

There was no advantage in increasing the length of autoradiograph exposure from the standard 24 hours to 72 hours. The level of background signal due to non-specific hybridisation continued to rise with time whilst the autoradiograph signal of those samples with high levels of HBV DNA reached a plateau.

In a study of 93 samples, 36 known to be HBsAg negative and the remainder selected from samples of unknown HBV serological status, none were positive by HBV DNA dot blot or HBV DNA polymerase assay. This confirmed that the conditions chosen for probe labelling, sample preparation and hybridisation did not give false positive reactions. Lin *et al.*,(1987) suggested that serum components in minimally prepared serum may prevent non-specific hybridisation more efficiently than bovine serum albumin based blocking buffers. In this study we did not see a reduction in background levels of hybridisation between our highly purified samples and our detergent treated samples and so were not able to confirm this finding. However, we did determine that phenol/chloroform extraction was not required for sample preparation.

The absolute sensitivity of the assay sensitivity was between four and 20 pg/ml which equated to approximately 0.1 pg to 0.5 pg /25 μl serum sample. The level of sensitivity in this assay compares favourably with those described in other studies.

3.2 A polymerase chain reaction for the detection of HBV

3.2.0 Objectives

- To develop a specific, sensitive *in-vitro* amplification assay (PCR) for the detection of HBV DNA.

- To develop sample preparation methods which minimise the risk of cross sample contamination whilst maintaining the sensitivity of the assay

- To optimise the PCR assay such that sufficient HBV DNA was amplified from a single copy of hepatitis B for subsequent nucleotide sequence analysis

3.2.1 Introduction

Whilst the HBV DNA dot blot assay is highly specific for the detection of HBV DNA in serum, the sensitivity of the assay is such that HBV DNA present at potentially infectious concentrations may not be detected. The development of the polymerase chain reaction has, with its extreme sensitivity, enabled one viral particle to be detected directly in serum samples. This is important where assays of serological markers fail to identify current HBV infections.

The reduction of sample processing may be an important factor in reducing cross sample contamination. Careful optimisation of assay conditions is required for reaction specificity. The following section describes the development and optimisation of a polymerase chain reaction for the development of HBV DNA in serum.

3.2.2 Result of a polymerase chain reaction carried out on phage lambda and HBV DNA.

HBV specific core primers 188/189 (table 2.6(i), Appendix 6, page 244) were used to amplify a 1.4 KB fragment from 1 ng of lambda phage 1150, which carried an HBV insert. The phage was diluted in water to a concentration of 1.7×10^7 copies of HBV DNA/ml. One microlitre of the sample was added to the standard PCR buffer as

recommended by the manufacturers of the *Taq* polymerase enzyme (Perkin Elmer Cetus). *Taq* polymerase (2.5 units /100 μl reaction volume) was added after an initial template denaturation step of 7 minutes at 91°C. Primers were used at a concentration of 30 pmols per 100 μl reaction. Three positive control samples, containing the phage with insert, were amplified. Four negative control samples were also amplified comprising two of phage minus the HBV insert and two of water only. The following cycling conditions were carried out (25 rounds); Pre-melt at 91°C for 7 minutes, denaturation at 91°C for 1.5 minutes, annealing at 25°C for 2.5 minutes and elongation at 70°C for 8.5 minutes.

The PCR product was visualised by agarose gel (1.5%) electrophoresis in the presence of ethidium bromide (0.5 ug/ml) (1 x TBE, Appendix 1). The size of the PCR product was measured by calibration against standard molecular weight marker (Lambda/HIND III digest).

The amplified fragment was shown to be of the expected size (1.4 kb) in the positive control. The main PCR product was surrounded by lower and higher molecular weight material which appeared as a smear above and below the main product band.

3.2.3 Amplification of HBV DNA extracted from 200 μl of serum

HBV DNA was purified from 200 μl of serum from two HBsAg positive Hepatitis B carriers whose HBV DNA polymerase status was known and from two HBV negative control sera.

HBV DNA was purified from serum after ultracentrifugation of the viral particles through a sucrose gradient. HBV DNA was released from the pellet by incubation with TN/NP40/2ME buffer (section 2.2.1) for 3 hours at 37 °C and then a 2 hour incubation with 1% SDS and 0.17 mg/ml proteinase K at 37 °C. The HBV DNA was purified away from the viral proteins by phenol/chloroform extraction and concentrated by ethanol precipitation. Sample DNA was resuspended in 50 μl of TE buffer. One microlitre and 0.1 μl of this were used as the source of template DNA in a PCR reaction mixture of 100 μl. A positive control for the PCR reaction of 10^6 copies of Lambda 1150/HBV was used.

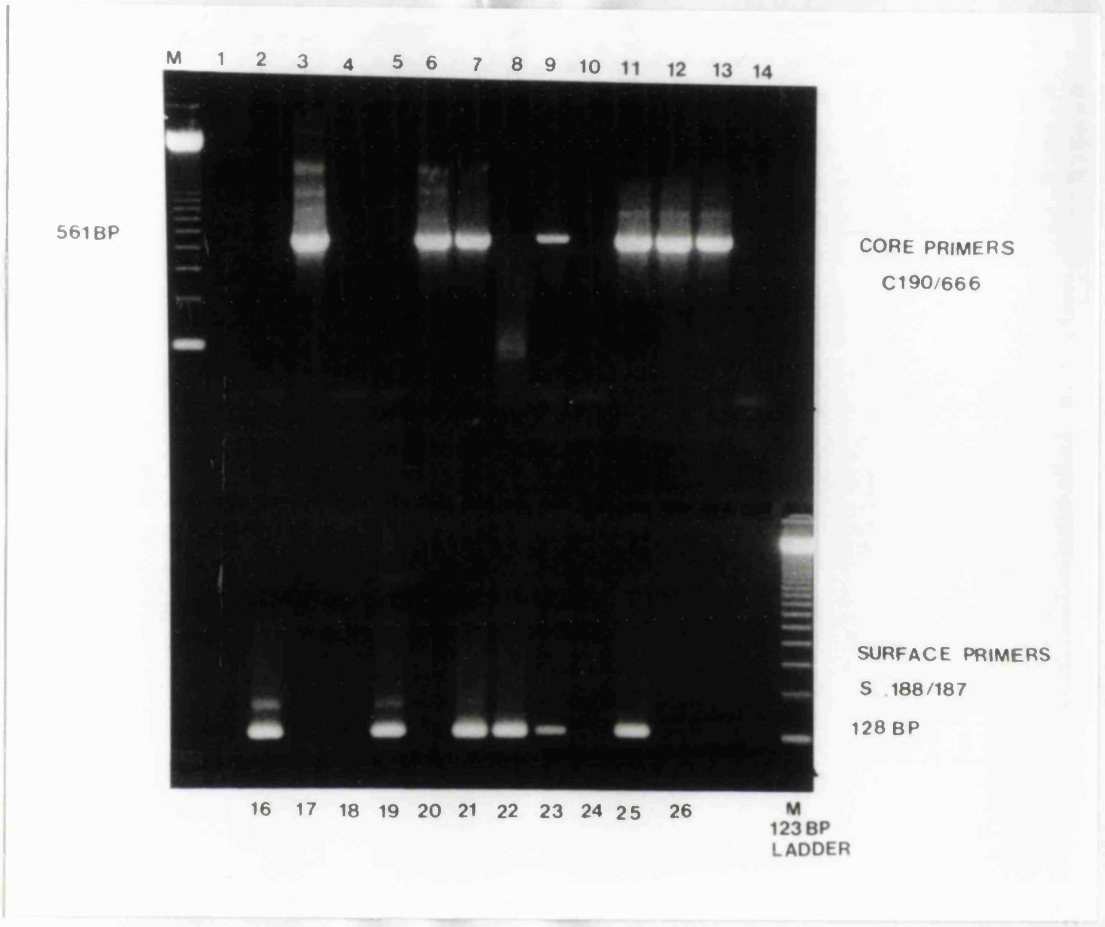
Negative controls of the lambda 1150 vector and water only were also included. The concentrations of reaction mix components and thermocycling conditions were as previously described. Ten microlitres of each sample were applied to a 1.5% agarose gel, the PCR product was separated by electrophoresis and visualised by ethidium bromide fluorescence.

The specificity of the product was confirmed by Southern blotting and hybridisation to a ^{32}P-labelled full length HBV DNA probe under stringent conditions. The higher and lower molecular weight material that was visible on the agarose gel was proven to be non specific. HBV DNA was amplified from the positive samples, a 1.4 kb product was detected in the positive control. Nothing was detected in the negative control samples, either directly on the gel or after Southern blot analysis. HBV DNA was amplified from both HBeAg positive samples when either 1 μl and 0.1 μl of sample were assayed.

The serum with the highest HBV DNA polymerase activity gave the brightest band of DNA on ethidium staining and also produced a second, smaller band of 580 bp which hybridised weakly to the probe in the Southern blot. The serum with the lower HBV DNA polymerase activity gave a less intense band of DNA at the correct size.

Non-specific amplification was a general feature of the unoptimised PCR and was present when different primer pairs were compared (figure 3.2(i)). However, the relationship between the PCR product band intensity and either HBV DNA as measured by dot blot or HBV DNAp was constant for all primer combinations that were used.

Figure 3.2(i) The visualisation of non-specific PCR products by Agarose gel electrophoresis.



Notes

Lanes 1 to 5 and 16 to 20 Samples from HBeAg negative HBV carriers

Lanes 6 to 14 and 21 to 26 Samples from HBeAg positive HBV carriers

3.2.4 The amplification of HBV DNA from non pelleted viral particles

HBV DNA was extracted from 100 μl of the same serum samples as used in section 3.2.2. Serum was incubated at 65°C for 1 hour with proteinase K (2.5 mg/μl), Sodium acetate (25 Mm) and SDS (0.5%). The HBV DNA was then phenol/chloroform extracted (Appendix 2, page 238) and ethanol precipitated (Appendix 2, page 238). The HBV DNA was resuspended in 20 μl of TE Buffer, pH 7.5, and incubated for 10 minutes in a boiling water bath to inactivate any residual proteinase K. The PCR was carried out on 1 μl and 0.1 μl of this sample. Positive and negative controls were as described above.

When the amplified HBV was visualised on an agarose gel a product of 1.4 kb was visible in the HBV DNA positive samples but not in the negative control samples. The specificity of this product was confirmed by Southern blot analysis.

3.2.5 Determination of the sensitivity of the polymerase chain reaction

The positive control used in this experiment was pHBV 130, the sensitivity of the assay was determined by titration of pHBV 130 in serum that was negative for all HBV markers. The control DNA was diluted to the point where it was no longer detectable by Southern blot after amplification. The cut-off of the assay, as determined by visualisation of the PCR product on an agarose gel, was 10^3 copies of HBV DNA in the sample. Using Southern hybridisation this improved to 10-100 copies of HBV DNA in the initial sample.

PCR was then carried out on fifteen serum samples chosen to reflect a range of HBV serological profiles. The samples were treated as previously described (section 2.6.6), 1 μl of the product was added to a 100 μl PCR reaction mix and thermocyled as described in section 3.2.3.

Two HBeAg positive samples were used as positive controls in addition to the plasmid. Water and a serum that was negative for all HBV markers were used as negative controls. These were co-processed with the samples. An additional negative control, of PCR reagents only, was also included.

HBV DNA was amplified from all five HBsAg, HBeAg positive samples by this method, the PCR product was visible both after gel electrophoresis and by Southern hybridisation. HBV DNA was amplified from one of five HBsAg-anti-HBe positive samples, the product was not visible on the agarose gel but was detected by Southern hybridisation. The sample which was HBsAg positive, but with neither HBeAg nor anti-HBe detectable in the serum was also positive. HBV DNA was not amplified from any of the five samples in which anti-HBc as the only serological marker of infection. The results of this assay are shown in Appendix 6, table 3.2(i) (page 256).

Several approaches were taken to try and improve the sensitivity of the assay and to increase the yield of the PCR. This would remove the requirement for Southern blotting PCR products and hence reduce the total assay time. The processing time could also be reduced if sample preparation were simplified.

3.2.6 Prevention of non specific amplification by increasing the stringency of the PCR.

The conditions of the PCR reaction were altered as follows: *Taq* polymerase was added to the reaction before the pre-melt step, this step was changed from 7 minutes at 91 °C to 5 minutes at 94 °C. This step immediately precedes the first denaturing stage of thermocycling. The denaturing temperature was raised to 94 °C for 1.5 minutes. Annealing was carried out at 45 °C for 2.0 minutes. The elongation time was reduced to 3.5 minutes at 72 °C. A final elongation step was introduced after the final cycle of 7 minutes at 72 °C. These modifications prevented some non-specific amplification and increased the yield of specific product, although the absolute sensitivity of the assay was not altered.

3.2.7 Nested PCR

The number of amplification cycles were increased in an attempt to increase the sensitivity of the assay. Increasing the number of cycles beyond a maximum of 30 gave an unacceptably high level of non-specific amplification in a single round PCR assay.

A method suggested by Mullis and Faloona, (1987) and described by Simmonds *et al.*, (1990), where two pairs of primers were used in a two stage amplification process, was adapted for use with HBV.

Sample preparation was as described in section 2.6.6. The number of cycles in the first round was increased to 35. One microlitre of the product of this amplification was carried forward to a second PCR reaction mixture. This contained a second pair of primers located within the region amplified in round 1, but was otherwise identical to that used in the first round. Thermocycling conditions were also as used in round 1.

The sensitivity of the assay was increased by 10-100 fold and HBV DNA amplified from as little as 5 copies of starting material could be detected by agarose gel electrophoresis. However, the product yield was not uniform for all concentrations of starting material, suggesting that the assay was not being carried out under optimal conditions.

3.2.8 Double nested PCR

Ten HBsAg positive samples were analyzed by 'double nested' PCR. A positive control sample which was diluted to a concentration of 10 HBV copies and a water only negative control were included in the assay.

One microlitre of serum sample was added to a standard reaction mixture containing core primers 188/189 (Appendix 6, table 2.6(i), page 244). Primers 188/189 amplify a 2.7 kb fragment which includes the 3' terminal of the surface gene, the X gene and the precore/core gene. A second round of amplification was then carried out with precore primers 439/440 (Appendix 6, table 2.6(i), page 244) or core primers 190/666 (Appendix 6, table 2.6(i), page 244). One microlitre of this reaction was carried forward into a third reaction mix which contained precore primers 439/441 (Appendix 6, table 2.6(i),page 244) or core primers 664/666 (Appendix 6, table 2.6(i), page 244).

The PCR products were analysed after the third round of amplification by agarose gel electrophoresis and ethidium bromide staining. The product of the second round of

amplification in those samples which gave a positive result was also analysed.

The inclusion of a third round of amplification allowed precore HBV DNA to be amplified directly from 1 μl of serum in two of five HBsAg-anti-HBe positive samples and all five HBsAg-HBeAg- positive samples.

Amplification with primers to the precore region detected 1 copy of control HBV DNA, with primers to the core region 10 copies of control HBV DNA could be detected. These results are summarised in Appendix 6, table 3.2(ii)(page 257). HBV DNA was not amplified from the negative controls with either of the primer combinations.

The introduction of a tertiary round of amplification increased the sensitivity of the assay and produced sufficient PCR product for further analysis (eg by sequencing).
The successful amplification of this large fragment allowed extensive sequence analysis of samples where only a small volume of the sample was available for analysis and the viral copy number was very low.

3.2.8.1 Optimisation of Magnesium Chloride concentrations in PCR reaction buffer
The magnesium chloride content of the PCR buffer was altered over a range of concentrations (0 mM to 20 mM) in an attempt increase the yield of PCR product and sensitivity of the assay. Relative quantities of DNA generated were assessed by inspection of their fluorescence under UV illumination.

The $MgCl_2$ titration was carried out 100 copies of pHV 130 with all primer pairs in a single round format PCR. The results are summarised in Appendix 6, table 3.2(iii)(page 258). The optimum concentration of $MgCl_2$ for the control HBV DNA diluted in water was between 1.0 mM and 3.0 mM. At these concentrations there was maximum amplification of the sample and a lower level of non-specific amplification. The optimum $MgCl_2$ was determined in this way for all primer pairs used in subsequent experiments and in all cases a reaction buffer containing 2.0mM $MgCl_2$ gave the most specific and sensitive assay.

3.2.8.2 Titration of primer concentration

The optimum primer concentration was determined by dilution of the primers in water before addition to a standard PCR buffer solution. The magnesium concentration in this experiment was 2.0 mM. One hundred copies of pHBV 130 were amplified in each case. Two negative control samples of water only and DNA purified from negative serum were included in the assay.

The optimum concentration of primers for amplification was between 1000 ng/ml and 3000 ng/ml. A concentration of 2400 ng/ml was chosen for all first round primers, as this concentration would ensure that the maximum amount of PCR product whist limiting non-specific amplification. This concentration was reduced to 1200 ng/ml of each primer for second and third round reactions; this strategy both conserved primer stocks and ensured that biotinylated primer used in the final round was exhausted.

The temperatures of dissociation were calculated according to the method of Suggs *et al.*,(1981). The first round amplifications were carried out at a low stringency ($>T_d$ -15). The stringency was increased in the subsequent nested or double nested rounds of PCR ($<T_d$ -5). Thermocycling times and conditions are summarised in Appendix 6 tables 2.6(iii)(page 249), 2.6(iv)(page 250) and 2.6(v)(page 251).

3.2.8.3 Analysis of the effect of sample diluent on the sensitivity of the polymerase reaction

The resuspension of HBV DNA extracted from serum in TE buffer (pH 7.5) is advantageous for longer term sample storage. EDTA is a chelating agent and its presence will reduce the effective concentration of $MgCl_2$, therefore the following experiment was carried out to analyze the effect of TE buffer on a PCR reaction.

Twenty four microlitres of TE were added to 100 μl PCR reactions and $MgCl_2$ added to the PCR reaction mixture at concentrations of between 2.0 mM and 10 mM. Two concentrations of HBV DNA were analyzed at each $MgCl_2$ concentration (10 and 1000 copies).

1000 copies of HBV were amplified in the presence of TE buffer at $MgCl_2$ concentrations < 10 mM. HBV DNA was only amplified from 10 copies of HBV in the presence of TE Buffer at $MgCl_2$ concentration greater than 6.0 mM. These results are summarised in Appendix 6, table 3.2(iv)(page 259).

3.2.8.4 Optimisation of sample extraction conditions

Three buffers were compared for the extraction of HBV DNA from serum samples with the buffer recommended by Larzul *et al.*,(1988).

One hundred microlitres of each extraction buffer was added to an equal volume of sample. The sample buffers and extraction conditions are shown in table 3.2(v). Proteinase K was inactivated by incubation of the sample in a boiling water bath for 10 minutes. HBV DNA was extracted from five HBsAg, anti-HBe positive samples, a dilution of a positive control at a final concentration of 100 copies/ml and 1 serum that was negative for all HBV markers. One of the five samples was HBV DNA dot blot positive.

The extracted HBV DNA was analyzed by double nested PCR with core primers (table 2.6(i) Appendix 6, page 244) 188/189 (round 1), 439/666 (round 2) and 439/441 (round 3). The PCR product was analyzed by agarose gel electrophoresis.

Treatment of the samples with buffer 1 gave the highest frequency of positive results. None of the 5 samples analyzed gave a visible signal after nested PCR, however, HBV was amplified from all samples after double nested PCR. The PCR amplification from the positive control was successful with each buffer, HBV was not amplified from any of the negative controls. Increasing the concentration of proteinase K did not increase the sensitivity of the PCR nor did changing the detergent used from SDS to NP40. The inclusion of sucrose and 2 ME increased the sensitivity of the PCR. The results are summarised in Appendix 6, table 3.2(vi)(page 260). These samples were also analysed after heat treatment as described in section 2.6.4. HBV DNA was amplified by nested PCR from all five samples by this method.

Table 3.2(v) A comparison of buffers and conditions for the extraction of HBV DNA from 100 μl serum samples

Sample buffer number	proteinase K (mg/ml)	NaAc	EDTA	SDS	Tris/ HCl pH 7.4	2 ME	Sucrose	NP 40	Sample extraction conditions	
									Time (minutes)	Temperature (°C)
1[A]	0.150	-	12 mM	1.0%	60 mM	200 mM	27%	-	120	57
2[B]	1.0	25 mM	2.5 mM	0.6%	-	-	-	-	120	56
3[C]	2.5	25 mM	2.5 mM	0.5%	-	-	-	-	60	70
4[D]	2	-	12 mM	-	60 mM	200 mM	-	0.45%	120	57

Notes

A Modification of Ljungren and Kidd., (1991)

B Carman *et al.*, (1989)

C Larzul *et al.*, (1988)

D Modification of Ljungren and Kidd ., (1991)

3.2.8.5 Removal of non-specific IgG and IgM rheumatoid factors with RF absorbent™

Five HBsAg-anti-HBe positive serum samples (Appendix 6, table 3.2(vii), page 261) were incubated with RF-absorbent to remove IgM and IgG antibodies (section 2.6.5). One of five was HBV DNA dot blot positive, four of five were HBV DNA dot blot negative and assumed to have low levels of viraemia. The adsorption process was carried out according to the manufacturers instructions. The samples were then digested with sample extraction buffer 1 (Appendix 1), purified by phenol/chloroform extraction (Appendix 2, page 238) and ethanol precipitated (Appendix 2, page 238). The samples were resuspended in 10 μl of TE buffer pH 7.5 and 5 μl of each sample added to the PCR reaction mixture. The PCR was carried out as previously described using core primers 189/188 (Appendix 6, table 2.6(i), page 244) for round 1 and primers 439/666 (Appendix 6, table 2.6(i), page 244) for round 2.

Pretreatment of samples by PEG precipitation was compared with RF adsorption. Both processes were followed by sample extraction and purification as previously described. Samples which had not been pretreated underwent extraction and purification as previously described.

Pretreatment of the samples with RF-absorbent increased the sensitivity of the assay and, with this combination of primers, HBV was amplified in sufficient quantity for visualisation of the product on an ethidium bromide stained agarose gel after 2 rounds of amplification in all five samples analysed. The concentration was such that in all of these samples no further amplification would be required for further analysis of the product. A PCR product was visible in three of five (25 μl sample volume) and four of five samples (100 μl sample volume) that had not been pre-treated. Concentration of the serum by PEG precipitation did not result in an increase in sensitivity of the assay nor an increase in the yield. From these results we concluded that pretreatment of HBsAg-positive, anti-HBe positive serum samples with RF-Absorbent increased the sensitivity of the assay and allowed a smaller volume of sample to be usefully analyzed. The results are summarised in Appendix 6, table 3.2(vii)(page 261).

A further comparison was made in 11 HBsAg-anti-HBe positive samples between amplification of HBV purified from the RF pellet or supernatant from 50 μl of serum.

This was done to ensure that HBV was not lost as complexes precipitated by the RF absorbent. Five samples were positive in both the pellet and the supernatant for HBV, 3 samples were HBV negative in both the pellet and the supernatant, 3 samples were HBV positive in the supernatant only.

3.2.8.6 The amplification of HBV DNA from 1 μl of serum by nested PCR

The aim of this experiment was to determine if HBV could be detected when 1 μl of serum was added directly to the PCR. For this a second comparison of sample preparation methods was carried out. An HBsAg-HBeAg-positive sample was serially diluted in a negative control serum in one series (neat serum). In a second series HBV DNA was extracted from 50 μl of the same dilutions series as described in section 3.2.5. A third series was treated with a lysis buffer containing KCl 50 mM, Tris/HCl pH 7.5 10 mM, $MgCl_2$ 2.5 mM, gelatin 0.1 mg/ml, NP40 0.45% , Tween 20 0.45% (lysed serum)(Higuchi.,1989). Negative control sera and water only controls were included for each procedure. A positive control of 10 copies of pHBV130 was also included.

One microlitre of neat serum,1 μl of purified concentrated HBV DNA (equivalent to 1 μl of serum sample) or 3 μl of lysed serum (equivalent to 1 μl of neat serum) was added directly to the first round reaction. Samples were amplified in a standard nested reaction with surface primers (table 2.6(ii), Appendix 6, page 246) 185/186 in round 1 and primers 187/188 in round 2. The results were analyzed by standard agarose gel electrophoresis in the presence of ethidium bromide.

HBV was amplified from a single copy in neat serum, from > 10 copies in extracted serum and from > 100 copies in lysed serum. The apparent reduction in sensitivity of the purified serum assay may have reflected the loss of HBV during processing. Increasing the volume of purified DNA added to the assay may have restored the sensitivity. The advantage of purified serum is that larger relative volumes of samples can be added because of the concentration step involved in this procedure. The lysis method reduced the sensitivity of the assay even further, this may have been because the concentration of lysis buffer constituents carried over into the PCR reaction inhibited the *Taq* polymerase. This method had no advantages and was not continued. The results for the first and second round reactions are shown in Appendix 6, table 3.2(viii)(page 262).

3.2.8.7 A comparison of PCR sensitivity in neat and heat treated serum

The effects of serum contaminants which are known to inhibit *Taq* polymerase activity (Higuchi PCR Technology) may be negated by boiling the sample with water and including a nested round of amplification. The possibility that this may render complex purification techniques for the majority of serum samples redundant was therefore investigated.

An HBV DNA positive sample which was estimated to contain 10^9 viral genomes /ml was serially diluted in serum known to be negative for all HBV markers. Diluent 1 was freshly prepared clear, non-haemolysed serum, diluent 2 was serum that had been stored at $+4^{\circ C}$ for >1 month and contained a high proportion of solid matter, diluent 3 was a severely haemolysed serum. Each dilution was divided into three aliquots as follows: the first aliquot 20 μl of the sample was heat treated as described in section 2.6.4; the second aliquot contained 20 μl of the sample and was untreated: the third aliquot contained 1 μl of untreated serum. Nested PCR was performed on these samples with surface primer pairs 185/186 and 187/188 and the results analysed by agarose gel electrophoresis and ethidium bromide staining.

The results are summarised in Appendix 6, tables 3.2(ix)(page 263), 3.2(x)(page 264) and 3.2(xi)(page 265). First round products were not detected following the direct addition of either 1 μl or 20 μl of any of the sera. HBV DNA was seen in the first round after heat treatment with both the fresh and particulate samples but not the haemolysed samples. In all cases the sensitivity of the assay was restored to the level where a single viral genome was detected in the second round.

3.2.9 Discussion of results

Lambda 1150 HBVDNA was amplified using primers and amplification conditions recommended by Professor K Murray, Edinburgh University (personal communication). This method allowed the amplification of HBV DNA, but there was a high level of non-specific amplification. However, DNA of the correct size was visible after UV illumination and Southern hybridisation with an HBV specific probe.

PCR amplification was originally carried out on highly purified HBV DNA from HBV DNA dot blot positive samples. There was no inhibition of the amplification reaction due

to carry over of detergent or extraction buffer components as HBV DNA amplified equally well from neat samples and samples that were diluted tenfold. The sample preparation method was simplified by omitting ultracentrifugation, whilst retaining phenol/chloroform purification and ethanol precipitation. HBV DNA was successfully amplified from samples prepared in this way. Pretreatment of samples with RF absorbent allowed the more efficient detection of HBV DNA in HBsAg-positive-anti-HBe positive samples and reduced the volume of serum required for analysis. The inclusion of SDS and 2-ME in sample extraction buffer was important for successful sample extraction at 37 °C.

When the assay was optimised so that HBV DNA could be amplified from ten copies of control material after nested or double nested PCR, small volumes of serum (1 μl) were added directly to the PCR reaction mixture. The absolute sensitivity of this method was still greater than 10 000 copies/ml of HBV DNA.

The volume of serum that could be analysed without treatment with buffers or phenol/chloroform purification was increased to 20 μl by the boiling method. Vandenvelde *et al.*, (1993) determined that the inhibitory effect of denatured serum albumin could be removed by the inclusion of sodium octanoate to the sample during heat treatment. This group used only a single round of PCR and found that PCR carried out directly on serum was ten fold less sensitive than on phenol/chloroform extracted samples. Our findings confirm this. However, the application of nested PCR removed this discrepancy. Phenol/chloroform extraction does offer the advantage that HBV DNA can be concentrated from a larger sample volume.

Lou *et al.*, (1993) described the treatment of cervicovaginal lavage samples by microwaving or boiling in a heat block prior to amplification. Boiling of 100 µl samples in a thermal cycler for ten minutes was found to be less sensitive than heating for twenty minutes or microwaving. We did not increase sensitivity when samples were boiled for twenty minutes rather than 10 minutes in a water bath. This may have been due to several factors. In our study the water bath was already boiling when the samples were placed in it, smaller sample volumes were used and a heating block is unlikely to reach or remain at boiling point. In our hands the heat treatment method of sample preparation was equally or more sensitive than all the other methods described.

The concentration of $MgCl_2$ in the PCR reaction buffer was not critical. The reaction proceeded equally well between 1 and 3mM. At concentrations of more than 3 mM $MgCl_2$ non-specific amplification occurred. This feature was common to all of the primer pairs. The inclusion of a biotinylated primer did not effect the sensitivity of the reaction. The overall sensitivity of the PCR was improved by increasing the stringency of the assay so that the annealing temperature was closely related to the temperature of dissociation of the primers.

The sensitivity of the reaction was improved between 10 and 100 fold by the introduction of the nested PCR, which also improved the specificity of the assay. The success of this method was influenced by the size of the fragments being amplified; where the first round product was very large (eg 2.7 kb), a third (double nested) round of amplification was introduced. This gave a higher yield of product for further analyses eg sequencing.

The importance of a highly sensitive method for the detection of low levels of HBV DNA, such as PCR, has been emphasised by many groups. The detection of HBV DNA in individuals whose HBV serology indicated a lack of viral replication was shown by Theilmann *et al.*, (1989). In that study HBV DNA was detected by HBV DNA dot blot assay in two of twenty four HBeAg negative patients but by PCR in twenty one of those patients. As previously discussed Thiers *et al.*, (1988) detected HBV DNA in the serum of hepatitis-B-seronegative subjects. Both of these studies used a single round of amplification followed by Southern hybridisation analysis.

The requirement for the confirmation of PCR results by Southern hybridisation analysis was largely removed by the development of nested PCR. In early studies Southern analysis was important for the confirmation of product specificity and was also used to boost the sensitivity of the assay. The double hybridisation involved in the nested round of amplification helped to ensure specificity and increased the sensitivity of the assay. The nested round of amplification diluted any inhibtory agents which may remain in the first round PCR mixture.

The direct analysis of PCR product sequences represented an important advance over conventional methods of template preparation allowing large numbers of samples to be studied. This method was particularly relevant when analysing samples with a very low

viral titre.

Amplified DNA that is suitable for sequencing must be relatively abundant and pure. Excessive thermocycling may produce non-specific high molecular weight products and reduce the yield of the specific product ((Bell and DeMarini, 1991). We avoided this problem by reducing the number of cycles per round. Increasing the primer concentration from 50 pmol to 100 pmol retained the product specificity for more cycles (Bell and De Marini, 1991). A similar optimum concentration of primer was found in our study. One hundred pico moles of primer was used in the nested round of amplification.

The presence of *Taq* polymerase induced errors must also be controlled for in direct sequencing studies. The lack of an enzyme mediated proof reading function means that any errors will become incorporated and amplified. This potential problem was overcome by amplifying and sequencing all samples in duplicate. Almond *et al.*, (1992) addressed the question of sequence fidelity in a study of multiply amplified and cloned SIV sequences. This group showed that the analysis of 'quasi-species' populations by PCR was reliable and reproducible.

The method was therefore suitable for the analysis of patient derived samples and was used in studies described in this thesis.

3.3 Optimisation of PCR product sequencing

3.3.0 Objectives

- To optimise existing methods of template preparation so that HBV DNA amplified by PCR could be sequenced directly

- To assess the relative merits of sequencing a single and a double stranded template by the Sanger dideoxy termination method.

3.3.1 Introduction

Prior to PCR, nucleotide sequencing required the cloning of a target sequence in to a suitable vector to produce sufficient template DNA. In many cases template DNA was cloned into the single-stranded filamentous bacteriophage M13 which, when grown in *E. coli*, both amplified the sequence and produced a single stranded template. Pairs of vectors in which cloning sites hadopposite polarities enabled both strands of a double-stranded template to be cloned and analysed. The advent of PCR has, in many cases, avoided the need for cloning as sufficient DNA template was produced for direct sequencing.

The method for sequencing with chain terminating deoxynucleotide analogues (Sanger *et al.*,1977) was used for all the work described in this thesis. The chain terminating method requires the synthesis of a specific single stranded template. Template specificity was achieved by optimisation of the PCR procedure as described in section 3.2.

In this method a specific oligonucleotide probe was hybridised to the template. The probe was then labelled with an appropriate radiolabelled nucleotide, in our study ^{35}S dATP was used. The probe was elongated with nucleotides that were complementary to the sequence in four termination reactions. Each reaction included a quarter of the template/probe mixture, all four nucleotide bases and one dideoxy nucleotide. The incorporation of the dideoxy nucleotide at the growing end of the oligonucleotide chain prevented any further chain extension. The nucleotide sequence of the template was then deduced from the relative lengths of the newly synthesised DNA fragments.

Both double stranded and single stranded sequencing rely on the process described above.

However, for double stranded sequencing the second strand of the template was not removed and care was taken to avoid the template reannealing. In single stranded sequencing the second strand was removed before the probe was added to the template and did not interfere with the sequencing reaction.

3.3.2 Double stranded sequencing

Sequencing was initially carried out as described in the section 2.9 with surface primer 187 (Appendix 6, table 2.6(ii), page 246) using the reagents as described by Tabor and Richardson, (1988) supplied in the Sequenase 2^{TM} kit (United States Biochemical Corporation, Cleveland, Ohio). The ratio of primer to template (200 ng) concentration was approximately 10/1. Ten percent NP 40 was added to the anneal mix.

The quality of the sequencing was poor. Less than 50 bases of the sequence were readable and random termination made sequences very difficult to read. To improve the sequence clarity and increase the number of readable bases the ratio of primer to HBV DNA template was altered. Three sample concentrations were compared (100 ng, 50 ng, 25 ng) with 50 ng of primer 187. All other reaction conditions were constant. The primer template ratios were 10/1, 20/1 and 40/1 respectively.

The quality of the sequences was improved in all the above reactions with a mean of 80 bp (range 65 bp-90 bp) of a 123 bp template being read in all reactions.

Random termination was decreased at all three template concentrations. The primer template ratio within these limits did not effect the quality of the sequence. Reducing the absolute concentration of HBV DNA template improved the sequencing results. 100 ng of product was used in all subsequent analyses of DNA templates <150 bp long.

3.3.3 A comparison of two additives (DMSO, NP40) in sequencing anneal mixture

To further decrease premature termination of the sequencing reaction due to secondary structure formation, a comparison of the effect of two additives, 10% DMSO (Winship,1989) and 0.5% NP40 (Bachmann *et al.*,1990) was made.

Two concentrations of template (25 ng, 100 ng HBV DNA) were used with each agent in an otherwise standard sequencing reaction. NP40 did not improve the sequencing at

either template concentration, however the combination of 100 ng of HBV template with 10% DMSO eliminated premature termination. 97 bases of the possible 123 were sequenced under these conditions.

3.3.4 Comparison of sequencing reactions carried out on a template of 551 bp and a template of 150 bp.

One hundred nanograms of a 551 bp HBV DNA template was sequenced under the above conditions with primer 664. The sequencing failed. Less than 50 bp were discernable and premature termination prevented accurate reading of the sequence.

This may have been due to the difference in the number of DNA copies/ng for longer fragments of DNA as compared to that for shorter templates. This altered the effective molar ratio of the template to the primer and compromised the sequencing reaction.

3.3.5 Modification of the method for sequencing templates >500 bp

The concentration of template was increased to 200 ng and the concentration of primer to 50 ng. This reduced the primer/template ratio to approximately 10/1 as compared to 146/1 when 25 ng of template and 100 ng of primer were used. This improved the sequencing dramatically by preventing premature termination and allowed 200 bases of the sequence to be read.

3.3.6 Comparison between Sequenase™ labelling and termination mixes and modified versions of these solutions.

Modified Labelling mixture (L Mix B, Appendix 1) and termination mixtures (ddNTP Mix B, Appendix 1) were used in an attempt to increase the number of readable nucleotides in each sequencing reaction, particularly nucleotide regions that were close to the primer.

These modifications improved the sequences both qualitatively and quantitatively and were used in all subsequent analyses.

3.3.7 Single stranded sequencing of PCR products

Single stranded sequencing required the purification and separation of the double stranded PCR product. The removal of one strand of the template DNA prevented the sample reannealing and premature termination of the sequencing reaction. Variation in the quality

of the sequencing with the double stranded method may have been due to the purification process used to prepare template DNA. Standardisation of sample purification with a biotinylated solid phase to capture the PCR product combined with single stranded sequencing was used to increase the length of readable sequence, particularly in templates of >200 BP.

3.3.8 Modification of the recommended Sequenase™ protocol (USB) for single stranded sequencing using magnetic beads as a solid support (modification of Hultman et al.,1989)

The biotinylated PCR product was made single stranded as described in section 2.10.1, the template was resuspended in 20 μl of water and 5 μl of the sample used for sequencing. The recommended protocol for single stranded sequencing with Sequenase™ was modified for use with a biotinylated PCR product.

The primer annealing step was carried out over 5 minutes at 70°C and the reaction allowed to cool to room temperature over 30 minutes in a Techne PHC-3 Thermocycler. Primer labelling was carried out for 5 minutes at room temperature and the termination reaction at 50°C for 5 minutes. The sequencing solutions used were the same as those as for double stranded sequencing.

3.3.9 Discussion of results

Sequencing was carried out on either a gel purified or a biotinylated PCR product. The quality of double stranded sequencing of DNA fragments greater than 550 bp long was critically dependent on the ratio of template to primer (10 to 1); the presence of 10% DMSO in the annealing and termination mixtures and the precise timing of the reaction steps. Single stranded sequencing of biotinylated template did not require such accurate timing. The slow cooling of the anneal mixture allowed the maximum amount of probe to bind to the template which ultimately improved the quality of the sequencing.

A problem with some DNA sequences is the formation of secondary structures due to G-C rich areas. The formation of secondary structure was characterised by the appearance of 'four track stopping', where a band was seen in all four lanes of the autoradiograph, or compressions. Whilst the areas sequenced in this study were not heavily G-C rich the inclusion of deoxy-7-deazaguanosine triphosphate (7-deaza-dGTP), which forms weaker

secondary structures, improved sequence clarity.

The enzyme *Taq* polymerase was used by Innis *et al.*,((1988) for sequencing. The thermostability of this enzyme allowed the labelling reaction to be carried out at 37°C and the termination reaction to be carried out at 70°C for three minutes. Innis *et al.*,((1988) suggested that the higher temperature would prevent secondary structure formation and allow sequencing through G-C rich areas. In experiments carried out in this thesis (data not shown) the intensity of the signal on the autoradiograph was severely reduced and the method was not pursued.

Bachmann *et al.*,(1990) suggested that the addition of detergents to the annealing and termination reaction mixtures (0.5% Tween 20 or 0.5% NP-40 or 10% DMSO) may prevent short templates reannealing. The addition of 10% DMSO in that study reduced the intensity of the signal on the autoradiograph. However, the experiments carried out in this thesis did not confirm this and 10% DMSO was added to all sequencing reactions.

The addition of sodium acetate to the polyacrylamide gel running buffer created an electrophoretic gradient which caused the shorter DNA fragments to migrate more slowly through the gel in relation to the larger fragments. This allowed nucleotides to be analysed close to the 3' end of the sequencing primer. Pre-heating the gel before loading the samples also produced clearer results. Samples were loaded directly to the pre-warmed gel from the heating block on which they had been incubated at 80 °C for five minutes. This helped to prevent sample reannealing.

Sequences were clear and easily read with subpopulations of approximately 10% visible on the autoradiograph. Sequences of up to 250 bases were analysed in one reaction, allowing the analysis of complete PCR products. The opposite strand was sequenced to confirm results where compressions occurred or sequences were unclear.

3.4 Modification of a point mutation assay for the detection of single nucleotide substitutions in HBV.

3.4.0 Objectives

- To modify the Point Mutation Assay (Kaye *et al.* 1992) for the detection of mutations in codons 1 and 28 of the HBV precore region and codon 145 of the surface gene

- To produce a sensitive, quantitative assay for the detection of subpopulations in a mixture of viral genotypes

3.4.1 Introduction

Naturally occurring single nucleotide mutations which may have a profound effect on the expression of HBV antigens have been identified. These mutations have been found in the precore region at codon 1 (Okamoto *et al.*,1990) and codon 28 (Carman *et al.*,1989). Sequence variation in the surface region has been associated with subtype variation between *d* and *y* at codon 122 and *w* and *r* at codon 160 (Okamoto *et al.*,1987). Nucleotide variation in the A epitope of the surface region between codons 124 and 147 has also been associated with vaccine induced escape mutants (Carman *et al.*,1990).

A common feature of these mutations is their occurrence in regions which are otherwise highly conserved. Sequencing the region gives information about the presence or absence of the mutation but does not allow accurate quantification in cases of a mixed population and may lack sensitivity.

Syvänen *et al.*, (1990) described primer guided nucleotide incorporation for genotyping Apolipoprotein E (Apo E). Genetic polymorphisms within Apo E have been associated with variations in lipoprotein metabolism. Syvänen *et al.*, (1990) demonstrated that three forms of Apo E (E1, E2,E3) could be distinguished by nucleotide incorporation. In this assay the appropriate region of the genome was amplified by PCR. The amplified product was biotinylated by the inclusion of a biotinylated primer. This allowed the product to be captured onto streptavidin coated polystyrene particles or microtitre wells, made single stranded and annealed to a primer. Primer extension was carried out directly on the particles or in the wells. The results of Syvänen's study correlated well with those of

phenotypic analysis.

Kaye *et al.*, (1992) made significant improvements to this method. The exclusion of cold nucleotides in the primer extension mixture reduced levels of background incorporation when using Klenow DNA polymerase. Klenow polymerase had several advantages over both *Taq* polymerase and T7 DNA polymerase. It was cheaper than both and more stable than T7 polymerase. Nucleotide incorporation was shown to be lower with *Taq* polymerase. Consequently low levels of sensitivity in the PMA were achieved. The use of Klenow DNA polymerase did not result in higher levels of non-specific incorporation when compared with T7 DNA polymerase (S Kaye personal communication).

The availablity of all four radiolabelled nucleotides ([^{35}S] dATP. dCTP. dTTP, dGTP) widened the scope of the assay as compared with Syvänen *et al.*, (1990) and allowed a more accurate determination of levels of non-specific incorporation.

The application of this method to HBV is described below. The method was applied to mutations at codons 1 and 28 of the precore region and codon 145 of the surface region.

3.4.2 Results

The point mutation assay was used as described by Kaye *et al.*,1992 (section 2.11). Biotinylated HBV DNA was produced by PCR, 1200 ng/ml of both the biotinylated and the non-biotinylated primer were used in the second round of the PCR. This concentration was used for all primers in the amplification of DNA analysed by PMA. The levels of non-specific signal were determined by the amplification of HBV DNA from a single molecule.

This ensured that only one nucleotide could be incorporated correctly in a clonal amplification reaction. Any incorporation of the three alternative nucleotides would be due to either, incorporation errors during amplification, non-specific incorporation or inefficient removal of unincorporated nucleotides during the PMA. The levels of background incorporation in the assay of nucleotides 1 and 28 of the precore region with probes PMA 1, PMA 28a and PMA 28b were low (<2% of the total counts per minute per sample). A negative control sample and a positive control sample (single copy) were included in the PCR reaction and run along side the test samples.

3.4.3 Determination of the sensitivity and specificity of the PMA

The sensitivity of the PMA was determined by construction of a standard curve for a published mutation at codon 28 of the precore region. Mixed populations were made containing known relative concentrations of wild type and mutant virus, these were then PCR amplified. The PCR product was then assayed by PMA, the primer used in this case was the antisense primer which allowed the detection of the point mutation TGG-TAG. The antisense primer incorporates the complementary labelled nucleotide, in this case **C** where the wild type sequence exists (TGG) and **T** where the mutant type sequence exists (TAG).

The results correlated well with those obtained by Kaye *et al.*,1992 and showed that sub-populations of 5% were clearly detectable. Sub-populations of between 2 and 5% were detectable in most assays (table 3.4(i)).

The sensitivity of the assay was established with each probe and found to be similar for all mutations.

Table 3.4(i) Point Mutation Assay standard curve: codon 28 of the HBV precore region

Percentage of mutant type virus added	Incorporated nucleotide	Mean % nucleotide detected	Standard Deviation	Range of percentages of nucleotide detected	
				Max value	Min value
1%	G	0.66	0.42	1.5	0.3
	A	0.35	0.3	0.8	0
	T	1.2	0.141	1.4	1
	C	97.6	0.69	98.2	96
2%	G	0.59	0.84	2.4	0
	A	0.05	0.52	1.6	0
	T	2.0	0.92	3.9	1
	C	96.8	1.3	98	94.5
5%	G	0.26	0.19	0.6	0
	A	0.66	0.74	2.3	0
	T	5.35	1.18	6.8	2.9
	C	93.73	1.26	96.3	92

Table 3.4(i) continued **Point Mutation Assay standard curve: codon 28 of the HBV precore region**

Percentage of mutant type virus added	Incorporated nucleotide	Mean % nucleotide detected	Standard Deviation	Range of percentages of nucleotide detected	
				Max value	Min value
10%	G	0.95	0.51	1.6	0.2
	A	0.45	0.37	1.1	0
	T	12.46	1.55	15.7	10.6
	C	86.16	1.74	88.1	82
25%	G	1.85	2.039	6.2	0.2
	A	0.48	0.39	1.0	0
	T	31.06	5.60	39	24.4
	C	66.33	5.70	74.4	59
50%	G	0.79		1.2	0
	A	0.40		0.5	0.3
	T	53.98		5.6	51.4
	C	44.58		46.9	42

3.4.4 Discussion of results

The point mutation assay was used essentially as described by Kaye *et al.*,(1992). The sensitivity of the HBV PMA was determined for each probe by mixing two different genotypes. This varied between 1% and 3% for all of the probes assayed.

Several strategies for the analysis of point mutations in a nucleotide sequence have been developed. Benjelloun *et al.*, (1993) studied mutations at codons 28 and 29 of the HBV precore region using differential hybridisation. The significance of these mutations will be discussed later. The mutations were accurately detected by this method. However, the procedure required three hybridisation reactions on each sample and was not quantitative.

Norder *et al.*,(1990), used a method very similar to the amplification refractory mismatch system (ARMS) described by Newton *et al.*,(1989) to determine the genotype of HBV. Subtype variation of HBV is linked to single nucleotide changes in the S gene. The subtypes were discriminated by amplification or non-amplification with primers specific for each subtype. Lo *et al.*,(1992) used a similar system to analyse mutations of the precore region. Quantification was by comparative ethidium bromide fluorescence and did not provide accurate information on subtypic proportions.

In both of these studies (Norder *et al.*,1990 and Lo *et al.*,1992) samples were found which did not hybridise to any internal primers. This phenomenon occurred wherever the nucleotide substitution was different to that expected or where there was additional variation around the 3' end of the primer.

The methods described above did not provide accurate quantitative information where there were mixed populations nor did they identify all possible nucleotide substitutions. The competitive oligonucleotide priming (COP) reaction (Gibbs *et al.*,(1989) did not significantly overcome these problems. The method relied on the hybridisation of specific probes which only identified two specific, alternative mutations. Yotsumoto *et al.* (1990) applied a method involving the single track sequencing of cloned PCR products to HBV subtyping. This method was quantitative for one genotype substitution but not practical for large numbers of analyses.

The requirement for qualitative and quantitative analysis of single nucleotide substitutions was first addressed by Sokolov (1989), who demonstrated the accurate detection of single nucleotides in a known sequence near the 'Cystic fibrosis gene'. The sample was divided between four PCR reaction mixes. One of four α^{32}P dNTP's was added to each one. Two primers were added to the mixture , a 20mer and a 30mer, which hybridised upstream of the two points of interest. The sample was amplified over 30 rounds, the product was electrophoresed and analysed by autoradiography. The 30mer was converted to a 31mer only in the PCR mix with α^{32}P dGTP and the 20mer to 21mer with the labelled dCTP as expected. Comparison of the band intensity on the autoradiograph made this method semi-quantitative and identified all possible substitutions. The use of α^{32}P made this method unsuitable for routine use on a large scale.

Syvanen *et al.* (1990), first described the method of point mutational analysis used in this thesis. The method was both quantitative and qualitative and used low specific activity isotopes or non-radioactive makers. The assay format was improved by Kaye *et al.* (1992) so that it was performed in a 96 well microtitre plate. This facilitated the analysis of large numbers of samples.

The method was applied succesfully to HBV in clinical settings. The correlation between sequencing and PMA results in later studies showed that the PMA was sufficiently reliable to be used as a diagnostic tool without additional confirmatory procedures.

3.5 A cross sectional survey of HBV DNA in serum from HBV carriers.

3.5.0 Objectives

- To validate the HBV DNA dot blot assay as a measure of viral titre (section 3.1) by comparison with the established HBV DNA polymerase assay

- To determine the degree of concordance between HBV DNA assay by dot blot and DNA polymerase

- To determine the range of levels of HBV replication in HBV carriers attending the Middlesex Hospital hepatitis clinic

- To determine the effect of coinfection with HIV 1 on the level of HBV replication in chronic HBV carriers.

3.5.1 Introduction

The risk factors for the acquisition of HIV 1 and HBV infections among homosexual men are similar. Concurrent infection with the two viruses is relatively common. Taylor *et al.*,(1988) found that 56% of a cohort of homosexually active men who showed current or past infection with HBV were anti-HIV positive. Weller *et al.*,(1986) found a similar HIV prevalence (60%) in a cohort of HBeAg positive homosexual men.

In chronic HBV-carriers the level of viral replication correlates with the presence of detectable HBeAg in serum. Studies of HIV co-infection in HBV-carriers have examined the natural history of viral replication, determined by the rate of loss of HBeAg positivity. A trend towards the continuation of active HBV replication in HIV positive patients has been seen in previous studies (Weller *et al.*,1986, Krogsgaard *et al.*,1987).

We have studied the effect of HIV infection in chronic HBV carriers on the rate of spontaneous loss of serum HBeAg and other markers of HBV replication.

3.5.2 Patient population

The patients described in this study were hepatitis B carriers attending the hepatitis clinic at the Middlesex Hospital. All were diagnosed as having a chronic HBV infection

(HBsAg positive for greater than 6 months). This work formed part of a larger study on the interactions between HIV 1 and HBV in homosexual men (Gilson *et al.*, in prep).

3.5.3 Serological methods

All assays for hepatitis B markers (HBsAg,HBeAg/anti-HBe and anti-HBc) were carried out by colleagues in the diagnostic division. Anti-HIV 1 was detected by competitive EIA (Wellcozyme).

3.5.4 HBV DNA Analysis

HBV DNA was analysed in serum by HBV DNA polymerase assay (section 2.2.1) HBV DNA dot blot assay (sections 2.3 and 2.4). All of the samples were prepared as previously described with NP40 and 2 ME treatment. All analyses were carried using the Statistical Analysis System (SAS) software. The standard deviations were calculated at 95% confidence intervals. Chi-squared and students t-Test were employed for comparative analyses.

3.5.5 Results

All of the specimens tested were HBsAg positive, 48 were HBeAg positive, 13 were anti-HBe positive and in 8 neither HBeAg or anti-HBe were detectable.

Each sample was tested in duplicate for HBV DNA and DNAp. To quantify DNA in samples, a titration curve of probe against itself (unlabelled) was constructed (Appendix 6, table 3.5(i), page 266). The DNA content of positive and negative control specimens were determined against this curve during the same hybridisation experiment. These positive control samples were included on every filter for direct comparison both between filters and between different hybridisation experiments carried out under the same conditions. The mean concentration of DNA in the positive control sera used was 2.6 ng/ml.

Samples were deemed positive if the densitometric analysis indicated a level of HBV DNA present that was equal to or greater than the mean value + 3 SD of the negative control samples. The overall results indicated that, of 69 specimens tested, 56 were positive for HBV DNA (81%) whilst 49 were positive for HBV DNA polymerase (71%).

These data were then correlated with HBeAg/anti-HBe markers (table 3.5(ii)).

Calculation of HBV DNA concentration

A 25 μl aliquot of the positive control serum contained a mean of 65 pg/25 μl aliquot serum. Therefore the positive control contained 2600 pg of DNA/ml. Each sample was related to that as a ratio to calculate pg HBV DNA/ml. e.g.

$$\frac{\text{sample value of specimen 1 on filter 1} = 674931}{\text{mean positive control value on that filter} = 790251}$$

Therefore the specimen contained 0.854 x the concentration of HBV DNA in the positive control.

i.e. 0.854 x 2.6 = 2.21 ng HBV DNA /ml

Table 3.5(ii) Results of a cross sectional study to determine the presence of HBV DNA in serum samples from HBsAg positive hepatitis B carriers by HBV DNA dot blot and HBVDNA polymerase

	HBV DNA positive	HBV DNA negative
HBV DNA polymerase Positive	44 HBeAg positive 3 HBeAg negative	1 HBeAg positive 1 HBeAg negative
HBV DNA polymerase negative	3 HBeAg positive 6 HBeAg negative	0 HBeAg positive 11 HBeAg negative

These specimens have all been repeatedly tested. Where HBV DNA was present at levels close to the cut-off value in the assay the test was repeated. Repeat testing was also carried out on samples where non specific hybridisation close to the sample made interpretation of the blot inaccurate.

HBV DNA was detected in all but one of the HBeAg positive samples, 44 of these samples were positive for both HBV DNA and HBV DNA polymerase. HBV DNA only was detected nine samples, three were HBeAg positive and six were HBeAg negative. Seven of those samples would not have been detected had the autoradiographs been analysed by eye alone. The two samples which would have been detected were HBeAg negative samples. This was unusual as HBeAg negative samples would be predicted to have lower levels of HBV DNA than HBeAg positive samples. HBV DNA and HBV DNAp was detected in three HBeAg negative samples. Neither HBV DNA nor HBV DNAp was detectable in eleven HBeAg negative samples (table 3.5(ii)).

The proportions of HBeAg positive and negative sera in which HBV DNA was detected were similar for both HIV positive and negative groups. However, the levels of detectable HBV DNA and HBV DNAp were higher in the HIV positive group. The difference was just significant for HBV DNAp ($p=0.043$) but did not quite achieve levels of significance for HBV DNA ($p = 0.07$) . These results are summarised in table 3.5(iiia) and table 3.5 (iiib).

HBsAg levels were lower in HBeAg positive, anti-HIV 1 positive patients than in HBeAg positive, anti-HIV 1 negative patients table 3.5(iiic). Alanine transaminase levels were higher in HIV negative carriers than in HIV positive carriers (table 3.5(iiic)). However, the differences were not statistically significant. The study also showed that, with respect to HBeAg status there were significantly lower levels ($P=0.01$) of HBV DNA in HBeAg negative samples than in HBeAg positive samples (figures 3.5(i) and 3.5(ii)).

This gave a strong positive correlation between HBV DNA and HBV DNA polymerase levels ($r=0.666$ ($p<0.001$)). When the data was compared for mean values of these two parameters there was also a trend towards higher levels of both HBV DNA and HBV DNAp in HIV positive samples. The confirmed result is represented in the final analysis. The assay data for individual samples is shown in Appendix 6, table 3.5(iv)(page 265).

Table 3.5(iiia) **HBV DNA analysis in HBV carriers: anti-HIV positive and anti-HIV negative patients compared**

	Number	Mean (ng/ml)	Standard deviation	P value
HBeAg positive HIV positive	28	2.07	1.7	0.07[1]
HBeAg positive HIV negative	20	1.41	1.31	
HBeAg negative HIV positive	10	0.5	0.48	0.36[1]
HBeAg negative HIV negative	11	0.25	0.14	

Notes
1 Student t-test

Table 3.5(iiib) **HBV DNA polymerase analysis in HBV carriers: anti-HIV positive and anti-HIV negative patients compared**

	Number	Mean (cpm/200 μl)	Standard deviation	P value
HBeAg positive HIV positive	28	413	378	0.043[1]
HBeAg positive HIV negative	20	245	156	
HBeAg negative HIV positive	10	65	154	0.41[1]
HBeAg negative HIV negative	11	8	15	

Notes
1 Student t-test

Table 3.5(iiic) **Virological test results in HBV-Carriers: anti-HIV positive and anti-HIV negative patients compared.**

	HIV negative	HIV positive	p value
HBeAg seropositivity Number (%)	23/43 (53.3)	48/61 (78.7)	0.012[1]
HBeAg seropositivity: Samples assayed for HBV DNA and HBV DNAp only Number (%)	14/29 (48.2)	28/36 (77.7)	NS[1]
Serum HBsAg x 10^3 ng/ml: mean (SD)			
HBeAg positive sera	237 (682)	147 (133)	0.55[3]
HBeAg negative sera	16 (13)	9 (8)	0.09[3]
Alanine transaminase[2] IU/L: mean (SD)	125.7 (76.7) n=23	110.0 (101.9) n=26	0.5[4]

Notes

1 Chi-squared test

2 Including only HBeAg seropostive patients

3 Student t-test

4 Calculated from the difference between the meanvalue of each group

Figure 3.5(i) A comparison of HBV DNAp and HBV DNA levels in samples from HBeAg-positive-HIV-positive and negative hepatitis B carriers

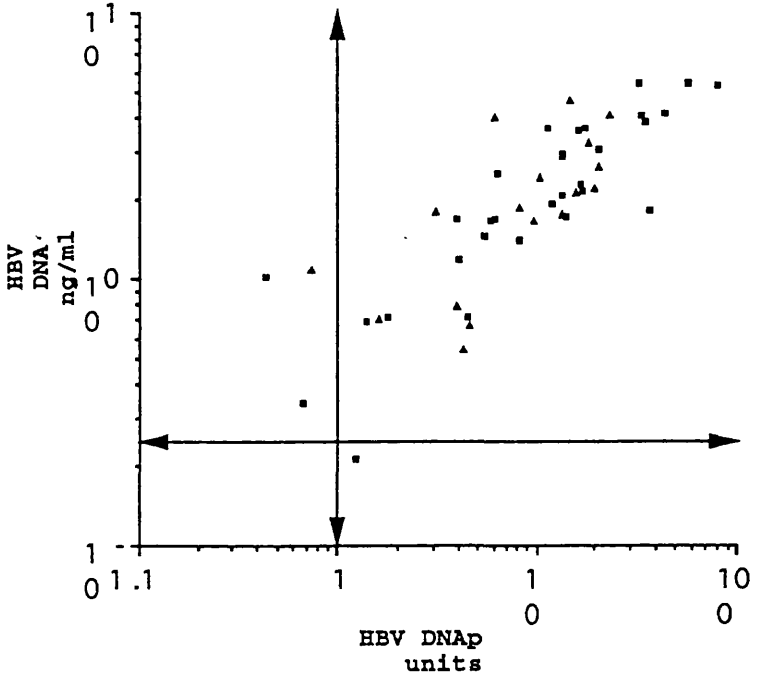
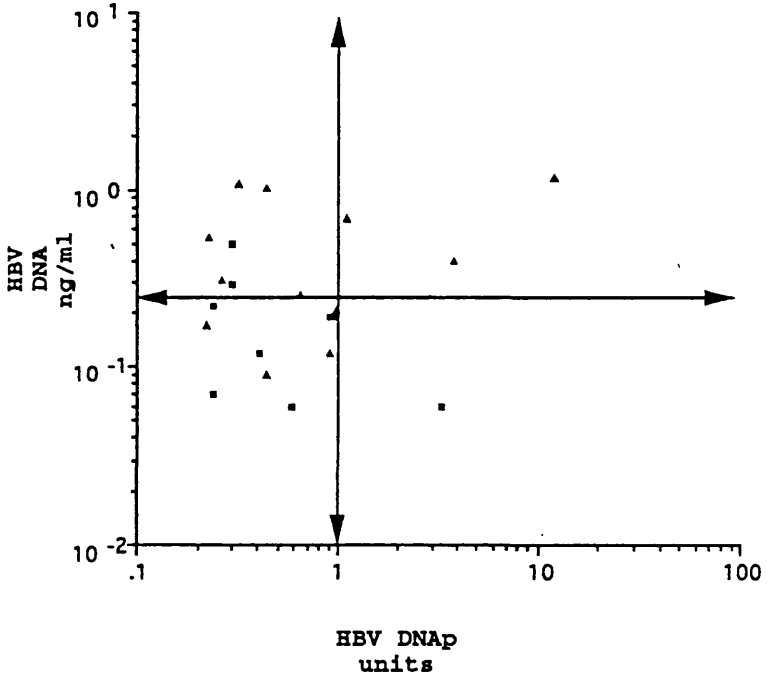


Figure 3.5(ii) A comparison of HBV DNAp and HBV DNA levels in samples from HBeAg-negative-HIV-positive and negative hepatitis B carriers



KEY

▪ HIV-positive samples

▲ HIV-negative samples

Negative cut-off value

HBV DNAp = 1 unit
HBV DNA = 0.269 ng/ml

3.5.6 Discussion of results

In this study we have examined the effect of coinfection with HIV 1 on the natural history of HBV replication. We have confirmed the finding of previous studies that coinfection with HIV and HBV is associated with increased levels of hepatitis B replication. The levels of both HBV DNA and HBV DNA polymerase were compared for HIV positive and negative HBeAg positive patients only using a student t-test. We have shown that HIV infection is associated with higher levels of HBV DNA polymerase activity in HBV carriers. Although the level of HBV DNA was higher in HIV positive patients it did not quite reach levels of significance. Krogsgaard *et al.*, (1987), in a smaller study, found a significant difference in HBV DNA levels in HIV positive and HIV negative HBV carriers. This study included both HBeAg and anti-HBe positive patients in a single analysis. This may have created a bias in the analysis of results as HIV positive patients may be more likely to be HBeAg positive than HIV negative patients and therefore have higher levels of HBV DNA.

The enhancement of viral replication in HIV infected carriers is accompanied by a decrease in inflammatory liver disease. In our study this was assessed indirectly by the level of alanine transaminase (ALT). In comparison with HIV negative HBV-carriers, ALT levels were lower in anti-HIV positive-HBeAg positive carriers but this was not statistically significant. This corresponded with the results of Krogsgaard *et al.*,(1987).

Monno *et al.*,(1991) examined the same parameters in a group of Italian HBsAg chronic carriers and could not demonstrate any modification of HBV or HDV infections by HIV. This discrepancy may be due to the group of patients studied. Only 8% of the group were homosexual men whilst 83.3% were intravenous drug users. Coinfection with HDV was common in both HIV positive and negative groups. This may have depressed HBV replication in the HIV positive group as HBV gene expression is inhibited by HDV infection (Rizzetto *et al.*,1985). HDV is less commonly associated with sexually acquired HBV and is rare in UK patients.

In earlier studies a significantly higher HBsAg carrier rate developed in HIV positive men who were then infected with HBV (Taylor *et al.*,1988). Whilst this was not related to the development of HBV related disease or jaundice it may be relevant to long term sequelae for example hepatocellular carcinoma.

In our study there were significantly fewer HIV positive, anti-HBe positive patients than HIV negative, anti-HBe positive patients indicating a reduction in the rate of loss of serum HBeAg in anti-HIV positive patients, implying a failure to clear sites of active replication. This may have been due to reduced immunological activity in HIV positive patients as indicated by the presence of lower ALT levels in that group.

There are conflicting reports of the effect of HBV on HIV disease progression. The rate of HIV replication and therefore T-cell depletion in HIV infected individuals may be related to *de novo* cellular protein synthesis in the infected cells Twu *et al.*,(1987). Thus, if HIV viral synthesis is further increased in cells that are infected with HBV accelerated T-cell depletion may occur. Evidence to support this theory was provided by Eskild *et al.*,(1992), who determined that evidence of past infection with HBV was associated with more rapid progression to AIDS. Taylor *et al.*,(1988) found that whilst there was a significant association between anti-HIV and HBV markers there was no evidence for accelerated progression. In our study there was no effect on HBV on progression to AIDS (data not shown).

The data presented here indicate that HIV may reduce inflammatory liver disease in HBV infection but increase or prolong hepatitis B viral replication.

3.6 No evidence for an effect of zidovudine on hepatitis B virus replication in homosexual men with symptomatic HIV infection (Gilson *et al.*,1990)

3.6.1 Objectives

- To determine the effect of zidovudine treatment on HBV replication by comparing levels of HBV replication over time in patients undergoing zidovudine treatment with those in a control group of untreated patients

3.6.2 Introduction

The replication cycle of hepatitis B includes a reverse transcription step (Summers and Mason, 1982). Zidovudine is a reverse transcriptase inhibitor licensed for the treatment of human immunodeficiency virus (HIV 1) infection.

Zidovudine triphosphate inhibited the HBV DNA polymerase (HBV-DNAp) *in vitro* in a liquid phase HBV-DNAp assay at concentrations of approximately 0.05 μM to 0.1 μM (Nordenfelt *et al.*,1987, Berk *et al.*,1992). Inhibition was shown to be equally effective for virus of either *ad* or *ay* and calculated 50% inhibition constants varied between 0.2 μM and 0.3μM in these studies. The *in vitro* evidence of an inhibitory effect of zidovudine on HBV replication was an indication that there may be an *in vivo* effect.

The effect of zidovudine, prescribed routinely for the treatment of symptomatic HIV 1 infection, on markers of HBV replication in a group of HIV seropositive chronic HBV carriers was therefore studied.

3.6.3 Patient population

Fourteen consecutive male homosexuals with chronic HBV infection were studied who were receiving zidovudine therapy for symptomatic HIV infection. Chronic HBV infection was defined by HBV surface antigen (HBsAg) seropositivity for at least six months. The median age of the patients was 32 years (range 28-48).

The median documented duration of HBV carriage was 39 months (range 6-74) prior to starting treatment, median duration of HIV 1 seropositivity was 36 months (4-74). Three patients had known HIV 1 seroconversion dates (duration of infection 34, 38 and 61 months respectively taken from the mid point of the date of the last HIV negative to the

first HIV positive sample). Patients began treatment between August 1987 and January 1989.

The clinical indications for treatment with zidovudine were the onset of any Centers for Disease Control (CDC) group IV disease. Indications for classification were as follows:

Pneumocystis carinii pneumonia (PCP)	Three patients
Kaposi's sarcoma (KS)	One patient
KS and PCP	One patient
KS, PCP and cytomegalovirus (CMV)	One patient
Oral candida	Three patients
Oral candida and hairy leukoplakia	One patient
Constitutional symptoms	Four patients

The median interval between the onset of the CDC group IV disease and the start of treatment with zidovudine was 5 weeks (range 3-24 weeks). The starting dose of zidovudine was 1200 mg/day in eight patients and 1000 mg/day in five patients, but in one patient with severe chronic liver disease a reduced starting dose of 400 mg/day was given. The median observed duration of continuous treatment was 15 weeks (range 2 weeks to 18 months).

All patients were HBeAg seropositive at the time of starting treatment and remained so throughout follow-up. Median values of laboratory tests at the start of treatment are shown in table 3.6(i).

A comparison group of untreated patients with chronic HBV infection were studied, in order to control for spontaneous short-term changes that may occur in serum HBV-DNAp and HBV-DNA. Ten HBeAg-seropositive homosexual men (median age 29, range 26-49 years) were tested for HBV-DNAp and DNA at weekly intervals during a four week period. All patients were asymptomatic; five were HIV seropositive (CDC Group II). Laboratory tests are shown in table 3.6(i).

Table 3.6(i) Median values for laboratory tests at start of treatment

Laboratory measurement	Treated patients	Untreated patients
Haemoglobin	12.4 g/dl (range 10.2-15.4.)	15.0 g/dl (range 13.7-16.0)
White blood cell count	4.9 x 10^9/l (range 2.2-6.3)	4.8 x 10^9 /l (range 3.4-5.9)
Lymphocyte count	0.85 x 10^9/l (range 0.7-2.7)	1.8 x 10^9 /l (range 1.3-3.4)
CD4 Lymphocyte count* (normal range 0.27-2.16)	0.075 x 10^9/l (range 0.06-0.11)	
Alanine aminotransferase (normal range 5-40).	41 U/L (range 17-148)	103 u/l (range 54-251)

Notes
*(for 4 patients only)

3.6.4 Methods

HBsAg and HBeAg were assayed by solid phase immunoradiometric assay (Tedder *et al.*,1981). Anti-HIV 1 was detected by a commercially available EIA (Wellcozyme). Serum HBV DNA and HBV DNAp activity were assayed as described in sections 2.2 and 2.3 respectively

3.6.5 Statistical methods

HBV DNA and HBV DNAp were measured at two weekly intervals before and during zidovudine treatment, with all the samples available from each patient being analysed in one assay.

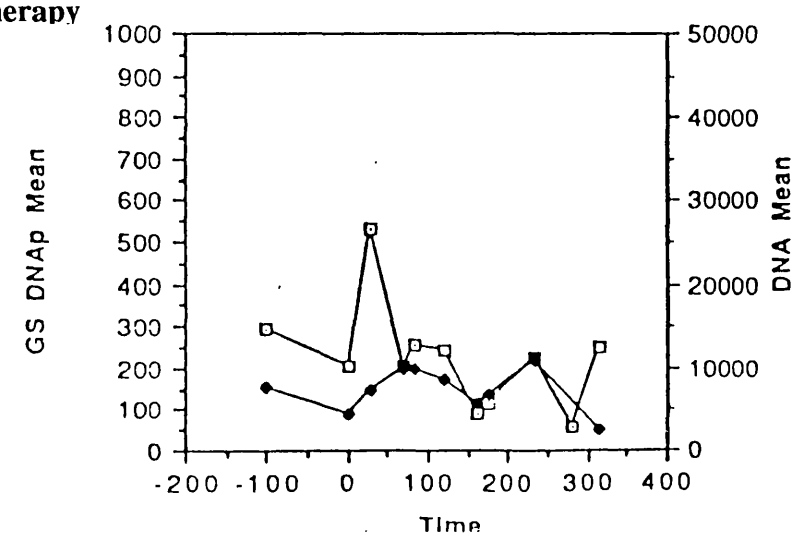
The values obtained during the treatment period were expressed as percentages of the pretreatment samples. Three samples were taken over a 90 day period before treatment, only one measure was taken for the untreated group at week 0. This was taken into account when the standard error of the mean was calculated for the untreated patients at time 0. Data was analysed over a 12 week period with an F-test in the treated group. A similar analysis was carried out for the untreated group.

3.6.6 Results

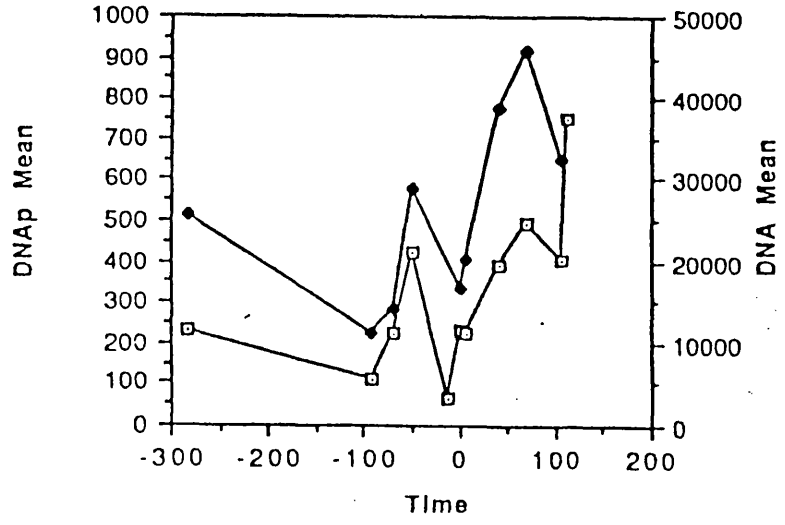
All of the patients remained HBeAg seropositive. HBV DNA and HBV DNAp fluctuated over time but behaved similarly (figure 3.6(i)). There was no significant change over time in either HBV DNA or HBV DNAp. There was also no difference in the level of fluctuation of either HBV DNA or HBV DNAp between the treated and untreated groups.

Figure. 3.6(i) Examples of the changing levels of HBV DNA and HBV DNA polymerase in serum over time of patients undergoing antiviral therapy

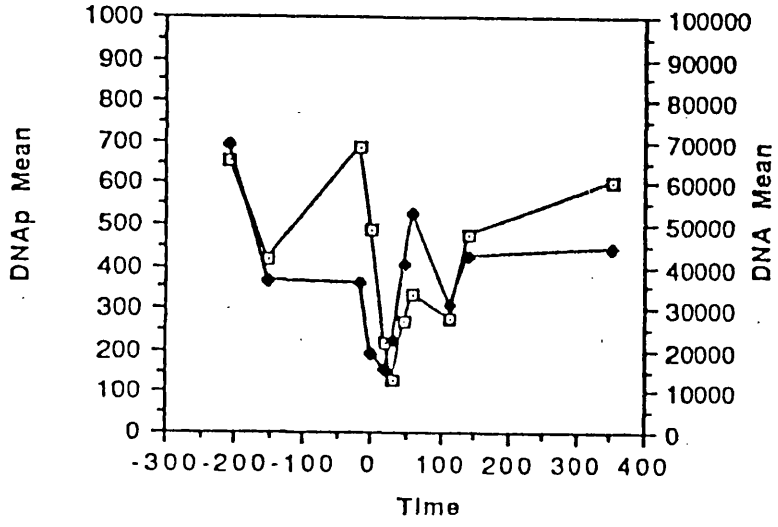
Patient 1



Patient 2



Patient 3



HBV DNAp
HBV DNA

3.6.7 Discussion

This was an observational study made possible by the treatment of symptomatic HIV disease in a group of chronic HBV carriers who received routine zidovudine therapy. In order to establish an effect for zidovudine on HBV replication it was necessary to establish the pattern of short term fluctuations in HBV DNA and HBV DNA polymerase levels. The comparison group comprised both singly (HBV alone) and dually infected carriers (HBV and HIV). There was no difference in the variation of HBV DNA polymerase and HBV DNA levels between the treated and the comparison group.

The study time was adequate for viral suppression to have commenced as other agents, which are active against HBV are associated with suppression of HBV replication within weeks (Weller *et al.*,1982).

The concentration at which HBV DNAp suppression has been observed *in vitro* (0.05 μM to 0.1 μM) was similar to concentrations of the drug observed in serum. However, this does not necessarily represent the amount of active metabolite which may be available in the liver. Thus the level of suppression may be decreased due to a lack of effective active metabolite concentration at the site of replication.

Inhibition of HBV DNA replication in human hepatoblastoma cell line 2.2.15 was demonstrated by Aoki-Sei *et al.*,(1991). This study compared the efficacy of 2',3'-dideoxyguanosine (ddG), 2',3'-dideoxyinosine (ddI), and 3'-azido-2,'3'-dideoxythymidine (zidovudine). In all cases higher concentrations were required to suppress replication of HBV compared to HIV. Zidovudine was found to be the least effective against HBV replication of the three drugs tested. Hence treatment regimes for HIV may be inappropriate for HBV, increasing the dose of zidovudine in these patients may increase the efficacy of treatment.

All of the men in the study had symptomatic HIV disease and this has been linked to a lower response to antiviral therapy and increased levels of HBV replication (Novick *et al.*,1985).

As liver biopsy prior to treatment was not carried out it was not possible classify the patients according to liver histology. The median ALT level of patients in this study was

41 U/l, only marginally outside the normal range of 5-40 U/l. Only two patients, included in the final analysis, had ALT levels greater than 70 U/l. In previous studies patients with chronic active hepatitis and higher liver transaminases were more likely to respond to antiviral therapy and to seroconvert from HBeAg to anti-HBe (Weller *et al.*,1985).

The results of this study are consistent with those of Féraye *et al.*,(1989), who studied a group of three AIDS patients. Marcellin *et al.*,(1990) found a similar lack of efficacy in asymptomatic HIV positive patients.

In contrast to this Berk *et al.*,(1991) observed an inhibtory effect in a group of patients who had not responded to alpha interferon therapy. In this study high levels of HBV DNAp activity prior to interferon treatment were associated with a response to zidovudine therapy. Only five of thirteen patients were anti-HIV positive and all were asymptomatic with CD4 counts of greater than 400 positive cells /mm^3. Again this contrasts with our study as CD4 counts (four patients tested) were below the lower limit of the normal range. Pretreatment with alpha interferon may have increased the efficacy of zidovudine in Berk's study. There is no indication of how long after cessation of alpha interferon therapy zidovudine treatment started in Berk's study. A synergistic effect of stimulation of the immune response and direct antiviral therapy can not be ruled out.

Our study failed to show a significant effect of zidovudine on HBV replication when administered at conventional doses in symptomatic HIV positive patients.

3.7 Hepatitis B reactivation or reinfection associated with HIV-1 infection: Detection of serum HBV-DNA by polymerase chain reaction

3.7.1 Objectives

- To amplify HBV DNA from serum samples of patients in whom reactivation of an existing infection or reinfection occurred in association with HIV infection

- To distinguish between reactivation or reinfection by analysis of amplified HBV DNA for sequence conservation between episodes of HBV infection

3.7.2 Introduction

The majority of patients undergoing an acute HBV infection clear HBsAg from serum. In most patients antibody to HBsAg develops. There may be a delay before the appearance of serologically detectable anti-HBs during which time anti-HBc may be the only serological marker of infection.

Immunosuppression is associated with reactivation of HBV infection in patients who have lost detectable HBsAg, with or without the development of detectable anti-HBs (Wands *et al.*,1975). This has also been observed more recently in patients with HIV-infection (Lazizi *et al.*,1988)

The following study describes the investigation of three patients in whom HBsAg fell to undetectable levels following acute infection and who failed to develop detectable anti-HBs. HBsAg then became detectable in serum again at which time all three were HIV-positive.

3.7.3 Patient population

Samples were studied from three patients attending the Middlesex Hospital. All were homosexual males whose cases have previously been described Waite *et al.*,(1988). The case histories are summarised below.

Case 1: A 33 year old man,recovering from an acute HBV infection in 1981 who lost detectable serum HBsAg (table 3.7(i)). This patient remained anti-HBc-positive but did

not develop anti-HBs. The patient was retrospectively tested for anti-HIV and found to have seroconverted between 1981 and 1983. He became HBsAg positive again in 1985 but remained asymptomatic. The initial HBV infection was subtype *ad*, the second HBV infection was subtype *ay* and it was concluded that this patient had become reinfected.

Case 2: A 31 year old man who developed an acute HBV infection in 1982 (table 3.7(ii)). The patient was found to be anti-HIV-positive in 1984. In 1987 the patient was found to be an asymptomatic carrier of HBV, retrospective testing showed that he had become HBsAg-positive in 1986. Both of the HBV infections were of HBsAg-subtype *ay*. This patient may have become reinfected due to continuing sexual contacts or reactivated a latent HBV infection.

Case 3: A 39 year old man whose serum was found to contain anti-HBc but no detectable anti-HBs or HBsAg (table 3.7(iii)).

The patient was treated for symptomatic HIV-1 infection and was found to be HBsAg positive on routine repeat testing. HBsAg was initially quantified at 2.5 ng/ml but this increased in successive samples. This patient had no evidence of an acute HBV infection between 1986 and 1988. Liver function tests were moderately elevated. HBsAg was not detectable in this period. The patient denied having any risk factors for reinfection, suggesting that a reactivation of his HBV infection had probably occurred. There were no HBsAg-positive samples from 1986 available for subtyping.

3.7.4 Methods

HBV serological markers were assayed by colleagues in the Division of Virology. Serum HBsAg was assayed by RIA. The specificity of the reaction was confirmed by neutralisation. HBsAg was quantified by serial dilutions in a reverse passive haemagglutination assay (RPH Hepatest, Wellcome Diagnostics Ltd). Anti-HBs and anti-HBc were assayed by in house RIAs. Anti-HBc was quantified by end point dilution in a passive haemagglutination assay (Corecell, The Green Cross Corporation). Anti-HBc IgM HBeAg/anti-HBe and anti-HDV were assayed as described by Tedder *et al.*,(1981). Anti-HAV and anti-HAV IgM were assayed with HAVAB and HAVAB-M (Abbott laboratories). Anti-HIV-1 was detected by competitive EIA, (Wellcozyme ,Wellcome diagnostics Ltd).

HBV DNA was detected by dot blot as described in section 2.2, HBV was also detected by PCR as described in section 2.6 A single round of amplification was carried out with core primers 189/190. The specificity of the product was confirmed by Southern blot analysis of the PCR product with a ^{32}P-dATP end labelled probe (3' end labelling kit,)

Restriction endonuclease digestion was carried out with Bgl II as described in section 2.4.5. Fragments of about the predicted size were produced by this digestion (33bp, 184 bp, 415 bp , 177 bp and 873 bp)as predicted by Microgenie analysis of the sequence, (Genbank issue 59, Beckman Instruments Inc, Palo Alto, Ca 94304). The 184 and 176 bp fragments were not easily distinguished from one another and the 33bp fragment comigrated with the primer band.

Samples were also amplified in a nested PCR with primers to the *a* determinant of the surface region. The amplified DNA was analysed by direct sequencing.

3.7.5 Results

In Case 1 HBV DNA was only detectable by dot blot in five of seven HBsAg positive samples from the second infection in this patient. HBV DNA was amplified by single and nested PCR with both sets of primers in both the first and second infections. It was detected by either single PCR, in six of seven samples or nested PCR in all seven samples. HBV was not amplified from two HBsAg negative samples taken after the second viraemic phase.

Sufficient sample was available from the second period of HBsAg positivity for repeated HBV DNA amplification. The derived sequence was compatible with subtype *ayw* and was consistent with the serological subtyping (Appendix 7). An amino acid substitution of valine for threonine was identified at codon 118 in the surface region. The X/precore/core region did not contain any mutations and was highly conserved when compared with the consensus sequence.

In Case 2 HBV DNA was detectable by both dot blot and PCR in five of ten HBsAg positive samples that were tested. HBV was detectable by dot blot, single and nested PCR in three of five serum samples from the first series of HBsAg positive samples. HBV DNA was amplified from the remaining two samples by nested PCR only. HBV

was not detected during the HBsAg negative period by any method but was detected by all three assays when HBsAg became detectable in the serum again.

HBV DNA was amplified from one sample, taken during the initial period of HBsAg postivity and another sample during the second period of HBsAg positivity. The nucleotide sequence was conserved between the two samples and was consistent with serological subtyping for subtype ayw. Nucleotide sequence analysis of the precore region revealed the appearance of a mutation at codon 29 only of A for G in the latest sample.

In Case 3 HBsAg was not detected in the samples available to the study until 16.12.88. The patient was assumed to have had a prior HBV infection because of the presence of anti-HBc in the serum. HBV DNA was detected by single and nested PCR in the first available HBsAg negative sample on initial analysis. This could not be repeated as there was insufficient sample available.

The conditions under which the assay was carried out made it unlikely that contamination of the PCR was responsible for this result. Three additional HBsAg negative samples were HBV DNA positive, (one of three by nested PCR only, two of three in both the single round and nested assay). Two of four HBsAg positive samples were PCR positive only, the remaining two samples were positive by dot blot.

One further sample was positive once with primers to the precore region but this result was not repeatable. HBV DNA was amplified and sequenced from twosamplesduring the HBsAg negative period (samples 23/7/87, 4/5/88 (data not shown)) and one sample from the second period of HBsAg positivity (Sample 15/2/89). All of the samples were identical.

Table 3.7(i) **Results of studies carried out on Case 1**

Date	HBV DNA			HBsAg ng/ml	HBsAg sub-type	Anti-HBs	HBe Ab/Ag	Anti-HBc+	AST* / ALT
	Dot Blot	PCR							
		Single	Nested						
7.06.80	NT	NT	NT	-		-	NT	-	NT
23.07.81	-	+	++	400	ad	NT	Ab	10,000[1]	NT
01.10.81	NT	NT	NT	-		-	ND	NT	22*
15.09.83	NT	NT	NT	-		-	ND	1,600	20*
23.08.84	-	-	-	-		-	ND	200	16*
27.11.84	-	-	-	-		NT	Ab	12,800	39*
22.02.85	-	-	-	-		NT	Ab	3,200	20*
28.05.85	-	+	++	50	ay	NT	Ab	3,200	30*
29.08.85	++	++	++	10^4		NT	Ag	12,800	79*
10.12.85	++	++	++	10^4		NT	Ab	6,400	53*

Table 3.7(i) continued Results of studies carried out on Case 1

Date	HBV DNA			HBsAg ng/ml	HBsAg sub-type	Anti-HBs	HBe Ab/Ag	Anti-HBc[+]	AST[*] / ALT
	Dot Blot	PCR							
		Single	Nested						
11.03.86	++	++	++	5.1×10^3		NT	Ab	6,400	46[*]
14.10.86	++	++	++	1.6×10^3		NT	Ab	>25,600	94
03.03.87	++	++	++	1.3×10^3		NT	Ab	NT	228
09.06.87	-	-	++	0.5		NT	Ab	NT	17
04.08.87	-	-	-	-		-	Ab	+	16

Notes

1 Anti-HBc IgM 80 units,all subsequent specimens <4.5 (arbitrary units; negative cut-off 10 units).
2 Anti-HBc IgM weakly reactive
3 Anti-HBc IgM positive
4 Anti-HBc IgM negative on this and all subsequent specimens
AST Aspartate aminotransferase
ALT alanine transferase
N Neither detectable. NT not tested. Ab/Ag antibody/antigen . [+] Anti-HBc Reciprocal titre.

Table 3.7(ii) Results of studies carried out on Case 2

Date	HBV DNA			HBsAg ng/ml	HBsAg sub-type	Anti-HBs	HBe Ab/Ag	Anti-HBc[+]	AST*/ALT
	Dot Blot	PCR							
		Single	Nested						
04.02.82	-	-	+	1	ay	NT	NT	-	37
11.02.82	-	-	+	15		NT	NT	NT	19
23.02.82	NT	NT	NT	4×10^2		NT	Ag	-	NT
05.03.82	NT	NT	NT	2×10^4		NT	Ag	+/-	44
10.03.82	+	++	++	4.1×10^4		NT	Ag	+	71
19.03.82	+	++	++	8.2×10^4		NT	NT	25,600[2]	166
26.03.82	+	++	++	8.2×10^4		NT	Ag	+	557

Notes

2 Anti-HBc IgM weakly reactive

3 Anti-HBc IgM positive

4 Anti-HBc IgM negative on this and all subsequent specimens

[+] Anti-HBc Reciprocal titre

AST Aspartate aminotransferase

ALT alanine transferase. N neither detectable. NT not tested. Ab antibody. Ag antigen.

Table 3.7(ii)continued Results of studies carried out on Case 2

Date	HBV DNA			HBsAg ng/ml	HBsAg sub-type	Anti-HBs	HBe Ab/Ag	Anti-HBc[+]	AST*/ALT
	Dot Blot	PCR							
		Single	Nested						
21.12.82	NT	NT	-	-		-	ND	3,200	NT
06.11.84	-	-	-	-		-	ND	400	19
10.06.85	-	-	-	-		-	ND	200	NT
08.10.85	NT	NT	NT	-		-	ND	200	16
23.07.86	-	-	-	-		-	NT	<200	NT
10.12.86	NT	NT	NT	4.1×10^4	ay	NT	Ag	640,000[4]	NT
07.04.87	NT	++	++	4.1×10^4		NT	Ag	80,000	NT
31.12.87	+	++	++	2×10^4		NT	Ag	20,000	NT
07.03.88	+	++	++	4.1×10^4		NT	Ag		NT

4 Anti-HBc IgM negative on this and all subsequent specimens Anti-HBc[+] Reciprocal titre

Table 3.7(iii) Results of studies carried out on Case 3

Date	HBV DNA			HBsAg	HBsAg sub-type	Anti-HBs	HBe Ab/Ag	Anti-HBc (Reciprocal titre)	AST*/ALT
	Dot Blot	PCR							
		Single	Nested						
02.06.86	-	+[5]	++[5]	-		-	ND	+	
26.02.87	-	-	-	-		-	NT	+	
23.07.87	-	-	++	-		-	NT	+	
03.12.87	-	-	-	-		-	NT	+	
09.02.88	-	+	++	-		-	NT	+	
04.05.88	-	+	++	-		-	NT	+	
13.10.88	-	-	-	-		-	NT	+	
10.11.88	-	-	-	-		-	NT	+	
16.12.88	-	+	+	+	NT	-	ND	+	
13.01.89	-	+	+	+		-	ND	+	
15.02.89	+	++	++	+		-	Ag	+	

Notes 5 Result not repeatable. AST Aspartate aminotransferase. ALT alanine transferase. N neither detectabl NT not tested. Ab antibody. Ag antigen.

3.7.6 Discussion

In this study the apparent resolution of an acute HBV infection followed by a second period of HBs antigenaemia has been described in three patients.

In Case 1 the presence of different serological subtypes in the initial and subsequent infections suggested reinfection. To date there is no evidence for subtype variation during the course of a single chronic or acute HBV infection where a single HBsAg subtype was initially observed. Reinfection with HBV is unusual and in this case may be related to infection with HIV between the two phases of active HBV replication.

In Case 2 the initial and subsequent infection were of the same subtype. The nucleotide sequences of HBV DNA amplified from the first and second periods of HBsAg positivity were identical. Although HBV DNA was not amplified from the serum samples between the two periods of antigenaemia, reinfection is unlikely. The patient became HBsAg positive again 56 months after his initial infection and denied sexual contact for over two years before then.

In Case 2 reactivation of an existing infection would appear more likely. However, an amino acid substitution of a aspartamine for glycine was only present in a sample from the second period of positivity. This is unusual as this mutation is more commonly associated with a mutation at codon 28 which is associated with HBeAg to anti-HBe seroconversion in some patients. Thus the presence of this mutation may indicate a longterm chronic infection infection. The possibility of reinfection was not excluded because similar mutations have been shown to be transmissible.

Reinfection was not suspected in Case 3, where the detection of HBV DNA between the first and second HBsAg-positive periods of infection indicated reactivation. Nucleotide sequences of the amplified samples were conserved throughout the infection.

The two patients who reactivated an existing infection were HBeAg positive and had no symptoms associated with the reappearance of HBsAg. HBV DNA was amplified in the absence of either HBsAg or HBeAg. Moreover the time course of the infection made it unlikely that the HBV DNA in all four samples was due to partially degraded HBV DNA from the initial period of infection. The patient who became reinfected had two episodes

of acute hepatitis but did not develop HBeAg.

Reinfection with HBV carrying mutations in the surface gene was described by Carman *et al.*,(1990) in which where passively and actively immnunised children of HBeAg positive mothers developed HBsAg. In that report HBV DNA amplified from the serum of one child indicated that the circulating HBsAg was of a variant type with a mutation at codon 145 in the A determinant of the surface gene. Mutations at this point were not seen in any of our cases, although this does not exclude the possibility of other HBV variants as only a small section of the genome was sequenced.

HBV DNA has been detected in the absence of conventional markers of HBV infection in lymphoid cells of three patients with AIDS and AIDS related complex who were HBsAg negative. One of the patients had no markers for current or past HBV infection whilst two had both anti-HBc and anti-HBs (Laure *et al.*,1985). Similarly HBV DNA has also been detected in the bone marrow of seven children with leukaemia lacking all markers of HBV infection (Pontisso *et al.*,1986). A common feature of both of these studies is the involvement of immunosuppressed patients. This may prevent the development of a protective, neutralising antibody response in such patients.

The development of DNA amplification procedures has shown HBV DNA in serum in the absence of conventional serologic markers and in the presence of anti-HBc only (Thiers *et al.*,1988, Lau *et al.*,1989, Liang *et al.*,1990). These and other studies indicate that a subclinical carrier state is possible and may be associated with a range of serological markers whist the patients remain infectious. Although the amplification of HBV DNA from serum does not necessarily indicate ongoing replication or the production of infectious particles.

Chimpanzees can be infected with HBV from the serum of seronegative individuals. Thiers *et al.*,(1989) demonstrated this with HBV isolated from a seronegative individual which elicited a serological response in experimentally infected chimpanzees.

We have also analysed samples from patients with anti-HBc alone in a cross sectional study (data not shown) who were identified through pre-vaccination HBV screening. HBV DNA was amplified from three of 103 samples tested, two of the HBV DNA

positive samples were also HIV positive. This indicated that whilst this serological pattern is unusual it may be related to the patients HIV status. However, the numbers were too small to draw any firm conclusions.

In summary three cases are described in which HBV reinfection (Case 1) and reactivation of an HBV infection (Cases 2 and 3) occurred. There was no evidence for nucleotide sequence variation in the regions of the HBV genome that were analysed which might account for this. Thus these unusual patterns of infection may be related to HIV status of the patients, all of whom became HIV positive between periods of active HBV infection.

3.8 Conservation of precore and core sequences of hepatitis B virus in chronic viral carriers (Hawkins *et al.*,1994)

3.8.1 Objectives

- The amplification of HBV DNA directly from serum samples from HBV chronic carriers

- To analyse the amplified HBV DNA by direct sequencing

- To document the degree of sequence conservation in the x/precore/core regions and determine whether the previously documented precore mutations were present in this population

- To detect the presence of any other mutations in the precore region which might effect HBeAg translation

- To analyse HBV DNA for associated mutations in the adjacent or overlapping core and X regions.

3.8.2 Introduction

The precore and core regions of the hepatitis B virus (HBV) genome encode the viral nucleocapsid or core protein. Initiation of translation can take place at one of two inframe translation initiation codons; at the precore initiation codon (nucleotide position 3130) or at a second initiation codon near the start of the core gene (position 1).

HBeAg is expressed when initiation occurs at the precore initiation codon and a 19 amino acid polypeptide is cleaved. A high degree of conservation of the precore and core region sequences at both nucleotide and amino acid levels has been reported (Tiollais *et al.*,1985).

The detection of HBeAg in serum is associated with active viral replication (Hoofnagle *et al.*,1987). Seroconversion from HBeAg to anti-HBe is associated with a fall in replication, measured by serum HBV-DNA or DNA-polymerase activity. In some HBV carriers with detectable serum anti-HBe, high levels of serum HBV-DNA and DNA-

polymerase are found (Lok *et al.*,1984). Carman and *et al.*,(1989) found that in 7 of 8 HBeAg seronegative patients with circulating HBV-DNA, sequence variation had occurred which would prevent translation through the precore to the core region, and suggested that this may explain the absence of detectable serum HBeAg. The mutations identified changed the coding for the two terminal amino acids of the precore region (codon 28 and codon 29) into stop codons. Santantonio *et al.*,(1991) described an HBV sequence with a mutation of the first precore initiation codon (codon1) which would also prevent HBeAg translation.

Cloning and expression of the precore codon 28 variant showed that HBeAg expression was not necessary for the productive replication of HBV (Ulrich *et a*l.,1990).

The association of precore mutant viruses with high viral replication *in vivo* in the presence of serum anti-HBe has been reported to occur more often in patients with chronic active hepatitis than chronic persistent hepatitis (Nauomov *et al.*,1992). One study of Japanese patients found evidence of this mutation in patients with mild or no liver disease (Okamoto *et al.*,1990). However, no causal link has yet been established between precore mutations and chronic liver disease.

In our study the PCR technique was used to amplify HBV DNA with direct sequencing of the PCR product. The assay was used to examine the precore region (position 3129 to 3215), part of the adjacent X region (position 3075 to 3153) and the first 168 of the 549 bases of the 5' end of the core gene from a selected group of asymptomatic homosexual or bisexual male HBV carriers.

3.8.3 Patient population

Serum samples were obtained from homosexual or bisexual male patients with chronic HBV infection attending the Middlesex Hospital. Sufficient serum was available from 65 of 69 patients described in section 3.5. Samples were studied from 11 anti-HBe positive patients 1 patient with neither HBeAg nor anti-HBe detectable in the serum and 53 HBeAg positive patients. The age, duration of infection, HBe/anti-HBe status, serum HBV DNA level and HBV DNAp activity, the presence of anti-HIV 1 and the level of alanine aminotransferase (ALT) in the patients studied are shown in table 3.8(i).

3.8.4 Methods

Serological markers of HBV infection were studied as previously described, HBsAg, anti-HBc (both total and IgM fraction) and HBeAg/anti-HBe were all assayed by in-house solid phase radioimmunometric assays (RIA) (Tedder *et al.*,1980). HBV DNA polymerase activity was detected as described in section 2.2, HBV DNA was assayed by dot blot hybridisation as described in section 2.3. HBV DNA was initially amplified directly from 1 μl of serum as described in section 2.6.3. In samples where HBV DNA was not amplified by this method HBV DNA was purified from 50 μl of serum according to the method described in sections 2.6.6. In five cases insufficient serum was available for purification and HBV DNA was amplified from 1 μl of serum in a double nested PCR assay.

The double stranded sequencing method described in section 2.9 was used to analyse the PCR product. Sequences were compared using the Microgenie sequence analysis database (Genbank Issue 59, Beckman Instruments Inc, Palo Alto, Ca 94304).

Table 3.8(i) Characteristics of patients studied, including those from whom HBV sequences were and were not analysed

	Sequenced		Not sequenced	
	HBeAg+	HBeAg-	HBeAg+	HBeAg-
Number	33	9	20	3
Age, years: Mean (SD).	32.5 (5.68)	36.2 (6.80)	34.0 (1.60)	34.0 (7.40)
Duration of known HBs-antigenaemia, years: Mean (SD).	2.77 (1.48)	5.23 (3.00)	2.78 (1.39)	5.05 (3.61)
Anti-HIV 1 sero-positivity: No. positive (%)	19 (58%)	2 (22%)	11 (55%)	2 (67%)
Alanine transaminase: Mean (No. tested, SD), IU/L	87.2 (29, 62.9)	53.7 (8, 28.9)	100 (17, 92.2	19 (1, -)
HBV-DNA: Mean (No. tested, SD), ng/ml	1.9 (23, 1.2)	0.3 (7, 0.53)	2.3 (17, 1.6)	0 (3, -)
HBV-DNAp: Mean (No.tested, SD), counts /min/200 μl	333 (32, 298)	104.5 (8, 191)	416 (20, 488)	6 (3, 11)

3.8.5 Results

HBV DNA was amplified directly from 1 μl of serum in 50 of 53 HBeAg positive cases, in three cases insufficient template was produced for sequencing after nested PCR. A double nested PCR on these samples increased the yield of HBV DNA, allowing these products to be sequenced. None of the HBeAg positive samples required phenol/chloroform extraction and ethanol precipitation. One anti-HBe positive sample produced sufficient template for sequencing from 1 μl of serum. The other seven required either sample purification or double nested PCR. These results are summarised below.

HBeAg/anti-HBe status	PCR positive	PCR negative	Number sequenced
anti-HBe +	8	3	8
HBeAg +	53	0	33
Neither detectable	1	0	1
Total	62	3	42

PCR products from 16 HBeAg positive samples selected to cover a range of HBV DNAp levels were sequenced in the precore, the carboxyl terminal end of the X region and the amino terminal end of the core region; PCR products from a further 17 patients were analysed in the precore region only. PCR products from eight anti-HBe positive carriers were sequenced; one sample which was positive by DNA dot blot and after nested PCR on 1μl of serum in the precore, core and X regions, four samples in the precore region only and three samples in the core region only. Samples were sequenced in this way because it was not possible to amplify these samples with primers to the precore and core regions simultaneously.

There was a high degree of homology between all the samples studied in all regions. Analysis with the Microgenie sequence analysis database, showed the sequences to be most closely homologous to the published subtype *adw* (Ono *et al.*,1983).

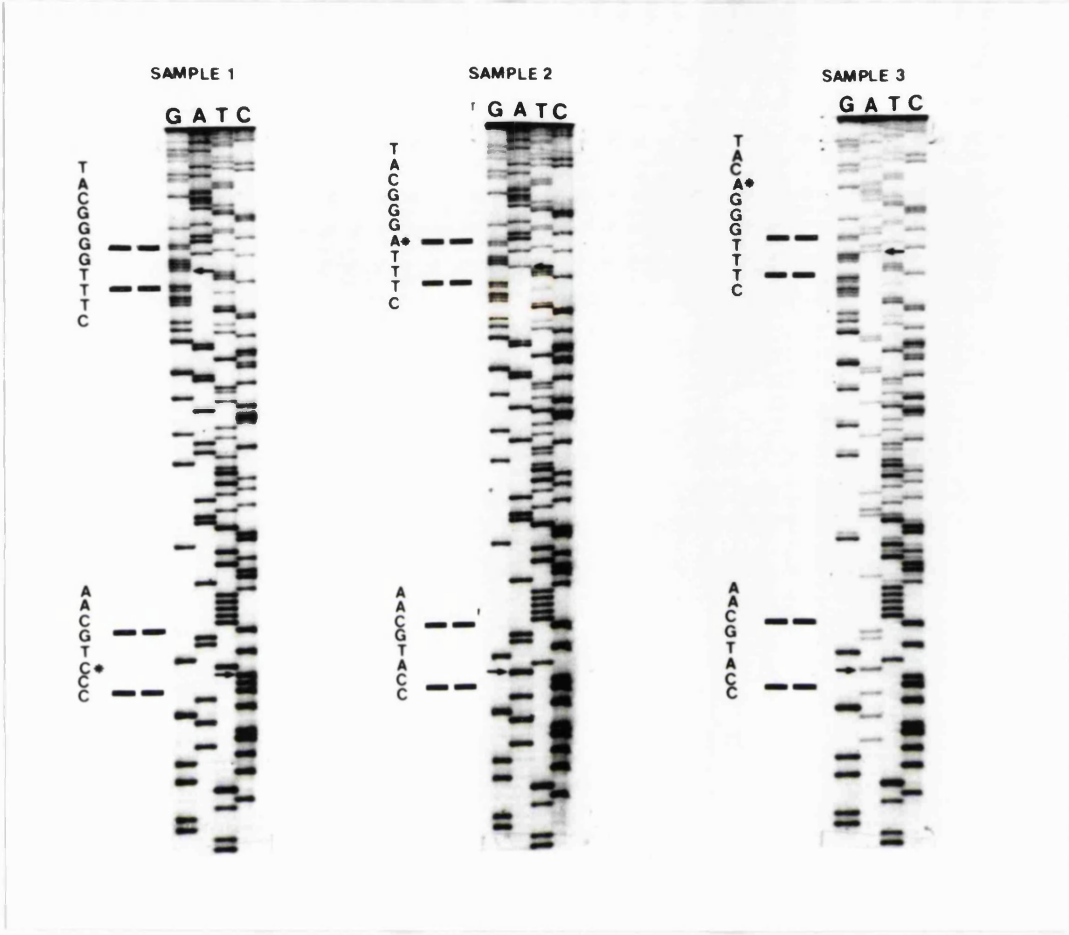
An autoradiograph showing mutations in the precore region which would prevent the translation of HBeAg is shown in figure 3.8(i). These mutations were detected in two of five anti-HBe positive samples studied (Appendix 7). In one sample the translation initiation codon, amino acid 1, of the precore region was altered from methionine to

leucine (ATG to CTG), this sample also contained a proportion of wildtype virus. In a later sample the wild type virus was not detected by sequence analysis. In the second sample codon 28 of the precore region was altered from a serine residue to a termination codon (TGG to TAG). Wildtype virus was not detected in this sample.

A mutation was detected in one of the HBeAg positive samples at codon 21 (lysine to asparagine (AAA to GAA). This substitution may effect the conformation of the protein as an uncharged amino acid was substituted for a positively charged residue. None of the HBeAg positive samples carried mutations that would prevent the translation of the precore region.

The carboxyl terminal of the X region was also highly conserved, three HBeAg-positive samples showed amino acid variation that did not correspond with published sequences in the Genbank database. The changes were as follows; codon 145, alanine to threonine in samples 14 and 23 and codon 132, phenylalanine to tyrosine in sample 25. The X to precore frame shift meant that the alteration of the precore initiation codon in one anti-HBe positive sample resulted in a silent sequence change to the X region. The core region showed sequence changes in only one of the 33 samples studied (Appendix 7).

Figure 3.8(i) An autoradiograph demonstrating nucleotide sequence variation in the HBV precore region[1]



Notes

1 Summary of nucleotide sequence variation

	Sample 1	**Sample 2**	**Sample 3**
Codon 1	CTG (leu)	ATG (met)$^+$	ATG (met)$^+$
Codon 28	TGG (trp)$^+$	TAG (stop)	TGG (trp)$^+$
Codon 29	GGC (gly)$^+$	GGC (gly)$^+$	GAC (asp)

\+ Wild type genotype

* Nucleotide variation associated with a genotypic variant

———> Nucleotide variation identified on the autoradiograph

3.8.6 Discussion of results

HBV DNA was amplified from all 53 HBsAg, HBeAg positive serum samples tested and from nine of 12 HBsAg positive, HBeAg negative samples. Of sixteen HBeAg positive samples sequenced through codons 129-154 of the X gene three had mutations not shown on the Microgenie database (Genbenk issue 59), two at codon 145 altering alanine to threonine and one at codon 131 altering phenylalanine to tyrosine. No non-silent codon changes were seen in the HBeAg negative samples. This study of HBV carriers shows that in the precore, X and core regions HBV sequences are highly conserved.

Both the nested and double nested PCR were used to amplify HBV DNA directly from serum in spite of HBV DNA copy numbers as low as 10^4 copies/ml. Amplification by PCR direct from serum was used where very limited volumes of serum were available. This method also limited the opportunity for contamination.

In the precore region only three mutations were identified in 40 samples sequenced. One of these mutations from an HBeAg-positive carrier has not previously been published (AAG to AAC, lysine to asparginine at position 21). Single base substitutions were identified in two anti-HBe-positive sera. These were in the precore translation initiation codon (codon 1) and codon 28 and corresponded to those previously reported (Carman *et al.*,1989, Santantonio *et al.*,1991). The mutation of methionine to leucine at codon 1 of the precore region was reported by Santantonio *et al.*,(1991) in only 1 of 42 anti-HBe positive samples from patients with chronic liver disease from an area endemic for HBV infection. The mutation at codon 28, by contrast, was found in 41 of 42 samples analysed. One anti-HBe-positive sample in the present study contained a mixed population of an initiation codon mutant and wild type.

The presence of high levels of circulating HBV DNA has been associated with chronic active hepatitis and anti-HBe seropositivity in HBV carriers (Akahne *et al.*,1990, Ulrich *et al.*,1990, Brunetto *et al.*,1991, Omata *et al.*,1991, Liang *et al.*,1991). Sequences from 7 of 8 such patients analysed in the precore region demonstrated mutations at codons 28 and 29 (Carman *et al.*,1989).

An important difference between the study described here and previous studies was the degree of HBV related disease. The population examined in this study was characterised

by relatively mild liver disease whilst most others have involved patients with symptomatic hepatitis.

Most infections were probably acquired in adulthood by sexual contact in a non-endemic area. Although liver biopsies were not performed, all patients were asymptomatic, transaminase levels were not markedly elevated, and only two had signs of chronic liver disease. Again, in contrast to previous series only one of the HBeAg-negative samples studied had sufficient HBV-DNA in serum to be detectable by dot-blot.

While the number available for study was small, it suggests that the precore mutation is not a prerequisite for HBeAg to anti-HBe seroconversion. Studies of anti-HBe-positive patients with chronic liver disease from endemic areas is likely to identify those who have been infected longer, including those infected early in life. Naoumov *et al.*, (1992) has shown that the mutant virus may be present as a subpopulation of the viral load, as observed in the serum of one patient studied here. Where the subpopulation is less than 10% of the viral load the techniques used would be unlikely to detect it. Thus it was not possible to say that patients studied here did not have a smaller subpopulation of other sequences.

As would be expected from the results of sequencing of the precore region, the carboxyl terminal of the X gene was also highly conserved. The core gene sequences showed greater than 91% homology between themselves and the published sequence for subtype *adw*. One sample, in which anti-HBc could not be detected, had several silent mutations but all except one have been noted in other published sequences.

The high level of sequence conservation in samples chosen to cover a wide range of HBV DNA and HBV DNA polymerase activities, indicated either; that these regions do not directly influencing HBV replication or they are vital for replication and any variation here would produce non-viable progeny. The latter is unlikely given the increasing body of evidence that sequence variation occurs frequently in some HBV infected groups.

3.9 The transmission of hepatitis B virus precore mutants associated with fulminant and acute hepatitis.

3.9.1 Objectives

- To amplify HBV DNA from patients with fulminant hepatitis B and their sexual partners with primers to the X/ precore/core genes and the A determinant of the surface gene

- To assess the frequency of mutations in precore codons 1 and 28 with the point mutation assay

- To establish whether HBV with mutations at these points may be transmitted between sexual partners

- To examine the possibility that HBV with precore mutations may be associated with a fulminant hepatitis

- To study the degree of sequence homology between sexual partners in a variable region (the 5' end of the X gene) and a conserved region (the precore region and the A determinant of the surface gene) of the HBV genome

- To study the effect of viral concentration in sexual partners on the clinical course of HBV infection of the infected patient

3.9.2 Introduction

As described in section 3.9 HBeAg in serum is associated with viral replication (Hoofnagle *et al.*,1981) and the appearance of anti-HBe in serum is generally associated with reduced viral replication. Lok *et al.*,(1984) and Bonino *et al.*,(1986) noted continued high levels of HBV replication , as evidenced by detectable circulating HBV DNA , in the presence antibodies to HBeAg (anti-HBe). This atypical HBeAg negative form of hepatitis was noted more frequently in patients of Mediterranean origin. Several studies have indicated that the precore mutant virus is transmissible *in vivo* by different routes.

Transmission between spouses who developed a fulminant hepatitis was identified by Yotsumoto *et al.*, (1992) and Carman *et al.*,(1989). The precore mutant virus has also been associated with nosocomial transmission (Liang *et al.*,(1991) in an epidemic of fulminant hepatitis in a hospital. Conservation of sequences between partners and patients was taken as evidence of the source of infection.

Adult patients of Mediterranean origin often present without detectable HBeAg and may have already lost the wildtype virus of their original infection. There is very little direct evidence as to which virus type (mutant or wildtype) patients were originally infected with.

The possible role of precore mutant virus in the development of fulminant hepatitis has been widely addressed. A number of studies (Omata *et al* (1990), Liang *et al* (19991), Kojima *et al* (1991), Kosaka *et al* (1991), Hasegawa *et al* (1994), Uchida *et al* (1993)) identified the precore mutant virus in patients with fulminant or severe hepatitis but not in patients with acute, self limited infection.

Carman *et al.*,(1991) described the association of mutant virus with acute and fulminant hepatitis in Greek patients. In many cases in that study mutations of both codons 28 and 29 in the precore region were present. The double mutant type was associated with more severe disease.

Sexual transmission of the precore mutant virus has not been clearly demonstrated in a contolled study. The possible effect, with regard to clinical outcome, of infection with this mutant virus has also not been confirmed. The significance of the second mutation at codon 29 remains unclear.

In the following study we describe sequence analysis of the HBV precore region amplified from serum samples. We have studied samples from six Greek patients with fulminant hepatitis and their sexual partners. We have included as a control group six patients with an acute , self limited infection and their sexual partners.

We have analysed the precore codons 1 and 28 by the point mutation assay (Hawkins *et al.*,1994) to give a sensitive measure of viral heterogeneity in both the patient and the

source of their infection.

3.9.3 Patient population

Samples were taken from Greek patients and their long term sexual partners. Two serial samples were collected from six patients with fulminant hepatitis who had been infected by their partners. A control group of six patients, matched for age and sex, were also studied. These patients, who were also assumed to have been infected by their HBsAg positive sexual partners, developed an acute uncomplicated hepatitis. The HBV serological markers for all patients studied are summarised in tables 3.9(i), 3.9(ii).

3.9.4 Methods

Serological markers were determined by Professor Hatzakis and colleagues in Greece using standard serological assays for the detection of HBsAg, HBeAg/anti-HBe and core IgM/IgG. The level of liver enzyme (ALT) was assayed by standard automated processes.

Serum HBV DNA was assayed by a commercially available (Abbott, Genostics^{TM}) DNA liquid hybridisation assay by Professor Hatzakis.

Nested and Double nested PCR was carried out on all samples with primers to the precore/core region and the *a* determinant of the surface region.

The amplified HBV DNA was analysed by direct sequencing of the PCR product and by PMA of codons 1 and 28 of the precore region and codon 145 in the *a* determinant of the surface gene.

In some cases although a PCR product was amplified the sequencing was very difficult to interpret. There appeared to be several different sequences present in the same sample. Single molecules were amplified from these samples for further analysis.

The single molecule amplification was achieved by serially diluting the sample in PCR reaction mixture and amplifying four aliquots at each dilution (Simmonds *et al.*,1990). Analysis of the frequency of positive samples at each dilution allowed the sample concentration (i.e. the number of molecules of HBV present in each PCR reaction mixture) to be determined. When DNA was amplified from only one of four samples at

a specific dilution, the probability was that this reflected amplification from a single molecule.

Having determined the concentration of the sample it was diluted to the point where amplification would take place from a single molecule and in this way several molecules could be obtained.

All samples were sequenced at least twice from two independent amplifications. The PMA for mutations of precore codons 1 and 28 was carried out in duplicate on the product of each amplification reaction and repeated where necessary.

3.9.5 Results

Serological data and PMA analysis of codon 28 of the precore region are summarised by patient groups; Serological and clinical data for both groups of patients and their partners are shown in tables 3.9(i) and 3.9(ii).

HBV DNA was amplified from five of six patients with fulminant hepatitis (study group) in at least one serum sample and all of their partners. HBV DNA was also amplified from five of six patients with an acute hepatitis B infection (control group) and all six of their sexual partners. HBV DNA sequences were analysed from codons 105-154 in the X gene and codons 1-29 of the precore region.

Serum samples from the partners of patients with a fulminant hepatitis and partners of those with acute hepatitis were titrated and their end point dilution of HBV DNA compared (table 3.9(iii), Appendix 6, page 275). These varied over a similar range (10^0 to 10^{-4}) in samples from both groups.

Mutations of codon 1 in the precore region were not detected in any of the samples assayed. HBV DNA was amplified from serum samples in five of six cases of patients with fulminant hepatitis and all six of their sexual partners. Four of five samples carried nonsense mutations of precore codon 28 (tryptophan to a stop codon TGG to TAG) (table 3.9(iv).

A sample from one patient had the wild type virus. All six sexual partners carried the

mutant virus, in two cases as part of a heterogeneous population with the wild type virus. Four of the patients with fulminant hepatitis died, all carried the mutant type virus.

HBV DNA was amplified from five of six patients with an acute HBV infection and all six of their sexual partners. Four of five patients carried precore mutant virus, one as part of a mixed population with the wild type virus (table 3.9(v)). One sample was homologous for the wild type virus at this point. All six partners carried the precore mutant type virus, in two cases as a mixed population with the wild type virus.

The results of PMA analysis of codon 28 in the precore region are summarised for the patients with fulminant hepatitis in table 3.9(iv) and the patients with acute hepatitis in table 3.9(v).

Table 3.9(i) Samples from patients with fulminant hepatitis and their sexual partners

Case ID	Sample ID	ALT IU/l	Bil mg/dl	HBsAg	IgM anti-HBc	anti-HBs	HBeAg / anti-HBe	anti-HDV	outcome
382	382.1	4521	30.2	-	+	+	-/+	-	survived
	382.1Y							-	
306 SP-382	306BD	119		+	-	-	-/+	-	
1434	1434.1	NA	NA	-	+	+	-/+	-	died
	1434.2							-	
418 SP-1434	418	51		+	-	-	-/+	-	
1574	2500	NA	8.15	-	+	+	-/+	-	died
	2533							-	
489 SP-1574	2946	62		+	-	-	-/+	-	

Table 3.9(i) continued **Samples from patients with fulminant hepatitis and their sexual partners**

Case ID	Sample ID	ALT IU/l	Bil mg/dl	HBsAg	IgM anti-HBc	anti-HBs	HBeAg / anti-HBe	anti-HDV	outcome
2342	9748	5769	8.80	+	+	-	-/-	-	died
	9795	1898	21.60	-	+	-		-	
791 SP-2342	9880	20		+	-	-	-/+	-	
2373	10013	3522	24.0	+	+	-	-/+	-	transplantation
	10174					-	-/+	-	survived
799 SP-2373	10025	NA		+	-	-	-/-	-	
2400	10184	6000	11.30	-	+	-	-/-	-	died
	10202								
803 SP-2400	10206	NA		+	-	-	-/+	-	

Table 3.9(ii) Samples from patients with an acute HBV infection and their sexual partners

Case ID	Sample ID	ALT IU/l	Bil mg/dl	HBsAg	IgM anti-HBc	anti-HBs	HBeAg/ anti-HBe	anti-HDV	outcome
2423	10324	3538	14.6	-	+		-/+	-	
809 SP-2423	10400	33		+	-	-	-/+	-	survived
2401	10183	6300	7.6	+	+		-/+	-	
824 SP-2401	10500	74		+	-	-	-/+	-	survived
2587	11869	3647	12.6	+	+		-/+	-	
895 SP-2587	12088	NA		+	-	-	-/+	-	survived
2626	12171	2045	16.0	+	+		-/+	-	
905 SP-2626	12380	2		+	-	-	-/+	-	survived
1898	5627	1065	11.0	+	+		-/+	-	
685 SP-1898	7640	40		+	-	-	-/+	-	survived
1628	3018	3561	14.3	+	+		-/+	-	
680 SP-1628	7591	39		+	-	-	-/+	-	survived

Table 3.9(iv) Samples from patients with fulminant hepatitis and their sexual partners

Case ID	Sample ID	Percentage of incorporated nucletide				Wild type/ Mutant	HBeAg/ anti-HBe	outcome
		G	A	T	C			
382	382.1	1.0	98.8	0	0	Mutant	-/+	survived
	382.1Y	NT						
306 SP-382	306BD	0.1	99.6	0.3	0.1	Mutant	-/+	
1434	1434.1	NT					-/+	died
	1434.2	NT						
18 SP-1434	418	4.9	88.3	2.9	3.7	Mutant	-/+	
1574	2500	3.5	93.4	3.1	0	Mutant	-/+	died
	2533	0.6	99.3	0.1	0	Mutant		
489 SP-1574	2946	0.7	98.9	0	0.3	Mutant	-/+	
2342	9748	0.8	98.8	0.3	0.1	Mutant	-/-	died
	9795	NT						
791 SP-2342	9880	1.1	97.9	0.1	0.9	Mutant	-/+	
2373	10013	98.1	0.9	0.4	0.7	Wild-type	-/+	transpla-ntation
	10174	NT					-/+	survived
799 SP-2373	10025	67.5	28.3	3.5	0.6	Mixed	-/-	
2400	10184	0.4	99.4	0	0.2	Mutant	-/-	died
	10202	NT						
803 SP-2400	10206	5.5	90.8	1.9	1.7	Mixed	-/+	

Table 3.9(v) Samples from patients with an acute hepatitis B infection and their sexual partners

Case ID	Sample ID	Percentage of incorporated nucletide				Wild type/ Mutant	HBeAg/ anti-HBe	Outcome
		G	A	T	C			
2423	10324	NT					-/+	
809 SP-2423	10400	0.3	99.4	0.2	0	Mutant	-/+	survived
2401	10183	2.4	1.7	92	3.8	Mutant	-/+	
824 SP-2401	10500	0.38	98.7	0.4	0	Mutant	-/+	survived
2587	11869	19.9	79.8	0.3	0	Mixed	-/+	
895 SP-2587	12088	0.8	99.0	0.1	0	Mutant	-/+	survived
2626	12171	1.1	98.0	0.5	0.3	Mutant	-/+	
905 SP-2626	12380	86.3	12.8	0.8	0.1	Mixed	-/+	survived
1898	5627	98.0	1.5	0	0.4	Wild-Type	-/+	
685 SP-1898	7640	40.0	59.3	0	0.3	Mixed	-/+	survived
1628	3018	0.70	99.2	0.1	0	Mutant	-/+	
680 SP-1628	7591	0.2	99.5	0.3	0	Mutant	-/+	survived

Full sequence data for all samples that are available as an appendix (Appendix 7) to this thesis but are not included in this volume. The samples were generally well conserved between patient partner pairs. In two samples there were single nucleotide differences in the X region between patients and their partners.

The presence of a double mutation at codons 28 and 29 in the precore region was conserved between patients and partners in all three cases where it was observed. Sequence variation in the precore region occurred between only one patient and their partner.

Mixed sequences were detected in two samples from patients with fulminant hepatitis which were resolved by the analysis of amplificate from single molecules. In the first case five different X gene sequences were detected when nine amplificates were analysed, two molecules carried inserts of one and three codons respectively. Four precore sequences were also found in this sample. The X gene was homologous in all amplificates from the second case however, four different precore sequences were detected.

The samples from patients with an acute hepatitis were well conserved, both as a group and between partners. The only mutations detected in the precore region of these samples were at amino acids 28 and 29.

Double mutations of codons 28 and 29 were found in a similar proportion of patients in both groups, three of five patients with fulminant hepatitis and two of five patients with acute hepatitis.

None of the patients or control group had evidence of a mutation at codon 145 either as a mixed or homogenous population.

The surface region of these samples was well conserved in both groups of patients and partners studied. There was single nucleotide variation between patients with an asymptomatic infection and their partners. However, there was more variability between the patients with a fulminant infection. These mutations are summarised in Appendix 6, tables 3.9(vi)(page 276) and 3.9(vii)(page 277).

3.9.6 Discussion

We have analysed HBV DNA which was amplified from the serum of Greek patients with fulminant hepatitis and their sexual partners. We have also studied patients with acute hepatitis and their sexual partners.

We have confirmed that sexual transmission of the precore mutant virus does occur. This conclusion is supported by the fact that samples were taken early in the HBV infection in both groups of patients. HBV is typically detectable in the serum of patients with a fulminant infection for only a short time. In the group studied here HBV DNA was detectable in five of six first samples but only one of six second samples. This indicated a rapid decline in circulating HBV and a probable cessation of viral replication. It is unlikely that these patients would have developed the precore mutant type virus at the time of the first sample and even less so that they would have cleared the wild type virus if they had been infected with it. The sensitivity of the PMA is such that a declining wild type virus population of greater than 3% could be detected.

The wild type virus was at undetectable levels in eight of twelve partners studied indicating that their partners were initially infected with the mutant virus. In transmission studies by Tong *et al* (1991), chimpanzees infected with a precore mutant virus produced anti-HBe. Therefore the presence of anti-HBe in the serum of patients, where wild type virus has not been detected, is not without precedent. This may reflect an antibody response to degenerate HBcAg. Alternatively wildtype virus may be present at levels which are below the level of sensitivity in the PMA but are sufficient to stimulate an immune response.

There was a high level of conservation of viral genotypes between patients and their sexual partners. The limited amount of sequence variation between partners was most frequent in patients with a fulminant infection. These patients were also the group in which mixtures of sequences were more commonly found. One patient with a fulminant hepatitis showed predicted amino acid sequence variation between the first and second samples (TCA to TCT to AGT, phenylalanine to leucine to lysine). This may be explained by rapid viral replication taking place under extreme immune pressure.

Alternatively, the partner may have had a mixture of viral genotypes present at very low

levels. Transmission may have occurred during a period of increased viral replication when quasi-species may be more numerous in the partner. Populations present at less than 10% of the total viral load would not generally have been detected by sequencing. HBV sequence variation between partners where sexual transmission was the most likely route of transmission has been reported. Aye *et al.*,(1994), reported differences of between zero and eight nucleotides between partners samples in the precore/core region. The results of Aye's study were in agreement with our findings and showed that the nucleotide variations that were seen were synonymous transitions.

In our study the partners were the patients' only admitted sexual contact at the time of infection. Other members of these families did not become infected, which helps to confirm the sexual route of transmission.

In this study we included a control group of patients with acute self limited hepatitis B infection. Precore mutant virus was found in four of the five patients from whom HBV DNA was amplified. Our results contrast with those of Hasegawa *et al.*,(1993) and earlier studies in which precore mutant virus was not detected in patients with acute hepatitis. However, the results of a study carried out by Uchida *et al.*,(1993) in which the precore mutant virus was found in patients with fulminant acute hepatitis corresponded with our findings. Similarly Tur-Kaspa *et al.*,(1992) did not find any correlation between the presence of precore mutant virus and either HBV disease severity or ethnic origin.

Carman *et al.*,(1990) also found the precore mutant virus in patients of Mediterranean origin with fulminant, symptomatic or asymptomatic hepatitis and suggested the presence of a double mutant (precore codons 28 and 29) may be related to the severity of disease. Our results did not confirm this hypothesis as the double mutant was found in both groups of patients.

The apparent discrepancies between studies examining the prevalence of precore mutant virus may be explained by the geographical distribution of HBV strains. Féray *et al.*,(1993) showed a low prevalence of precore mutant virus in French patients with a fulminant hepatitis. Li *et al.*,(1993) suggested that the prevalence of precore mutant virus may be related to the nucleotide sequence of codon 15 in the precore region. The precore codon 28 mutation disrupts the hair-pin loop formation of the precore region that is

essential for pregenome packaging in a genotype designated type A but not in a genotype designated type D. Genotype A is prevalent in France where precore mutations are rare and genotype D is prevalent in China where precore mutant viruses are more common. The sequence in our samples is CCU (type D) so that the precore mutation produces a viable virus.

Two of the patients in this study had an HBeAg positive infection despite the absence of detectable wild type virus. This indicates that an alternative mechanism for the cessation of HBeAg production exists. The partners of these patients carried a mixed viral infection.

The point mutation assay was important for the detection of subpopulations which were not detectable by direct sequencing. This clarified situations were the partner and patient appeared to have different viral types. Having found the precore mutant virus in both the study and control groups we looked for differences in the viral load but did not find any. The levels varied over a similar range in both groups.

We have confirmed the sexual transmission of HBV with a defective precore region. The mutant virus may be involved in the development of a more severe hepatitis but is not exclusive to such patients.

3.10 Novel application of a point mutation assay; evidence for transmission of hepatitis B viruses with precore mutations, and their detection in infants with fulminant hepatitis B.

3.10.1 Objectives

- To study the role of hepatitis B viruses with precore mutations in neonatal hepatitis and to compare the sequence identity between mothers and infants.

- To analyse HBV DNA amplified from the serum of infants with neonatally acquired fulminant hepatitis and their mothers with primers to the x/precore/core genes and the A determinant of the surface gene.

3.10.2 Introduction

In section 3.9 evidence was presented for the transmission of precore mutant hepatitis B virus. Perinatal transmission of HBV infection is strongly associated with HBeAg positivity in the mother (Okada *et al.*,1976; Beasley *et al.*,1977; Wheeley *et al.*,1990). Perinatal transmission of HBV from mothers whose serum contains anti-HBe has also been documented and may be associated with a fulminant course in the infant (Sinatra *et al.*,1982; Deinhardt *et al.*,1985).

Precore mutations have been associated with the development of fulminant acute hepatitis B in neonates (Terazawa *et al.*,1991). However, the nature of the association between transmissibility, the severity of disease and precore mutants remains uncertain.

In our study the point mutation assay (PMA) was applied (Syvanen *et al.*,1990; Kaye *et al.*,1992) to detect single nucleotide mutations in the precore gene of HBV to determine whether the transmission of HBV infection from four HBV carrier mothers and one healthcare worker, all of whom were anti-HBe seropositive, was associated with the presence of precore mutations and whether they may be associated with the development of fulminant hepatitis B in infants.

3.10.3 Patient population

Serum samples were examined from all cases of clinical hepatitis B in infants investigated in the West Midlands region of the United Kingdom between 1974 (when antenatal

screening for HBV infection was introduced) and 1990. The clinical and serological details of four of the six mother-infant pairs have been published (Case 1; Case 2, 1st and 2nd infants; Case 3) (Beath *et al.*,1992). Sera from all the mothers were HBe-antigen negative and three of the six babies developed fulminant hepatitis B. Clinical and serological details are summarised in table 3.10(i).

The affected infants not previously described include a third infant of the mother in Case 2. This infant was given one dose of HBV vaccine at birth but was not followed up. At 10 weeks of age the infant developed acute hepatitis, which followed a fulminant course. The infant was given a liver transplant, but died in the early post-operative period. At presentation HBsAg was detected in the infant's serum. Anti-HBc (total) was present in the serum but anti-HBc-IgM was not detectable.

The infant in Case 4 was the second child of an anti-HBe seropositive HBV carrier mother of European origin. This infant developed acute hepatitis B which followed an uncomplicated course to recovery. The 7 year old sister of this infant had no history of clinical hepatitis but was found to be immune to HBV infection.

One serum sample was available from a healthcare worker (HCW) who had been implicated in a work-related transmission event. The HCW was found to be an anti-HBe seropositive HBV carrier, with HBV DNA in serum detectable by liquid hybridisation assay. The patient to whom the healthcare worker was believed to have transmitted infection, a regular blood donor, was HBsAg-negative and anti-HBc negative three months prior to abdominal surgery. Three months after surgery, the patient developed acute hepatitis, but by the time of presentation was HBsAg negative, anti-HBc and anti-HBcIgM positive. The patient made a full recovery.

3.10.4 Methods

HBV serological markers were detected by a commercial RIA and passive haemagglutination assay. Anti-HBc IgM was detected by capture radioimmunoassay.

HBV DNA was detected by a liquid hybridisation assay (Abbott Genostics). HBV DNA PCR was initially carried out on 1μl of serum as described in section 2.6.3 in a nested reaction with core primers 188/189 and primers 439/441. When HBV DNA could not be

amplified by this method 50μl serum was treated as described in section 2.6.6. In three cases insufficient serum was available for purification and 1 μl of sample was amplified in a double nested reaction (section 2.6.9) with precore primers 188/189 in round 1, primers 439/666 in round 2 and 439/441 in round 3.

The amplified HBV DNA was sequenced as described in section 2.9, codons 1 and 28 of the precore region were also assayed by PMA (section 2.11) using probe-primers PMA 1, 28a and 28b respectively.

Table 3.10(i) Summary of Cases, HBV serology and liver function tests

Case	Mother/ infant pairs	Clinical summary	HBsAg	HBeAg/ anti-HBe	HBV DNA (pg/ml)	anti-HBc-IgM[b]	AST[c]
1	Mother/ infant	Acute hepatitis B at age 15 weeks-recovered	1:8,000 1:8,000	Ab Ab	2.4 nt	- +	2,380
2a	Mother/ first infant	Immune-vaccinated	>1:8000 +(RIA only)	Neither nt	nt nt	nt nt	
2b	Mother/ second infant	Not immunised;fulminant hepatitis B, died	1:8000[d] >1:8000	Ab Ab	1.7 nt	- +	751
2c	Mother/ infant	One dose vaccine at birth, fulminant hepatitis B, liver transplant but died	1:2000 1:8,000	Neither Ab	206 nt	- -	3,340
3	Mother/ infant	Fulminant hepatitis B, liver transplant survived	1:12800 +(RIA only)	Ab Ab	0 nt	- +	2,117
4	Mother/ infant	Acute hepatitis B at age 3 months-recovered	1:2000 1:8000	Ab Ab	nt nt	nt nt	

Notes

Ab antibody. nt not tested. AST aspartate transaminase. b All sera had detectable total anti-HBc

c Liver function tests on the day of presentation for cases of neonatal hepatitis only. d Cord blood sample

3.10.5 Results

The PCR results are summarised in table 3.10(ii). HBV DNA was not amplified from the mother and infant in Case 1 and from the infant in Case 3 by either nested or double nested PCR. There was insufficient serum available to attempt purification of these samples. In all other Cases the HBV DNA amplified from these samples was generally conserved. None of the samples showed mutations of codon 1 in the precore region by direct sequencing or PMA. The full sequences of the X precore regions are shown in Appendix 7.

All of the samples from Case 2 had mutations in codons 28 and 29 of the precore region. The mutation of codon 28 introduced a premature stop codon which prevented the translation of HBeAg. The sense probe-primer was used for the PMA of codon 28 in this Case as the additional mutation at codon 29 prevented efficient hybridisation of the anti-sense probe-primer. An additional sample taken between the second and third pregnancies gave similar results.

The sample from Case 3 had no mutations in the precore region. In Case 4 both the mother and infant carried a subpopulation virus of mutant for codon 28.

Ninety seven percent of the sample from the healthcare worker contained a single mutation at precore codon 28 of the sample. The codons for amino acids 122-154 of the X region were also sequenced, Case 2a carried a silent mutation at codon 142 (GTC to GTG), 1 amino acid substitution was detected in Case 4 (isoleucine to methionine, ATT to ATG).

Table 3.10(ii) Analysis of point mutations in the hepatitis B virus precore region at amino acids 1 and 28 amplified by PCR from serum samples of mothers and infants implicated in transmission events.

Case	Mother/ infant pairs	HBeAg/ anti-HBe	PCR	% mutant[1] (% W.T.) aa 1	% mutant[1] (% W.T.) aa 28 Anti-sense primer	% mutant[1] (% W.T.) aa 28 Sense primer
1	mother	Ab	-	nt	nt	nt
	infant	Ab	-	nt	nt	nt
2	mother	Neither	+	<3 (94)	70 (8)	96 (<3)
	1st infant[2]	nt	+	nt	78(<3)	90 (3)
	mother	AB	+	<3 (95)	82(<3)	95 (<3)
	2nd infant	Ab	-		nt	nt
	mother	Neither	+	<3 (95)	40(<3)	94 (<3)
	3rd infant	Ab	+	<3 (95)	25(<3)	93 (<3)
3	mother	Ab	+	<3 (93)	<3 (98)	nt[3]
	infant	Ab	-	nt	nt	nt
4	mother	Ab	+	<3 (96)	9(86)	nt[3]
	infant	Ab	+	<3 (100)	86(80)	12 (80)

Notes

1 A mutant sequence is defined as :Amino acid 1: substitution of A to C. Amino acid 28: substitution of G to A. The limit of sensitivity of the assay was a 3% subpopulation

2 Cord blood sample.

3 Use of the PMA with sense primers was not required (see text).

nt Not tested.

WT Wild type viral sequence

3.10.6 Discussion

HBV DNA was amplified from three of four cases where hepatitis B infection was transmitted from an HBsAg/anti-HBe positive mother to her child. The amplified DNA was sequenced and analysed specifically for mutations of codons 1 and 28 in the precore region by PMA. The PMA was designed to detect mutations at codons 1 and 28 of the precore region. Existing methods for the detection of mutations in viral sequences include direct sequencing of PCR products and sequencing cloned PCR products.

However, these methods are time consuming and expensive. Other methods such as the amplification refractory mutation system (Newton *et al.*,1989) and the differential amplification system (Boucher *et al.*,1990) have been used for the analysis of point mutations.

A significant advantage of the PMA over all of these methods is that large numbers of samples can be analysed in a standard 96 well microtitre plate format. The assay provides both qualitative and quantitative data, being able to detect small subpopulations of as little as 3% of the total hepatitis B viral load. The sensitivity of this application of the PMA compares well with results previously obtained for HIV-1 mutations by Kaye *et al.* (1992).

Sequence diversity can be a limiting factor for the PMA as illustrated in Case 2. Other mutations at or near the 3' end of the target sequence (codon 28) can hinder probe hybridisation, in this case a mutation in the adjacent codon (codon 29). This problem was overcome by using a sense probe to hybridise upstream of the point mutation site. High background signals caused by non-specific amplification can be avoided by careful optimisation of the PCR and use of a nested or double nested format.

The precore and part of the X region were sequenced directly to confirm the results of the PMA and to identify other sequence variations. In all Cases direct sequencing and the PMA results were concordant.

No samples were detected in this study with a mutation at codon 1 of the precore region, although a mutation at this position has been previously reported in association with the precore codon 28 mutation (Santantonio *et al.*,1991).

Samples from both the mother and the infant in Cases 2 and 4 had mutations at codon 28 in samples. In Case 2 the viral population was homogenous for the mutant type virus in both the mother and the infant. A second mutation was present at codon 29 which altered glycine to arginine. In Case 4 this mutant formed a part of a mixed population with wild type virus. A second codon change leading to amino acid variation was also found in this sample in the X region at codon 126 causing the substitution of methionine for isoleucine. No nucleotide sequence variation was detected within the mother infant/pairs consistent with perinatal transmission. This confirms previous evidence indicating that transmission of mutant viruses does occur from mother to infant (Terazawa *et al.*,1991).

The mutation of a tryptophan to a stop codon at codon 28 has been associated with high levels of circulating HBV-DNA particularly in patients from HBV-endemic areas and usually in association with evidence of severe chronic liver disease. All the mothers studied here, including two with mutant viruses, were asymptomatic with no evidence of clinically significant liver disease.

Most reports of precore mutants have been in patients who are anti-HBe seropositive. One of the patients studied here (mother in Case 2), had neither HBeAg nor anti-HBe detectable during her first and third pregnancies, but was anti-HBe positive at the time of the second. Samples from all time points contained identical HBV sequences with precore mutations, at a prevalence in excess of 95%.

Most instances of vertical transmission of HBV arise from HBeAg positive carrier mothers, and most HBV infected infants are asymptomatic. In the West Midlands region, there is approximately one case of clinical neonatal or early infant hepatitis for every 750 pregnancies in anti-HBe positive mothers (E H Boxall, personal observation).

Here we report on three infants with fulminant and two with uncomplicated hepatitis B. One of the fulminant cases was certainly associated with a precore mutant virus in mother and infant. In the two other fulminant infections HBV DNA could not be amplified from the infant and therefore the genotype could not be determined. However, in one the mother carried the mutant (Case 2) but in the other (Case 3) the mother did not. For one of the two infants with uncomplicated hepatitis it was not possible to determine the HBV genotype from either mother or infant (Case 1). For the other infant, DNA sequences

from both mother and infant were of a mixed genotype.

These findings confirm that although fulminant hepatitis in infants may be associated with precore mutations, they are not always present, even where transmission has occurred from an HBeAg negative mother. The lack of a consistent association between vertical transmission and the presence of precore mutant viruses means that infection with such viruses is not a predictor of transmission in all cases. This data supports the recommendation that all infants born to HBsAg carrier mothers should be immunised, and this should be the policy regardless of the presence of precore mutants and the HBe status of the mother.

Case 2 may also illustrate that HBV immunisation is protective against disease caused by HBV mutant strains. The first baby in Case 2 was given HBIg at birth and received a complete course of four doses of vaccine which led to seroconversion for anti-HBs. A sample of cord blood did contain HBV DNA with precore mutations, but the baby did not develop hepatitis and at follow up has remained HBsAg and anti-HBc negative. The second infant in Case 2 received only a single dose of HBV vaccine at birth and the third none; neither received hyperimmune globulin and both developed fulminant hepatitis B.

Precore mutations are not always associated with high levels of HBV DNA in serum, nor do they have to be the result of a long period of HBV carriage. In Case 3 only a low level of circulating HBV DNA was detected in the serum sample from the mother who had probably been a carrier for only approximately 15 months (Beath *et al.*,1992). Despite the low level of HBV-DNA the infant was infected. An adverse outcome of vertically transmitted hepatitis B has been associated with recent maternal seroconversion to anti-HBe (Ewing *et al.*,1985), but it is not known whether this is related to the presence of precore mutations.

Analysis of the serum from a healthcare worker, who had been implicated in transmission of HBV infection to a patient during a surgical procedure, revealed a mutation at aa28 of the precore region. This case suggests that anti-HBe seropositive HBV carriers cannot be regarded as presenting no risk to their patients. Unfortunately no samples were available from the infected patient in order to confirm, by sequence homology, this presumed transmission. More recently two other cases of transmission from healthcare workers who

were anti-HBe positive HBV carriers have been identified who also had precore mutant virus (Zuckerman *et al*, in press. Hawkins and Zuckerman, unpublished observations). One of these (Zuckerman *et al*, in press) is described in section 3.13.

The study has confirmed the association of precore mutants with some, but not all cases of transmission of HBV from HBeAg negative mothers to their infants and similarly with some cases of fulminant disease. Transmission from a healthcare worker with anti-HBe may also have been associated with precore mutants.

Section 3.11 Hepatitis B virus escape mutant following liver transplantation

3.11.1 Objectives

- To study HBV sequence variation associated with reactivation of hepatitis B infection following liver transplantation

- To analyse variation in the *a* determinant of the surface gene in response to hyper-immune globulin prophylaxis

- To assay the development of mutations in codons 1 and 28 of the precore region and codon 145 of the surface gene *a* determinant by PMA

3.11.2 Introduction

The hepatitis B virus (HBV) envelope comprises three proteins; the major protein (226 amino acids), the large protein (389-400 amino acids) and the middle protein (281 amino acids).

The major surface protein is highly immunogenic and carries the variable subtype determinants as well as the highly conserved, group specific *a* determinant. This determinant is the target for neutralising antibody following natural infection. Antibody against this determinant provides protective immunity following vaccination with both plasma derived and recombinant vaccines. Laboratory assays for HBsAg which are based on monoclonal antibodies are also dependent on this determinant.

Although some monoclonal antibody based assays may not detect the mutant type surface antigen, it is detected by polyclonal or haemagglutination assays. Thus both the routine detection and prevention of hepatitis B rely on the conservation of the *a* determinant.

The A determinant spans amino acids 124 to 147. Variation in the viral genome at codon 145 has been identified in apparently immune infants with high titre anti-HBs following vaccination (Carman *et al.*,1990).

Sequence variation in the *a* determinant has also been identified in patients undergoing monoclonal anti-HBs therapy to prevent re-infection of a transplanted liver. McMahon

et al.,(1992) detected the same codon 145 mutant sequence in a patient after a liver transplant carried out for chronic HBV-related liver disease. Wild type virus had been isolated prior to transplantation.

Graft infection is common following liver transplantation for chronic HBV related liver disease and frequently causes rapidly progressive liver failure (Samuel *et al.*,1991a, O'Grady *et al.*,1991). Various histological appearances have been reported, ranging from massive hepatic necrosis to cirrhosis and frequently a characteristic condition, fibrosing cholestatic hepatitis (Davies *et al.*,1992). The reduction of graft infection and disease is achieved by the administration of polyclonal anti-HBs (Samuel *et al.*,1991a).

Three cases of three HBV infected patients who underwent liver transplantation but became, or remained, HBsAg positive despite anti-HBs treatment are described. HBV DNA amplified from serum samples taken before and after transplantation have been analysed by direct sequencing and by use of a point mutation assay (Hawkins *et al.*, in press).

3.11.3 Patient population

Samples were investigated from three patients all of whom had undergone liver transplantation for end-stage, chronic HBV related liver disease. The HBV infection recurred in all three patients after transplantation.

Case 1

This patient was an Italian female aged 59 transplanted for decompensated liver disease. Four samples were analysed from this patient, one pre-transplant sample and three post-transplant samples. Prior to transplantation the patient was seropositive for HBsAg, and anti-HBe but seronegative for anti-HCV and anti-HDV, HBcAg was distributed in both the nucleus and the cytoplasm of her explant. Transplantation (Tx) was carried out on 30/9/91, Hepatitis B immunoglobulin (HBIg) was administered preoperatively (5000 units per day). This was stopped 10 days after Tx as a maintenance level of greater than 100 IU/l was attained. HBsAg remained detectable (Amerlite™ monoclonal HBsAg assay) throughout the postoperative period.

The patient under went biopsy after two weeks and showed no evidence of HBV

infection. A second biopsy on 11/1/91 revealed the presence of nuclear and cytoplasmic HBcAg.

The patient remained anti-HBs seropositive despite HBIg therapy being withdrawn. The patient became anti-HBe positive at this time and remained so until she developed fibrosing cholestatic hepatitis, suffered liver failure and died after 24 weeks.

Case 2

This patient was a 49 year old Japanese man with a history of chronic hepatitis (1975) and cirrhosis (1980). The patient was transplanted for decompensated chronic liver disease on 24/3/91. He was seropositive for HBsAg and anti-HBe but seronegative for anti-HBc, anti-HDV and HBV DNA. The explanted liver contained HBcAg distributed in both the nucleus and the cytoplasm. He received HBIg prophylaxis as in Case 1; at day three anti-HBs was 44 IU/l and at day 10, greater than 1000 IU/l. A biopsy at week two revealed no evidence of HBV infection.

The patient was discharged and remained well for seven months, maintaining an anti-HBs titre of >1000 IU/l after HBIg treatment had been stopped.

At seven months the patient developed symptomatic acute hepatitis. This was confirmed by liver biopsy which revealed HBsAg and HBcAg. He was tested for serum HBV markers at this time and was HBsAg seronegative by monoclonal assay with an anti-HBs titre of 44 IU/l. He remained negative in all other samples except one by the Amerlite™ Assay.

HBsAg was detected in these samples after analysis by a haemagglutination assay (Hepatest™). The anti-HBs level began to decrease and became undetectable nine months later.

The patient died at 11 months from liver failure due to cholestatic hepatitis.

Case 3

Case 3 was a 46 year old Italian male transplanted for chronic liver failure. He was seropositive for HBsAg, anti-HCV and seronegative for HBeAg, anti-HBe and anti-HDV.

His explanted liver contained HBcAg which was distributed in both the nucleus and the cytoplasm. He received conventional HBIg prophylaxis. Levels of anti-HBs decreased with time after the transplant, 985 IU/l at two weeks and 75 IU/l at seven weeks. HBsAg was not detectable in these samples by either monoclonal or polyclonal assay. Serum HBsAg and HBeAg was first detected at week six. The patient underwent liver biopsy at three months which revealed fibrosing cholestatic hepatitis and he died at six months of liver failure.

3.11.4 Methods

During the clinical management of the patients HBsAg was assayed initially by a monoclonal-antibody based assay, (Amerlite™, Amersham, UK) and then by a polyclonal-antibody based reverse passive haemagglutination assay (Hepatest™ Murex diagnostics UK Ltd). HBeAg/anti-HBe and anti-HBc were assayed by commercial immunoradiometric techniques.

Liver biopsy was examined by standard histological techniques Biopsy material was assayed for HBsAg and HBcAg using polyclonal antibodies.

Nested PCR was performed on heat treated serum (section 2.6.4) with primers to amplify the *a* determinant of the surface gene (primers 163/363 round 1, primers 365/366 round 2) and the two major subtype determinants for *d/y* and *w/r*. The precore region was also amplified as previously described. The PCR product was biotinylated as described in section 2.7 and analysed using the single stranded sequencing method described in section 2.10.2. The point mutation assay was employed to detect mutations of amino acid 145 in the surface gene *a* determinant and amino acid 28 of the precore region (section 2.11).

3.11.5 Results

Case 1

HBV was amplified from all Case 1 samples. Point mutation analysis of amino acid 145 showed that there was a small subpopulation of virus with a mutation at this point. In this case glycine was converted to arginine by the mutation of G to C at nucleotide 1902. The level of mutant virus detected varied between 2.7 and 5.4% and was present in pre and post-transplant samples. These results were confirmed by a repeat assay.

The nucleotide sequences were otherwise well conserved in the surface region.

Comparison with reference sequence pHBV 130 allowed the subtype of this sample to be determined as *ayw*.

The precore region, which was amplified from all four samples from this patient, was also analysed by PMA for mutation of codon 28 (stop codon for tryptophan). The mutation was detected in all pre and post transplant samples. The post-transplant samples had an homogeneous viral population of mutant type (greater than 98%). The pre-transplant sample had a minor subpopulation of wild type virus present (3.1%). The results are summarised in table 3.11(i).

Sequencing of the precore region in both pre and post transplant samples showed that amino acids 28 and 29 were mutant. One additional mutation was detected in all samples at codon 126 of the X gene (isoleucine to leucine, **A**TT to **C**TT).

Case 2

HBV DNA was amplified from both pre-transplant samples and from 15/21 post-transplant samples. HBV DNA became detectable by PCR two months after transplantation. Comparison with reference sequence pHBV 130 allowed the subtype of this sample to be determined as *adw*.

PMA analysis of the pretreatment samples showed that there was a subpopulation (7.6% and 30%) of A determinant mutant type virus present. This was as described in Case 1 with the mutation of G to C at nucleotide 1902. This mutant type was detectable in 1/15 HBV DNA positive samples at a very low level (3.5%).

The presence of the mutant virus with the unusual nucleotide substitution of C for G was verified by serially diluting one sample so that the polymerase reaction was carried out on a single HBV particle. Five HBV particles were analysed in two PMAs The results are summarised in table 3.11(ii). Four molecules were homogenous for the wild type virus ie nucleotide G was detected and, one molecule was homogeneous for the alternative mutant type virus ie nucleotide C was detected. The resolution of the previously mixed sample into two distinct populations indicated that the amplifications in this assay were from a single molecule.

The same method was used to analyse six molecules amplified from a post-transplant sample, all of the molecules showed homogeneous PMA product of > 98% of G to A mutant type virus. Neither the wildtype virus nor the alternative mutant type virus were detected in this sample

The remaining post-transplant samples had the mutation G to A and this became the dominant population. The proportion of wild type virus fell to levels which were at or below the limit of sensitivity of the assay.

A sample from the cross sectional study of the surface region (section 3.13) with a mutation at codon 143 is also shown for comparison. One other sequence change was noted in the post transplant samples at surface region codon 128, (alanine to valine, GCT to GTT).

Sequences with a mutation of codon 28 of the precore region were detected in 27% of the viral load of the pre-transplant sample. Analysis of a post-transplant sample showed that the virus load was homogeneous for the wild type. In addition mutations at codon 29 in the precore region(glycine to arginine), codon 143 in the X region (alanine to phenylalanine) and a silent change at codon 142 of the X region (TGC to TGT).

Case 3

HBV was detected in the pre-transplant sample and all three post-transplant samples. There was no evidence of mutations in the s region either before or after the transplant (table 3.11(iii)).

The precore/ X region was sequenced and contained one mutation at amino acid 126, (isoleucine to threonine (ATT to ACT) in both the pre- and post-transplant samples. PMA analysis of precore region codon 28 indicated the presence of a mixed population of wild type and mutant virus in all samples.

Table 3.11(i) **Case 1: Results of tests for hepatitis B surface antibody and antigen, and point mutation assay of surface gene codon 145 and precore gene codon 28**

Time (weeks)	HBsAg detected by:		Anti-HBs	Surface gene codon 145 nucleotide sequence			Precore gene codon 28 nucleotide sequence	
	Amerlite[1]	Hepatest[2]		Mutant types		Wild type	Mutant type	Wild type
	Units	Reciprocal titre	IU/l	CGA	AGA	GGA	TAG	TGG
				percent viral load			percent viral load	
Pre-transplant	1663	>2048	0	5.4	<2.5	92	>97.5	<2.5
Post-transplan t4	516	8	25	5.4	<2.5	92	96.9	3.1
8	1663	>2048	0	4.5	<2.5	94	>97.5	<2.5
24	850	>2048	0	2.7	<2.5	95	>97.5	<2.5

Notes

1 Monoclonal-based chemiluminescence assay

2 Polyclonal-based reverse passive haemagglutination assay

Table 3.11(ii) Case 2: Results of tests for hepatitis B surface antibody and antigen, and point mutation assay of surface gene codon 145 and precore gene codon 28

Time (weeks)	HBsAg detected by		Anti-HBs	Surface gene codon 145 nucleotide sequence			Precore gene codon 28 nucleotide sequence	
	Amerlite[1]	Hepatest[2]		Mutant types		Wild type	Mutant type	Wild type
	Units	Reciprocal titre	IU/l	CGA	AGA	GGA	TAG	TGG
				percent viral load			percent viral load	
Pre-transplant	720	512	0	30.1	<2.5	70.6	26.7	72.8
Post-transplant 4	0	<8	>1000	HBV DNA not detected				
8	0	<8	>1000	<2.5	96.7	3.2	NT	NT
16	0	<8	>1000	NT	NT	NT	NT	NT
20	0	<8	706	NT	MT	NT	NT	NT
24	0	<8	638	NT	NT	NT	NT	NT
28	0	1024	13	<2.5	95.8	2.8	NT	NT
36	0	1024	0	3.5	90.6	5	NT	NT
44	0	512	0	<2.5	>97.5	<2.5	>97.5	<2.5

Notes: NT Not tested. 1 Monoclonal-based chemiluminescence assay. 2 Polyclonal-based reverse passive haemagglutination assay

Table 3.11(iii) Case 3: Results of tests for hepatitis B surface antibody and antigen, and point mutation assay of surface gene codon 145 and precore gene codon 28

Time (weeks)	HBsAg detected by		Anti-HBs	Surface gene codon 145 nucleotide sequence			Precore gene codon 28 nucleotide	
	Amerlite[1]	Hepatest[2]	IU/l	Mutant types		Wild type	Mutant type	Wild type
	+/-	+/-		CGA	AGA	GGA	TAG	TGG
				percent viral load			percent viral load	
Pre-transplant								
-18	+	+		<2.5	<2.5	>97.5	10.8	89.0
-10	+	+		<2.5	<2.5	>97.5	59.2	39.9
Post-transplant 2	-	-	985	NT	NT	NT	NT	NT
4	-	-		NT	NT	NT	NT	NT
5	+	+	75	NT	NT	NT	NT	NT
7	+	+		<2.5	<2.5	>97.5	35.6	63.1
14	+	+		<2.5	<2.5	>97.5	59.2	39.9

Notes
NT Not tested. 1 Monoclonal-based chemiluminescence assay
2 Polyclonal-based reverse passive haemagglutination assay

3.11.6 Discussion

We have analysed serum samples from three patients who sustained reinfection of their graft following liver transplantation, despite receiving prophylaxis with HBV hyperimmune globulin. All three patients died of recurrent HBV related liver disease between 6 and 11 months after transplantation. In all cases the histology revealed fibrosing cholestatic hepatitis.

Pre- and post-transplantation HBV sequences were amplified from serum using a PCR assay and analysed with the point mutation assay.

In two patients (Cases 1 and 2), nucleotide sequences with a mutation affecting codon 145 of the surface gene were identified prior to transplantation, as subpopulations of the total viral load. The mutation observed at nucleotide 1902, substitution of A for G or C for G, has been reported previously. Both nucleotide substitutions are predicted to result in the same amino acid change, from arginine to glycine. The substitution of a purine with a purine i.e. a transition, is more likely to occur than a transversion in which a purine would be replaced with a pyrimidine, for example C for G. The more frequently observed sequence variation at codon 145, the transition of A for G, only occurred after transplantation. It was perhaps more stable when compared to the transversion observed in pre-transplant samples as it (A for G) became the dominant genotype within a short period post-transplant.

Sequence variation at amino acid 145, causing alteration to the *a* determinant, has been associated with vaccine failure and graft infection despite HBIg prophylaxis.

HBV DNA amplified from samples of Case 2 also exhibited sequence change at codon 145 both before and after transplantation. In this case Pre- and post-transplant sequences were different (G to C, and G to A respectively). This switch may reflect situation where the G to A mutant sequence was already present at the time of transplantation but was below the level of delectability of the PMA. Alternatively, the mutation may have occurred *de novo* post-transplantation. The observation that HBV DNA was not detectable until several months post-transplantation even by PCR amplification, would be consistent with either hypothesis.

The third patient was the only one without any evidence of sequence variation at codon 145. In this case, HBsAg and HBeAg became detectable six weeks post-transplant, as the anti-HBs titre declined. Reactivation of HBV infection with infection of the graft was of wild type virus despite immune prophylaxis.

Graft infection in Cases 1 and 3 was predominantly or entirely wild type. Short courses of HBIg were administered in both cases because of early evidence of graft infection. Infection of the graft has therefore been demonstrated in patients with (Case 2) and without (Cases 1 and 3) mutations affecting codon 145.

The results of serological tests for HBsAg were consistent with the sequence analysis. In Cases 1 and 3, HBsAg was detected by both monoclonal and polyclonal assays before and after transplantation, consistent with the predominance of wild type A determinant sequences. In Case 2, the only one to have predominantly or exclusively mutant sequences affecting codon 145 post-transplantation, HBsAg was only detected by a haemagglutination assay. These results confirm that if patients are to be followed serologically after transplantation, to detect reactivation of HBV infection, it is essential to use assays which are not dependent on the conservation of the *a* determinant.

Samples from these patients also contained mutations in the precore region affecting codons 28 and 29. Sequence variation in codon 28 from tryptophan to a stop codon has been associated with a broad spectrum of histological changes ranging from mild to severe liver disease in HBeAg seronegative patients, mostly from high endemicity countries (Carman *et al.* 1990).

An associated mutation of the adjacent codon 29 has also been described. All three patients described here were, by virtue of requiring liver transplantation, suffering from severe chronic liver disease, all were HBeAg negative, and all were from countries where HBV is endemic. The finding of precore mutant sequences in all three patients was therefore consistent with previous reports.

Whether the presence of the precore mutation was causally related to their severe liver disease remains unclear.

The presence of precore mutations may also have been related to the reactivation of HBV infection post-transplantation if, as has been proposed, mutations in the precore region are associated with loss of immune regulation of chronic HBV infection.

From the evidence presented here graft infection may occur whether patients have low levels of HBV DNA, detectable by PCR only, as in Case two or high levels of HBV DNA ,detectable by standard hybridisation assays, as in Cases 1 and 3 pre-transplant. Mutations of the precore region may be associated with increased levels of HBV replication leading to rapid graft infection as shown in Cases 1 and 3.

A further study was carried out to determine the prevalence of mutations in the HBsAg *a* determinant among 64 UK HBV carriers who were described in section 3.5 (data not shown). Only one sample had an amino acid substitution the *a* determinant (codon 143 alanine to threonine). Codon 145 was conserved in all samples that were analysed indicating that this mutation rarely occurs in that population.

The identification of mutations in the surface region at codon 145 specifically but at other points in the *a* determinant may allow more appropriate prophylaxis as polyclonal HBIg may be ineffective in this situation. This could involve the use of cocktails of monoclonal antibodies which have been raised against mutant and wild type genotypes.

3.12 The application of sequence analysis to the detection of transmission events in clustered outbreaks of HBV infection.

3.12.1 Objectives

- To apply the study of sequence homology in the precore and surface genes to the investigation of clustered out breaks of hepatitis B infection.

3.12.2 Introduction

Perinatal and sexual transmission of HBV between anti-HBe positive individuals was demonstrated in sections 3.9 and 3.10. The regions of the HBV genome chosen for analysis in these studies were sufficiently well conserved to follow HBV transmission. The area of the surface genome analysed encompasses the highly conserved A determinant and the less conserved regions immediately upstream of it Lauder *et al*,. (1993). Similarly the area around the precore region contains both highly conserved and more variable regions.

These regions show sufficient divergence between subtypes and between individuals of the same subtype to allow confirmation of the source of infection in patients studied. The possibility of applying this type of analysis to the study of transmission events generally was therefore investigated.

The assay of HBsAg serological subtypes within HBV outbreaks is generally used to determine possible routes of transmission. However, this method is of limited use in such situations as the subtype alone may not distinguish the patients under investigation from HBV infected individuals in the general population.

An advantage of direct sequencing of amplified HBV DNA over routinely applied serological analysis is the ability to distinguish between subtypes *w/r* as well as *d/y*. In some cases this may represent a viral population in which *d* and *y* subtypes (Ashton-Rickardt and Murray, 1989) and *w* and *r* (Kanagawa *et al.*,1992) may be exhibited by the same virus. In cases where serological subtyping is inconclusive, evidence for transmission may be based on the HBV DNA sequence alone.

Hospital acquired infections have been noted between healthcare workers and patients

during surgical procedures and between patients through shared equipment (Oren *et al.*,1989).

In the following study HBV DNA sequences from 15 samples were analysed to determine which, if any, were linked to an epidemiologically identified clustered outbreak of HBV infection. Samples were coded before analysis so that the study was carried out under blinded conditions. This allowed us to asses the possibility of applying the method to situations where epidemiological data was not available.

3.12.3 Patient population

Fifteen samples from 15 patients were analysed. These patients had been selected to include nine HBsAg positive, anti-HBe positive patients and six HBsAg positive, HBeAg positive patients. The group included five patients who had all been involved in an HBV outbreak.

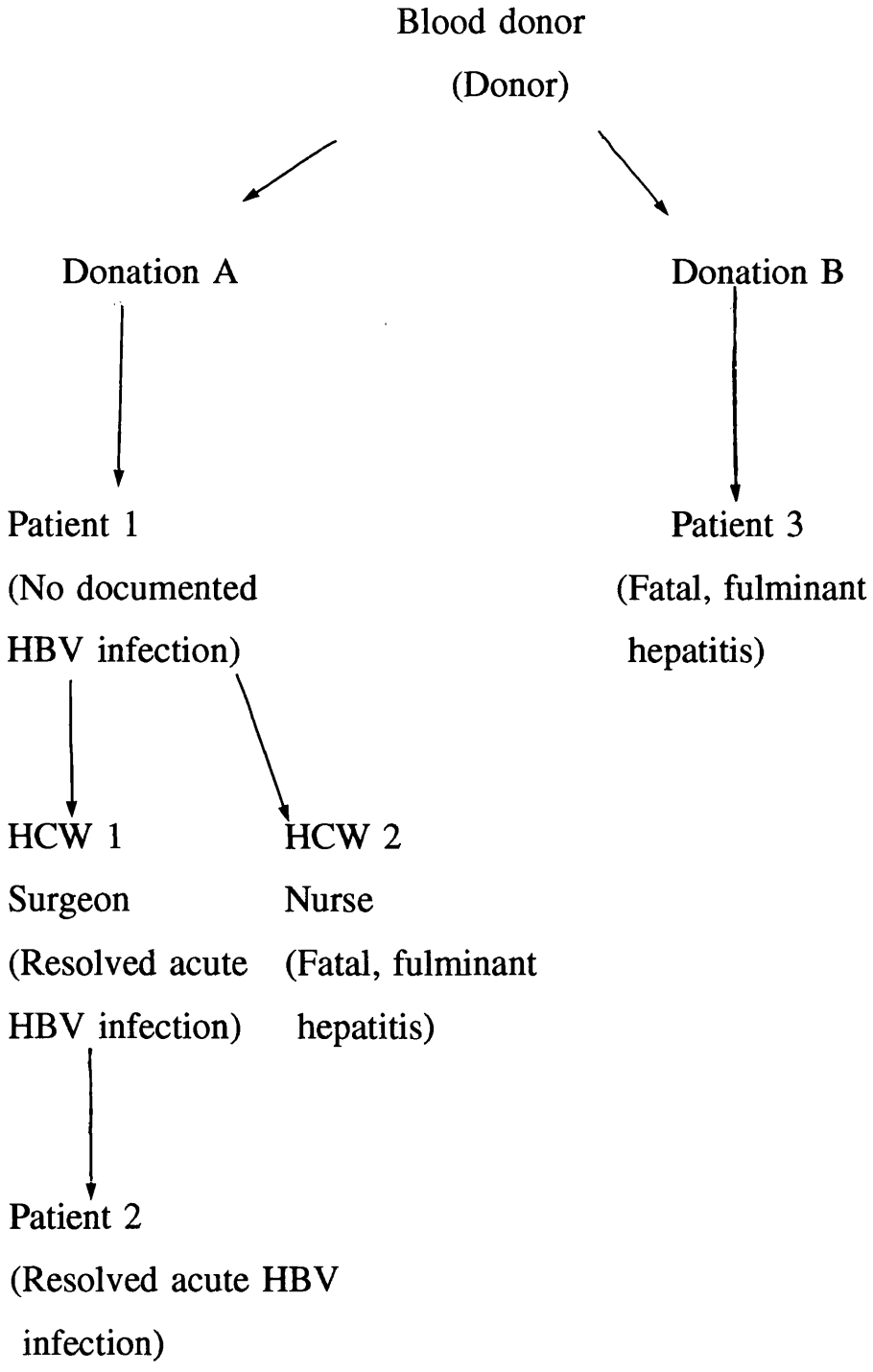
The first patient (patient 1) (figure 3.12(i)) had breast cancer and received blood from the implicated donor. She underwent a bilateral oophorectomy and died of metastatic disease. There were no specimens available from this patient who did not have a documented hepatitis.

Two health care workers (HCW 1 and HCW 2) were involved in the clinical management of this patient. HCW 1 was the surgeon who operated on patient 1. He developed an acute symptomatic HBV infection within two months of performing the operation and recovered fully. HCW 2 was the nurse involved in the care of the patient. This included procedures involving the drainage of pleural effusion and laying out of the body. Within two months HCW 2 developed an acute HBV infection and died of fulminant hepatitis.

The surgeon operated on Patient 2 whilst incubating HBV. Patient 2 developed an acute HBV infection and recovered.

Patient 3 was a 75 year old woman who underwent an orthopaedic procedure during which she received a blood transfusion from the implicated donor. She developed an acute HBV infection two months later and died from fulminant hepatitis.

Figure 3.12(i) Relationship between individuals involved in an hepatitis B outbreak



3.12.4 Methods

HBsAg detection was performed by EIA (Wellcozyme; Murex Diagnostics Ltd, UK.) and quantification carried out by reverse passive haemagglutination assay. Subtypes were determined by RIA and HBeAg/anti-HBe were assayed according to methods described by Tedder *et al.*,(1981).

Antibody to HCV was assayed by ELISA (Ortho HCV ELISA Test System, Third generation, Raritan, new Jersey, USA and Murex anti-HCV) Markers for HDV and HAV infections were tested for by ELISA (Murex Diagnostics Ltd, UK.).

HBV DNA was amplified from 20 μl of serum as described in section 2.6. The X/precore/core region and *a* determinant of the surface region were amplified as previously described .

Direct sequencing of both regions was carried out as described in section 2.10. The samples were coded and assayed by PCR, surface gene sequencing being completed before the code was broken. Codon 28 of the precore region was analysed by PMA as described in section 2.11.

The Kimura two-parameter method was used to analyse variance between the outbreak samples compared with that between control samples. The Fitch-Margoleish method was used to construct an unrooted phylogenetic tree.

3.13.5 Results

HBV DNA was amplified from 14 of 15 samples tested, with primers to the surface and precore regions.

The surface region had one silent nucleotide substitution in codon 138 (TGC to TGT) in five samples which were all of the same subtype (*ayw*). This group had a significantly less variation between themselves than was present between the other nine samples assayed ($p < 0.05$)(Figure 3.12(ii)). This was also the case when a similar comparison was made between this group and seven unrelated control samples of the same subtype.

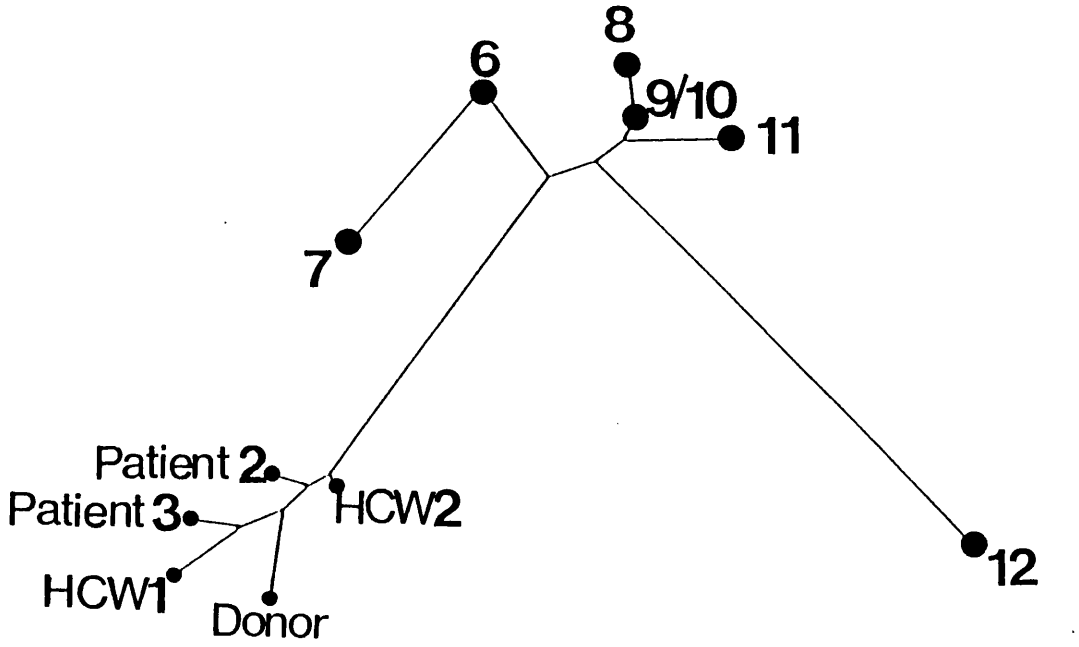
The branch length, in an unrooted phylogenetic analysis, between the putative cluster and

control samples helped to confirm this as a related group of samples. Thus, it was possible to conclude that these individuals formed a discrete group. The remaining nine samples were all highly conserved but none were identical. In all cases the inferred subtype by sequencing corresponded with the results of serological subtyping.

Once the code was broken it was revealed that the five individuals with the novel mutation had all been involved in the clustered outbreak of HBV described above. Precore sequencing revealed further mutations common to the group, codon 126 of the X gene (isoleucine to leucine), codon 28 of the precore region (tryptophan to a stop codon), and codon 29 of the precore region glycine to arginine. PMA analysis of codon 28 revealed a homogeneous mutant population in these samples. Serological analysis of HBeAg/anti-HBe using standard techniques did not reveal HBeAg in any samples. Modification of the assay did show a minor amount of HBeAg in the serum of HCW 1.

There was some sequence variation within the group in both regions studied. In the surface region (Appendix 7) this was restricted to published variants. The outbreak samples were compared with control samples of the same subtype as it was important to be able to distinguish groups from other samples of the same subtype (Appendix 7). A similar comparison of the X and precore regions was made between the outbreak samples and *ayw* control samples (Appendix 7).

Figure 3.12(ii) Unrooted phylogenetic analysis of *ayw* cluster and control samples



- Donor, HCW 1, HCW 2, Patient 2 and Patient 3 are as described in figure 3.12(i)
- Samples 6 to 12 are unrelated control samples of the same subtype (*ayw*)

3.12.6 Discussion of results

Five samples from a clustered outbreak of HBV infection were identified in a blind study from nine other non-related samples by sequence analysis of the 3' end of the X gene, the precore region and the *a* determinant of the surface gene.

The outbreak samples shared the same HBsAg subtype (*ayw*) and also contained a novel silent motif in the surface gene. Three codon changes in the X precore region determined amino acid substitutions at codon 126 (isoleucine-leucine), codon 28 (tryptophan to stop codon) and codon 29 (glycine to arginine). The non-related samples did not have any conserved novel changes.

HBV DNA sequence analysis has been applied to many cases of transmission and as in our study most have described a limited amount of sequence variation between related samples. The presence of multiple sequences (section 3.9, Kaneko and Miller, 1989) in some individuals which are not apparent after direct sequence analysis may partly explain this. One variant may become dominant and therefore apparent only after transmission.

HBV has been shown to be generally stable on transmission. In common with other groups (Lin *et al.*,(1990) our study has shown that passage of the virus over three transmission generations did not increase sequence variation in the regions of the genome that were studied.

HBV DNA sequencing of the precore and core genes has been used to exclude patients from epidemiologically defined outbreaks (Oren *et al.*,1989, Liang *et al.*,1991).

These reports describe the epidemiological and molecular investigations respectively of a nosocomial outbreak of hepatitis B in six patients, five of whom developed a fulminant hepatitis. The proposed mode of transmission was via a multiple-dose vial of heparin and normal saline flush solution. The index case was, as in our study, an HBsAg positive, anti-HBe positive carrier. Precore HBV DNA was analysed by sequencing. The source plus five patients were nearly identical to one another and contained unique mutations in that region. The sequence in the sixth patient did not have these mutations. In addition Patient 6 had 60 nucleotide differences in the 822 bp HBV DNA sequence spanning the X/precore/core region when compared with the other five patients. Thus, Patient 6 was

only excluded from the out break by HBV DNA sequencing.

An alternative approach is the analysis of short hypervariable sequences (Lin *et al.*, 1991). A 100 nucleotide sequence between the core gene and the pre-S region was amplified. Sequences were highly conserved between children and their HBV infected parents. Furthermore, all infected siblings in nine families also had identical HBV sequences which were not homologous with any recognised genotypes. However, as in our study differences were detected between individuals presumed to have been infected from the same source. This may reflect the relatively high mutation rate of HBV ($1\text{-}5 \times 10^{-5}$) (Orito *et al.*,1989) which is comparable with that of RNA viruses.

In another study, the same group (Lin *et al.*,1990) showed that the less variable S region was completely conserved within HBV infected members of Chinese families. This emphasises the influence that different rates of mutation throughout regions of the HBV genome may have on genotypic analyses. This must be controlled for when this method is used to identify HBV outbreaks.

We have tried to address this by analysing part of three different regions (X, precore and surface) which have different levels of conservation.

We have shown that subtype analysis by sequencing can be used to augment serological subtyping. An alternative approach using multiple primer sets to identify all four HBsAg subtypes in cases where serological subtyping had failed was described by Echevarria *et al.*,(1994). The main disadvantage of this method is that it would not allow the detection of a cluster among other samples of the same subtype.

The relationship of the precore mutation to disease severity has been discussed elsewhere. In our study the same mutation was associated with four different clinical patterns;chronic carriage (Donor), symptomatic acute infection (HCW 1) and fatal acute fulminant hepatitis. The two patients that died had apparently little in common, one was an elderly patient and the other a 22 year old nurse.

A possible dose effect was considered. However, Patient 3 had become infected via transfusion and HCW 2 through her care of Patient 2. Patient 1 had also received a blood

donation but did not develop a symptomatic hepatitis.

In this study we have shown that the analysis of short conserved regions of the genome can be used to support epidemiological and serological evidence in the investigation of transmission events.

Chapter 4 General discussion

Summary

This thesis describes the development and application of assays for the detection and analysis of HBV DNA. The effects of dual infection with HIV and HBV on the natural history of HBV have been examined. We have studied chronic HBV carriers and HBV infected patients who had either reactivated a previous HBV infection or had become reinfected. HBV DNA sequence variation in the X/precore/core and surface regions was analysed in these patients. The effect of antiviral treatment for HIV infection on HBV was also assessed.

Carman *et al.*,(1989) and many others since have described specific mutations of the precore region associated with unusual serological profiles. We have studied acutely and chronically HBV infected patients to determine if these mutant viruses arise *de novo* and also if they are transmitted.

We have studied the evolution of immune escape mutants in three liver transplant patients receiving passive immunisation. In some cases of chronic HBV carriage liver transplantation is the only available treatment. The prevention of graft infection by immune-globulin prophylaxis in these patients is a priority but, in the cases described in this thesis, this was unsuccessful.

The development of sensitive and specific methods for the detection and analysis of HBV have all been crucial in the studies described above.

4.1 Optimisation of the HBV DNA dot blot assay

The detection of HBV DNA by a dot blot hybridisation assay was the method of choice where viral quantification was required. In previous studies this has involved extensive sample preparation from large sample volumes. The simplified method of sample preparation for assay by HBV DNA dot blot described in this thesis was modified from that of Harrison *et al.*,. (1985). Our method did not require the ultracentrifugation of serum samples and used only 25 μl of serum for each analysis.

The highly purified probe and stringent hybridisation conditions adopted did not produce

false positive reactions due to non-specific hybridisation of the probe to the serum sample despite the lack of sample purification. Using this method 0.1 pg of HBV DNA (2.5 pg/ml equivalent to approximately 7 x 10^5 viral copies) was detected, this was comparable with other published methods (Weller *et al.*,1984, Moestrup *et al.*,1985, Kroggsgaard *et al.*,1986). There was a good correlation of HBV DNA with HBV DNA polymerase confirming that the HBV DNA dot blot assay was largely detecting intact, replication competent virus particles.

4.2 A polymerase chain reaction for the detection of HBV

The HBV DNA dot blot assay was not sufficiently sensitive to allow the detection of low levels of HBV in serum present at potentially infectious concentrations of 10 to 100 particles. The development of the polymerase chain reaction has overcome that problem. Mullis and Falloona (1985), Saiki *et al.*,(1985) and Chisaka *et al.*,(1986).

HBV DNA was first amplified from serum by Larzul *et al.*,(1988) using a single round of amplification. Kaneko *et al.*,(1989) compared the sensitivity of this method with slot blot hybridisation and showed that PCR was the more sensitive assay by a factor of $>10^4$. This allowed HBV detection at the lowest level of infectivity (Prince *et al.*,1983).

In almost all PCR based assays the removal of serum proteins which are inhibitory to *Taq* polymerase and stripping of the viral coat have been achieved by the treatment of serum with SDS, proteinase K and in many cases 2 ME. A comparison of buffers in this thesis confirmed the requirement for these components in serum extraction buffers when samples are not boiled. Pre-treatment of samples with RF absorbent has not previously been described. This process improved the sensitivity of the assay, particularly when samples had low viral titre in the presence of serum anti-HBe and anti-HBc. Possibly excess protein was removed, which would otherwise prevent efficient viral lysis or serum protein digestion.

SDS and proteinase K have been shown to be inhibitory to *Taq* polymerase (PCR Protocols, 1992). Sample dilution can eliminate inhibition by SDS and boiling the sample after buffer treatment inactivates proteinase K (PCR protocols, 1992). However, the successful amplification of DNA directly from boiled serum showed that proteinase K and detergent based buffers are not necessary for the extraction of HBV DNA from

serum.

Complex extraction procedures involving phenol/chloroform may make them susceptible to cross sample contamination, particularly when viral titres of $>10^{12}$ /ml have been noted in serum. Successive simplifications of methods of extraction described in this thesis and by others (Zeldis *et al.*,1989, Liang *et al.*,1989, Boom *et al.*,1991) have shown that these procedures are not necessary.

In 1989, Larzul *et al.*,. described the thermal treatment of purified viral particles and of crude serum. The analysis of the crude serum was very insensitive when compared to the purified HBV particles, (greater than $2x10^5$ particles /ml). These results indicated that amplification was inhibited either by serum factors, for example serum albumin or by physical trapping of the virus in the clot of heated serum. Frickhofen and Young (1991) showed that heating 10 μl of serum with 150 mM NaCl for 45 seconds at 70 °C allowed the detection of 10^5 copies /ml of parvovirus B19. However, they also found that heating serum with water only abolished any amplification under the chosen thermocycling conditions. Our studies did not confirm this, suggesting that the salt treatment rather than heating was exposing the DNA for amplification. Alternatively this difference in findings may have been due the direct addition of the PCR reaction mixture to the heated serum by Frickhofen and Young (1991), without removal of the serum clot by centrifugation.

All of the above methods relied on the additional sensitivity of Southern hybridisation or dot blotting for PCR product detection. The development of the nested PCR for HBV by Larzul *et al.*,(1988) removed the need for blotting as it both confirmed the specificity of the product and increased the sensitivity of the assay.

Vandenvelde *et al.*,(1993) suggested that denatured serum albumin was the main inhibitory component of *Taq* polymerase in serum. The addition of 5% sodium octanoate to the serum, which was diluted in PBS prior to amplification, allowed the detection 100 HBV particles/ml by ethidium bromide fluorescence. The simplicity of this method is comparable with the boiling method but it is of a two fold lower sensitivity. There was no confirmation of product specificity other than by size fractionation and suboptimal amplification of viral titres $<10^4$ HBV particles /ml would not allow analysis of the PCR product by for example direct sequencing.

In our study the PCR products were sequenced by two methods; double stranded from a gel purified product and single stranded from a biotinylated product. The success of sequencing was determined by the quality and quantity of the template.

4.3 Optimisation of PCR product sequencing

Double stranded sequencing was more frequently used before the incorporation of biotin in to the primers at synthesis was widely available. This method was effective but crucially dependent on exact reaction times and temperatures. The tendency of the template to reanneal limited the number of sequencing reactions which could be performed simultaneously. The sequencing primer to template ratio was also very important for efficient sequencing.

Double stranded sequencing was also useful when non-specific amplification occurred. By excising precisely the band of product required extraneous material was separated from the specific PCR product. This was particularly applicable to the analysis of products from single round PCR. The inclusion of the nested round of the PCR, with one biotinylated primer rendered this process largely obsolete.

Single stranded DNA templates have been produced in a variety of ways. Santantonio *et al.*,(1991) used asymmetric PCR and direct sequencing to study the variability of the precore region in anti-HBe positive chronic carriers. The nested round of amplification was carried out using only one primer and this produced sufficient DNA for several sequencing reactions. Labelling one primer with biotin was an alternative method which allowed the template to be made single stranded.

In both of these methods the PCR product was purified before sequencing to remove PCR reaction mix components. The use of the biotinylated primer allowed the non-biotinylated strand to be recovered and analysed. This was useful when confirming sequence variation. The biotinylated PCR product was captured on to streptavidin coated beads and sequenced while attached to the bead. This allowed up to 220 bases of a sequence to be read from a single reaction and the detection of mixed populations. When combined with single molecule amplification this method was of comparable sensitivity to the analysis of cloned PCR products. Amplifying single molecules was time consuming and expensive, in some cases only one nucleotide change was being analysed in otherwise

highly conserved regions. Therefore the point mutation assay was developed.

4.4 Modification of a point mutation assay for the detection of single nucleotide substitutions in HBV.

Several strategies for the detection of point mutations in a nucleotide sequence have been developed. These have involved three main approaches: restriction analysis of the amplified product, which has been used to identify two different populations where a restriction site is created or removed by the point mutation; allele specific amplification and hybridisation of a labelled internal probe to the PCR product.

The information gained from restriction analysis is limited. Loss of the restriction site may be due to an alternative mutation so that the only information gained is that the product is not a specific genotype. However, the sample may not be the previously identified mutant either. Restriction with two enzymes specific for the wild and mutant type would at least clarify that situation. The quantification of restriction fragments by ethidium bromide fluorescence is crude and not suitable for the discrimination of less than ten fold differences in the concentration of DNA (Old *et al.*,1990).

Allele specific amplification has the same limitations. This method is only useful where heterozygous or homozygous mutant or wild type alleles are present (Saiki *et al.*,1986, Embury *et al.*,1987, Heim and Meyer,1990). Spurious hybridisation of primers to alleles of the alternative type may occur when sample DNA is present at high concentrations. Ault *et al.*,(1994) described a type specific PCR for JC virus, which was highly specific as both of the internal primers in each type specific reaction had 3' nucleotides at points of variation. However, whilst this increased the specificity of the assay between the two types the primers would fail to amplify from a virus that was neither type 1 or type 2, creating false negative results. The use of a third ubiquitous primer set would resolve this.

Allele specific PCR has been applied to the detection of HBV precore mutants (Lo *et al.*,1992). A limitation of this method is the requirement for specific annealing temperatures. These are difficult to achieve as the actual temperature of the PCR machine block may fluctuate with the air temperature. Non-uniformity of temperature across the block or between different PCR machines may effect primer hybridisation. Lo's method

also required four separate amplification steps as the sample was quantified before the type specific primers were added. This helped to avoid spurious amplification in samples with very high viral titre.

The detection of viral mutants required a system which differentiated between the four possible genotypes in a quantitative manner. The use of labelled probes or primers provided an alternative approach. Chehab *et al.*,(1990) used primers conjugated to fluorescin dyes to identify two alleles of the sickle cell anaemia mutation. A method which identified all four possible nucleotides was described by Sokolov (1989). In that assay primers were extended by a single radiolabelled nucleotide over 30 cycles. Labelled product was then analysed by gel electrophoresis and autoradiography. Essentially this was the same principle as that adopted by Syvanen *et al.*,(1990) and Kaye *et al.*,(1992). An adaptation of Kaye's method was used in this thesis. The major modification of Sokolov's method was the attachment of the PCR product to a solid phase and the measurement of the incorporated radiolabelled nucleotide by scintigraphy. This provided a quantitative assay for the detection of any nucleotide present at the point of interest in that sequence. The method was sufficiently sensitive to detect minor subpopulations.

The presence of variable concentrations of PCR product in different samples did not adversely affect the assay as each sample was compared within itself from a single amplification. The provision of specific product by the nested PCR reaction ensured that subpopulations of as little as 3% of the total load were detected.

The exact correlation between sequencing and PMA results showed that the PMA was sufficiently reliable to be used as a diagnostic tool without additional confirmatory procedures. Circumstances where the PMA failed were seen. However, these results were not ambiguous. They were clearly wrong and therefore discounted. The failures were either no signal at all or high levels of isotope present with in all four reactions. In either case the product was sequenced to determine the cause of the failure. In all cases additional mutations around the 3' end of the probe/primer prevented efficient hybridisation to the PCR product. This was overcome by the design of an alternative probe/primer which hybridised to the opposite strand of the template.

An example of this was in the analysis of codon 28 of the precore region. The probe was

designed to hybridise down stream of the nucleotide under analysis so that the start codon of the core gene was close to the 3' end of the probe/primer. The core protein is essential for encapsidation and therefore deemed likely to be highly conserved. However, a second mutation in the adjacent codon (precore codon 29) was later found to be commonly associated with that of codon 28. This prevented the probe binding and produced low levels of non-specific signal. By using the alternative sense probe/primer the sensitivity and specificity of the assay were completely restored.

None of the above methods could have been developed without data provided by sequencing and all are limited by the requirement for sequence conservation around the point of analysis.

4.5 A cross sectional survey of HBV DNA in serum from HBV carriers.

The cross sectional analysis of HBV DNA and HBV DNAp described in section 3.5 demonstrated that coinfection with HIV is associated with increased HBV viraemia in HIV positive HBV carriers when compared with HIV negative HBV carriers. This may reflect the effect of immune suppression by HIV which indirectly promotes HBV replication as determined by the presence of HBV DNA, HBV DNAp and HBeAg in serum. The evidence available indicates that HBV is not directly cytopathic (Yoffe and Noonan 1993). The pathogenesis generally being more related to the T- and B-cell responses of the host (Moriyama *et al.*,1990, Barnaba *et al.*,1990). Thus it may be reasonable to assume that HIV-positive patients with severely impaired immune systems would have a lower level of hepatocellular damage and unrestricted viral replication. The HIV positive patients described in our study were asymptomatic with respect to their HIV infection and this may explain the lack of a significant increase of serum HBV DNA in our HIV positive versus HIV negative group. However, both HBV DNA and HBV DNAp were higher in our HIV positive group. This was true for both HBeAg positive and HBeAg negative patients, although the numbers were small in the HBeAg negative group. HBeAg was also more prevalent in the HIV positive group.

In our study the HBV DNA assay was more sensitive than the HBV DNAp assay. The positive cut off value for the HBV DNA dot blot assay was calculated by taking the mean value of the negative control samples plus 3 times the standard deviation. This was used

because the measurement of HBV DNA by scanning densitometry favoured the creation of false positive results due low level non-specific hybridisation. Whilst this was visible by eye it could not be controlled for on the scanner.

The inclusion of appropriate negative serum controls in each assay minimised this problem which appeared to be related to non-uniformity of pressure around the dot blot apparatus. The possibility of false positive reactions due to bacterial contamination of the samples was reduced by careful separation of the probe away from the vector. The measurement of hybridisation by scintigraphy in later studies (data not included in this thesis) improved the analysis of the results and gave a more accurate quantitation. The HBeAg negative samples that were dot blot positive alone were also positive by PCR in later studies as were eight more dot blot negative samples. Similar results were reported by Imazeki *et al.*,(1985) with HBV detected in a higher proportion of samples by HBV DNA dot blot than by HBV DNAp. This highlighted the problem of using relatively insensitive assays in which the positive samples were correctly identified but the samples in which HBV DNA was not detected could only be said to have HBV DNA levels of less than 250 pg/ml of serum.

The standardisation of HBV DNA quantitation systems is difficult as a comparison must be made with a known quantity of DNA, usually the probe itself unlabelled. In our assay we related three positive control sera to a dilution series of the probe. However, the probe is likely to hybridise more efficiently to itself than to less homologous samples. This may have accounted for the discrepancy between the limit of detection on a series of plasmid dilutions (0.1 pg) and the positive control serum (5pg). Therefore the calculated HBV concentrations can not be regarded as absolute.

The strong positive correlation between the HBV DNAp and HBV DNA indicates that the relative concentrations of DNA in the samples were correct. Standardisation of results between different assay formats is a recurring problem with HBV DNA detection systems as demonstrated by Janssen *et al.*,(1993).

A comparison of two solution hybridisation assays produced a two to 17 fold difference in the concentration of HBV DNA in the same panel of sera. A multicentre quality control study co-ordinated by Dr E Boxall included a panel of 20 HBV positive and

negative samples. The HBV DNA concentration was measured by commercial liquid hybridisation assays and by in-house dot blot assays (data not shown). There was wide variation in the quantitation of HBV between the different centres but the results were concordant for the presence or absence of HBV DNA.

In our study the distribution of HBV DNA values compared with HBV DNAp values was skewed in both cases with a predominance of low values. There was some evidence of a bimodal type of distribution in the HBV DNA analysis with a peak at 501-1000 pg/ml and a second peak at 4001-5000 pg/ml. This may reflect the presence of two sub-populations in the group i.e. the HIV positive samples having generally higher values than the HIV negative samples. This was not reflected in the less sensitive HBV DNAp assay.

4.6 No evidence for an effect of zidovudine on hepatitis B virus replication in homosexual men with symptomatic HIV infection

The effect of zidovudine treatment on thirteen patients with detectable HBV DNA and HBV DNAp was studied. There was no significant change in either measure of viral replication during 16 weeks of treatment compared with the 13 weeks in the untreated comparison group.

The presence of a virally encoded reverse transcriptase in both HBV (Summers and Mason, 1982) and HIV (Wain-Hobson *et al.*,1985) provides a common target for anti-viral therapy. The inhibition of reverse transcription by chain terminators involves the prevention of DNA synthesis by nucleotide or nucleoside analogues which are incorporated in to the pregenome of HIV or the genome of HBV. The affinity of polymerases for zidovudine triphosphate varies and this may be important in determining the efficacy and toxicity of the drug. The effect of zidovudine on HIV RT is greater than on cellular polymerases thus at the concentrations used for therapy HIV RT is selectively inhibited (St Clair *et al.*,1987). In a similar way the concentration required to inhibit HBV polymerase is greater than for HIV RT (Aoki-Sei *et al.*,1991) and may not have been reached in the patients included in our study.

Both HIV and HBV lack a virally encoded thymidine kinase . The pro-drug is phosphorylated by cellular thymidine kinase to the active triphosphate. The up-take and

degree of phosphorylation of zidovudine may vary between cell types. Therefore one explanation for the apparent lack of activity against HBV may be lower concentrations of the active metabolite in hepatocytes as compared with lymphocytes. Active HIV expression within CD4 cells may lead to accelerated terminal differentiation and cell death (Zagury *et al.*,1986). Therefore, if new rounds of viral infection could be stopped by zidovudine, HIV disease progression may be prevented. However, the elimination of actively replicating HBV may not prevent HBV related disease. The integration of the HBV genome into the human genome has been associated with hepatocellular carcinoma (Shafritz *et al.*,1981). Also expression of HBV proteins from the integrated genome would continue to stimulate the cytopathic immune response. Viral integration has been demonstrated early in the course of a chronic infection (Scotto *et al.*,1983, Bartolomé *et al.*,1990) and may have taken place before the patient presents for treatment.

Most *in vivo* studies of the efficacy of zidovudine against HBV have shown very little effect of zidovudine on HBV. The only group which found a statistically significant effect studied a group of thirteen patients for one week (Berk *et al.*,1990). However, HBV DNAp remained detectable in all patients. In contrast to our study only five patients were HIV positive and all five were asymptomatic with CD4 counts of >400. The short course of treatment meant that the results of this study were difficult to interpret. Possibly the HIV disease state of the patient is a factor in determining the efficacy of zidovudine.

In our study the patients were severely immunocompromised and diagnosed as having AIDS. Given the lack of an effective immune response to HBV in chronic carriers, together with the effect of HIV co-infection the antiviral effect of zidovudine alone may not be sufficient to prevent HBV replication. Stimulation of the immune system may be required for clearance of the cells harbouring the virus while replication and reinfection is suppressed by zidovudine or a similar antiviral agent.

4.7 Hepatitis B reactivation or reinfection associated with HIV-1 infection: Detection of serum HBV DNA by polymerase chain reaction

The reinfection or reactivation of HBV in HIV infected individuals was studied in section 3.7. These patients developed anti-HBc but not anti-HBs, following apparent resolution of an acute HBV infection.

Serological subtype analysis and nucleotide sequencing allowed the identification of reinfection in Case 1, and reactivation in Cases 2 and 3. HBV DNA was detected in three HBsAg negative samples from Case 3. The A determinant of the surface region did not contain any novel mutations in any of the Cases described. Where samples were available sequences were conserved between periods of HBsAg antigenaemia.

The failure to detect HBsAg or anti-HBs during the course of an HBV infection may reflect sampling during the very early stages of the infection or during the window period when anti-HBc may be the only marker of infection. Also, in an immune patient anti-HBc may remain detectable for longer than anti-HBs.

In some "silent" carriers the level of HBsAg expression may be too low to be detected, either HBeAg or anti-HBe is generally present in the serum in addition to anti-HBc. In a minority of patients a mutant virus has been identified that produced HBsAg which was undetectable by monoclonal antibody based assays.

The serological profiles present in our patients were not consistent with an HBV type 2 infection (Coursaget *et al.*,1987, Buisson *et al.*,1992).

The detection of HBV DNA in two of our patients who underwent a reactivation of an existing infection indicated a "silent" carrier state. Although evidence has been presented (Lazizi *et al.*,1993) to show that HBV DNA may be detectable in serum for a maximum of 12 months in the absence of HBV replication, it is unlikely that residual HBV DNA from disrupted viral particles could be detected 11, 20 and 23 months after the initial period of infection in Case 3.

The presence of HBV DNA in serum has been associated with chronic liver disease in HBsAg negative HBV infections. The patients in our study did not have biochemical indications of chronic liver disease, although histological examination of liver tissue was not performed.

The reactivation of HBV infections has been described in association with mutations of both the pre-S (Raimondo *et al.*,1990) and precore regions (Raimondo *et al.*,1990). We did not find any mutations in either the precore or surface regions. The HBV sequence

conservation in our study may have been due to the very low level of viral replication during the HBsAg negative period. In other studies mutations in the surface gene have been associated with immune escape mutants (Carman *et al.*,1991).

In Case 1 the initial infection may have been a surface mutant type which allowed reinfection with a wild type virus. However, considering the rarity of such mutations in the UK, this seems unlikely. Unfortunately, HBV DNA was not available for sequencing from the first infection to confirm this.

The mechanism for reinfection, as demonstrated in Case 1 is unclear. One explanation may be the development of immune-tolerance to HBsAg so that a protective anti-HBs response was not stimulated against the second infection, allowing Case 1 to be reinfected. This may be analogous to the incomplete HBeAg specific T-cell tolerance which has been demonstrated in HBV transgenic mice. Mice which contained the whole HBV genome failed to develop anti-HBe, despite producing relatively large amounts of HBeAg in their serum and liver. A specific tolerance of proliferating and helper T-cells for HBeAg was detected in these mice (Milich et al.,1990).

The absence of an anti-HBs response in HBV infected individuals has been linked with the development of a chronic infection (Mishra *et al.*,1992). A response to amino acids 124 -147 (the A determinant) was detected in all cases of early acute infection but not in chronic hepatitis. Thus, failure to develop a response to the envelope protein may explain the development of the "subclinical carrier state" (Case 3) or allow reinfection (Case 1). This may occur more frequently in patients with a low viral titre.

All three Cases were HIV positive at the time of HBV reinfection or reactivation. As shown in section 3.5 the suppression of the immune system by HIV may enhance HBV replication and prolong the period of active viral replication in patients whilst reducing the inflammatory component of their liver disease as measured by transaminases. Therefore, immunocompromise may have prevented either the development or maintenance of an effective immune response in these three patients.

The absence of serum HBV DNA would correspond to the existence of a truly latent HBV infection with periods of reactivation associated with co-factors, for example HIV

infection. A situation which may be comparable to that which is known to occur with Herpes viruses (Lau, 1990, Laurence, 1990).

4.8 Conservation of precore and core sequences of hepatitis B virus in chronic viral carriers

In this study we have shown that precore and core sequences were well conserved in our group of asymptomatic HBV carriers. Mutations of the precore region, preventing HBeAg transcription were detected in two of five HBeAg negative patients included in the initial study. Later work carried out on five more of the same group of patients identified one more HBeAg negative patient with a precore termination mutation (data not shown).

The amplification reaction was optimised so that HBV DNA was present in sufficient quantity for analysis. The development of direct sequencing techniques from PCR amplified material required the analysis of a well conserved region to study the frequency of sequencing artifacts. The precore/core region which is overlapped by the X gene at the amino terminal and the polymerase gene at the carboxy terminal was suitable.

PCR primers were chosen to highly conserved regions within the X/precore/core genes. The primer used for sequencing was one of the inner amplification primer pair. Thus internal sequence variation in the PCR product would not prevent the sequencing reaction.

The primer extension step of the PCR with *Taq* polymerase (Saiki *et al.*,1988) is carried out at temperatures of 70-72°C. High primer annealing and extension temperatures increased specific amplification due to primers hybridising specifically to their complementary sequences. After 20 cycles the concentration of template would ideally have increased by a factor of 10^5 or 10^6. In practise this may be reduced because of inhibition of the enzyme or suboptimal primer annealing. The introduction of a second round of amplification using an inner pair of primers increased the concentration of PCR product and improved specificity of the reaction.

Whilst direct sequencing of PCR products provided a good method for consensus sequencing, single molecule amplification and sequencing was required to provide a method of comparable sensitivity to PCR product cloning and sequencing. The analysis of independent amplifications from each sample and both strands of the PCR product helped to identify both T7 polymerase and *Taq* polymerase induced sequencing artifacts.

The precore/core regions are important regions for both replication of the virus and pathogenesis of HBV disease. Therefore it was important to examine sequence variation in our carrier population to identify the background level of variation in asymptomatic carriers from an area where HBV is not endemic. Most other studies have looked at patients from areas where HBV in endemic. Two viral RNA transcripts are made from this region. The first begins at the first precore initiation codon and is translated to produce HBeAg. The second initiates at the core start codon and forms the HBV pregenome; HBcAg and polymerase are also synthesised from that region. Encapsidation of genomic RNA requires the recognition of the cis encapsidation signal which is located at the 5' end of genomic RNA within the precore region (Pollack & Ganem, 1993, Knaus & Nassal, 1993). The development of an infection, initially an acute infection is characterised by a cellular immune response directed against an HLA-A2 restricted determinant within the HBcAg (Penn *et al.*,1991).

Mutations in the precore region have been demonstrated to have a profound effect on viral replication. The most commonly detected mutation in that region is the substitution of A for G at nucleotide 1986 resulting in a nonsense mutation preventing the synthesis of HBeAg. This mutation has been shown in many groups (table 4.8(i)) of patients and was initially thought to be a more aggressive viral form. The "precore mutant virus" was linked with outbreaks of fulminant hepatitis particularly in regions of high HBV prevalence.

Li *et al.*,(1993) presented evidence that multiple genotypes of HBV existed one of which (genotype A) had the nucleotide sequence CCC at precore codon 15. The variant CCU (genotype D) was found in three subtypes (*adw, ayw* and *adr*) whilst genotype A (CCC) was restricted to subtype *adw*. A possible explanation for this phenomenon was brought about by the identification of a "stem-loop" structure that is critical for encapsidation of HBV (Knaus and Nassal.,1993). The development of a mutation at codon 1896 in that genotype would disrupt base pairing in the lower region of the stem-loop structure and decrease the viral replication capacity. In genotype A a stable C-G pair would be disrupted in the mutant type virus to form a C-A pair, which is less stable. Where genotype D existed the same mutation would convert a U-G pair to U-A, conferring greater stability.

In many cases a second mutation has been observed in precore mutant viruses at codon 1899 (Carman *et al.*,1989). This mutation is not predicted to disrupt "stem and loop" formation and occurs only rarely without the prior disruption of HBeAg transcription. In our study HBV DNA was amplified from 10 HBeAg negative individuals with primers to the precore region. Only two of the patients had a precore mutation at nucleotide 1896, both were genotype D.

The studies from countries where HBV is endemic contrast with those from both American and French patients with or without liver disease (Laskus *et al.*,1993, Féray *et al.*,1993) in whom precore mutant virus is rarely detected.

The variable prevalence of precore mutants may be explained by the geographical distribution of genotypes A and D. The study of French and Chinese patients by Li *et al.*,(1993) indicated that this was the case. Precore mutations were assayed by hybridisation with type specific probes for mutations at codon 28 alone or codons 28 and 29 together. Genotype A (CCC) was detected in 32 of 47 HBeAg positive French samples whilst genotype D was detected in all 16 Chinese HBeAg positive samples.

Where the genotype permits the mutation of the precore region the absence of HBeAg does not adversely effect replication (Chen *et al.*,1992). However, in experiments with WHV, neonatal woodchucks infected with precore mutant virus did not develop a chronic infection in contrast to animals infected with wild type WHV. Thus the presence of HBeAg may be important for the establishment of chronic infection. This data provided evidence for the theory that HBeAg may act as a tolerogen.

The precore mutant virus has been found as part of a mixed population with wild type virus in some HBeAg positive individuals (Chuang *et al.*,1993) and appears to have an association with chronic active hepatitis and increased levels of viral replication. We did not see any mixtures in our HBeAg positive patients In a study by Naumov *et al.*,(1992) all 11 patients with predominantly mutant virus (>95%) had CAH and HBV DNA detectable at levels of more than 20 pg/200μl. Patients with a mixture of mutant and wild type virus in their serum had lower levels of DNA (mean 15.9, SD 16.6). Thus the absence of HBeAg may be associated with an increased risk of HBV related disease in some cases. From these results and the association of high levels of HBV DNA detected

by dot blot in Mediterranean, anti-HBe positive carriers the precore mutation appeared to confer a replicative advantage.

The relationship between precore mutant virus with a premature termination codon and mutations at other codons in the region was also addressed by Carman *et al.*,(1992). Sera from Hong Kong Chinese patients with anti-HBe, elevated ALT and high level viraemia were compared with other patient groups, in particular one group with fluctuating HBeAg and anti-HBe. Mutations were detected in the precore region at codon 15, proline to serine, codon 28, tryptophan to a stop codon and codon 29, glycine to asparagine which was thought to occur only with the translation stop signal at codon 28. In the group studied by Carman *et al.*,(1992) mutations at codons 15 (nucleotide 1856) and 28 were mutually exclusive. Codon 15 mutations were found in both HBeAg and anti-HBe positive patients. This mutation of the first nucleotide may have a similar effect to that described above for the third nucleotide in the same codon. There was an association between infection with virus with mutations at codon 15 and a worse prognosis after seroconversion to anti-HBe (Carman *et al.*,1992). None of our patients had a mutation at nucleotide 1 of codon 15.

Mutations at any of a total of nine sites in the precore region would produce premature termination codons. However, the variation is restricted to codon 2 and 28, neither of which are detectable in all patients. This helps to confirm that specific selection pressures are constraining variation in the precore region.

Our study also highlights the fact that an alternative mechanism for the cessation of HBeAg production exists in some patients. Whether this occurs in all patients irrespective of the development of precore mutations is not clear. Mutations in the precore region have been detected before patients become anti-HBe positive and in Mediterranean patients correlate with high levels of viral replication. During a period of increased viral replication prior to HBeAg/anti-HBe seroconversion mutations may be more likely to occur. These may not be corrected if they are present in a gene which is no longer translated or if the mutations do not confer any replicative disadvantage.

We did not find precore mutations in virus amplified from any of our HBeAg positive individuals, 10% of whom would be expected to seroconvert in any one year (Weller *et*

al.,1986). Had the development of precore mutations been a prerequisite for seroconversion we would have expected to find it in our HBeAg positive patient group.

Our HBeAg negative carriers were asymptomatic, which, if these mutations are relevant to disease, may explain their absence in our study. All of the patients in this group except two were genotype A. The only patients which had genotype D also had a precore mutation at codon 28.

In common with other studies the precore region was otherwise well conserved with very few mutations in anti-HBe positive patients.

The HBV core region was also well conserved in our samples. The choice of patients in our study may have reduced the likelihood of viral mutations being present. Mutations in the core region appear to occur more frequently in patients with symptomatic HBV disease (Ehata *et al.*,1993).

In summary, our findings were consistent with published data regarding the distribution of mutations within the precore region and provide evidence for the infrequent detection of these mutations in UK anti-HBe positive chronic hepatitis B carriers.

4.9 The transmission of hepatitis B virus precore mutants associated with fulminant and acute hepatitis

In this study of Greek patients with fulminant and acute uncomplicated hepatitis B, most patients, regardless of the clinical course of their HBV infection were infected with virus carrying the mutation of codon 28 in the precore region. Similarly there was no clear association between an additional mutation at precore codon 29 and HBV disease.

As discussed in section 4.9 early studies reported an association between the precore mutant virus and severity of disease. However, later studies of populations where HBV is not endemic did not confirm this association.

Our study supports the sexual transmissibility of the precore mutant virus. There was a

high degree of sequence conservation between the cases of new infection and their presumed source throughout the X/precore/core region.

In five of the ten cases for which samples were available the codon 28 mutant type was the only viral type detectable by either direct sequencing or PMA. In two cases the increased sensitivity of the point mutation assay as compared to conventional sequencing allowed the detection of a minor viral population in the source which became the dominant genotype in their infected partner. The analysis of these patients by nucleotide sequencing alone would have failed to identify these transmissions. It appears that either the wildtype or the mutant may be transmitted where the source carries a mixed viral population. The presence of mixtures of wildtype and mutant virus was not associated with more severe disease as had been suggested by Raimondo *et al.*, (1993).

There was some sequence variation within patient partner pairs. This was most notable in patients with fulminant infection, where multiple genotypes were detected in two patients. The precore sequences were highly conserved with most variation, including deletions and insertions occurring in the X region. These mutations may have arisen de novo in the patients, as has been suggested for the codon 28 mutation (Carman *et al.*,1991) and do not necessarily indicate an alternative source of infection. Many of the sources of infection in our study would have been infected either perinatally or in childhood and may carry a variety of viral types undetectable by consensus sequencing but at sufficiently high concentrations to be transmitted. Sequence variation within patient/partner pairs has also been present in other studies examining the sexual transmission of precore mutant viruses (Aye *et al.*,1994).

HBV DNA amplified from two patients in our study had in-frame insertions, this initially made sequence analysis very difficult. Single molecule amplification was used as an alternative to cloning and provided multiple templates which contained a single variant for analysis .

The rate of development of mutations may be influenced by the immune response to the infection. In chronic carriers, where this is a suboptimal immune response, mutations may not occur or may take place slowly over a long period of infection. The accumulation of mutations may take years. For example Tran *et al.*,(1991) presented an HBeAg positive

HBV carrier in whom the precore mutant virus developed during a six year follow-up period. There was no indication as to how long the patient had been a carrier before follow-up. In patients with a strong immune response ie symptomatic acute hepatitis mutations may arise much more quickly.

Although wild type virus was not detected in seven of the twelve partners and two of the patients studied all had anti-HBe in serum. This result agrees with Tong *et al.*,(1991) who found that precore mutant virus did elicit an anti-HBe response when it was transmitted to a chimpanzee.

In our study wild type virus was not detected in two HBeAg positive patients. This may have been due to an undetected subpopulation of wild type virus. Alternatively this may reflect the presence of an actively replicating site from which HBeAg but not complete viral particles were released into the serum.

We have not shown any relationship between the viral titre in the partners and clinical outcome in the patient. Whilst the partner's viral titre was not measured at the time of infection the distribution of values in the later samples did not show any trend toward lower or higher concentrations in the study and control groups. As expected HBV DNA was amplified less frequently from patients with fulminant hepatitis than asymptomatic acute infection (Brechot *et al.*,1981).

4.10 Novel application of a point mutation assay; evidence for transmission of hepatitis B viruses with precore mutations, and their detection in infants with fulminant hepatitis B.

In this study we have demonstrated the transmission of precore mutant virus from HBeAg negative mothers to their infants. We have confirmed an association between these mutants and some but not all cases of neonatal fulminant hepatitis. We have also shown that transmission from a healthcare worker may have been associated with precore mutants.

Perinatal transmission from HBeAg positive mothers occurs in upto 90% of infants. Immunisation can reduce this to approximately 10% (Tong *et al.*,1989). The transmission

of HBV from anti-HBe positive mothers is rare. An estimate of one in 750 births i.e. <1%. has been suggested (E Boxall, personal communication). The babies born to such mothers rarely develop a persistent carrier state but exhibit an acute infection which may be symptomatic (Sinatra *et al.*,1982). Initially the need for immunisation of such infants was largely unrecognised. However, the frequency of transmission may vary between <1% and 20% depending on the rate of HBV infection and chronic carriage in the population. Whilst the risk of transmission is lower than for HBeAg positive mothers the outcome may be more severe.

Approximately 10% of infants born to Japanese anti-HBe positive carrier mothers develop an acute asymptomatic or fulminant hepatitis (Shimisu *et al.*,1991). This constitutes about half of all cases of hepatitis in infants and is therefore an important factor in infant mortality. Vanclare *et al.*,(1991) also reported fulminant hepatitis in infants born to anti-HBe positive, HBV DNA dot blot negative mothers.

Whilst such transmission events are rare in the UK frequently fatal outcomes in such situations indicate that these infants should be immunised. The infected infants may also be at risk of long term sequelae in cases where fulminant hepatitis does not occur. When a chronic infection is established the long-term carriage of maternally acquired HBV has been associated with the development of hepatocellular carcinoma (Sung *et al.*,1980).

The transmission of mixed precore mutant and wild type virus has been associated with an early elimination of the virus in the absence of a fulminant hepatitis (Raimondo *et al.*,1993). Whereas infection with wild type HBV alone was associated with the development of a carrier state. In our study precore mutant virus alone or as part of heterogenous population was associated with immune elimination. Additional mutations in Case 2 confirmed the transmission of precore mutant virus and demonstrated that this virus was infectious.

Schaefer *et al.*,(1993) demonstrated HBV DNA by PCR only in the serum of three babies born to anti-HBe positive carrier mothers, providing further evidence that dot blot negative, anti-HBe positive mothers do transmit HBV. None of the babies in that study were vaccinated and after 3 months they developed a fatal fulminant hepatitis. The detection of HBV DNA in maternal mononuclear cells indicates that infection with HBV in

maternal mononuclear cells may provide a mechanism for *in utero* infection.

In utero infection via infected cord blood leucocytes has been proposed (Shen *et al.*,1987) although HBV DNA was not detected in the mothers' serum. The infants in Shen's study both became symptomatic carriers. Immunisation in these babies would not affect the clinical outcome and may explain a proportion of vaccination failures in babies. In one case in our study (Case 2) although HBV DNA was amplified from cord blood, the baby was successfully immunised.

Virus with mutations in the precore/core region may stimulate an enhanced core-specific immune reaction. HBeAg is one of the major targets for the cytotoxic T-cell response. The expression of HBeAg may contribute to the rapid destruction of liver cells associated with a fulminant infection. A comparison of an HBV mutant associated with fulminant hepatitis (Hasegawa *et al.*,1994) and a cloned HBV with or without mutations at codons 28 and 29 produced by site directed mutagenesis, indicated that additional mutations throughout the HBV genome (precore/core codons 57,68,94,95,115,126,135,160, 198,210. Pre-S/S codons 110,113,160,193. Pol codons 45,144,156,169,177, 229,285,291, 293,330,335,342,458,644,704. X codons 85,94,127,128,132,133) conferred an increase in encapsidation of pregenomic RNA into core particles in the fulminant strain. Viral antigen synthesis, transcription and replication were analysed after transfection of either wild type or mutant full length HBV constructs into human hepatoma cells (HuH 7). The HuH 7 cells containing the fulminant strain demonstrated higher levels of core associated antigens but not surface antigens. The fulminant strain specifically appeared to retain more of the core antigen in the cell. This may have lead to an increased immune response to the core antigen and fulminant hepatitis. This helps to confirm the evidence presented in this and previous sections of this thesis that codon 28 and 29 mutations alone are probably not causally associated with fulminant hepatitis.

4.11 Hepatitis B virus escape mutant following liver transplantation

In this study we described three patients who underwent liver transplantation for chronic HBV related disease. Graft infection occurred in all three Cases despite HBIg prophylaxis. Nucleotide mutations were detected in the *a* determinant of the surface region at codon 145 in Cases 1 and 2 before and after treatment. In Case 1 these remained a minor subpopulation with a C for G transition. In Case 2 the mutant type

became the dominant population with a substitution of C for G before treatment and A for G after treatment. The wild type virus was present exclusively in Case 3 both pre and post-transplant. HBsAg was not detected in Case 2 post-transplant by a monoclonal antibody based assay.

The production of antibodies to a common, immunodominant antigenic A determinant has been shown to protect against reinfection by all HBV subtypes (Hileman *et al.*,1978). This property has been exploited in the design of vaccines which include the surface antigen. The *a* determinant has been mapped between codons 124 and 147 of the surface region. Monoclonal antibodies to that region have demonstrated a protective, neutralising activity against HBV for chimps (Iwarson *et al.*,1985). The position of cysteine residues at codons 124,137, 139 and 147 are proposed to maintain a double loop conformation of this region of the HBsAg. Antibodies to both loops have been demonstrated in serum from vaccinated individuals (Brown *et al.*,1984). Detergent treatment (Vyas *et al.*,1972) and site directed mutagenesis, in which residues 142 (proline) 124 and 147 (cysteines) are altered (Ashton-Rickardt and Murray, 1989) have provided further evidence that HBsAg *a* determinants are conformational.

Single nucleotide changes within the S region have been associated with specific reactivities in the form of HBV subtypes. Variation between lysine and arginine at codon 122 specifies subtype *d* or *y* and at codon 160 *w* or *r* (Okamoto *et al.*,1989). A further example of the dramatic effect of a limited number of nucleotide changes in a sequence was demonstrated by Ashton-Rickardt and Murray (1989). A combination of subtypes may be manifest on the same HBV molecule by the presence of concurrent mutations at codons 113, 122 and 134.

Mutations within the A determinant have been associated with HBV infection in children despite achieving apparently protective levels of anti-HBs mediated immunity (Carman *et al.*,1990). The development of a mutation in this region was noted in an Italian child who developed serious HBV related disease. The child had been vaccinated and developed HBsAg and HBeAg in the presence of anti-HBs. A mutation was detected at codon 145 of the surface region which converted a glycine residue to arginine in the second loop of the *a* determinant. This mutation was not present in HBV DNA amplified from the mother's serum, who was presumed to be the source of infection. The effect of

this mutation was confirmed *in vitro* by Waters *et al.*,(1993). The introduction of this mutation to a recombinant HBsAg destroyed wild type monoclonal antibody binding to the *a* determinant. The same mutation was identified by Harrison *et al.*,(1993), in an Asian child who had been vaccinated. In that case a mixed population of wild type and mutant virus was detected. We have also seen mixtures of viral of genotypes in Cases 1 and 2. However, these were detected before immune therapy and therefore arose naturally during the course of the HBV infection. The samples were otherwise well conserved in these regions. The unusual C for G mutation was confirmed by single molecule sequencing which did not reveal any other unusual subpopulations.

Sequence variation in surface region has been associated with monoclonal antibody treatment of HBV infected liver transplant patients (McMahon *et al.*,1992). HBV graft infection and survival was improved in patients. However, circulating HBsAg reappeared in 2.7% of patients studied. The rate was greatest in patients with cirrhosis due to hepatitis B and lowest in patients with fulminant hepatitis B. The lack of an established chronic infection in patients with fulminant hepatitis may reduce extrahepatic sources of infection in these patients. *De novo* infection with HBV in patients undergoing orthotopic liver transplantation may be associated with a less severe clinical course when compared with recurrent infection (Douglas *et al.*,(1993). Peripheral mononuclear cells were implicated as a possible source of reinfection by Feray *et al.*,(1990) in HBsAg negative patients. The detection of extrahepatic HBV DNA in acute HBV infection (Yoffe *et al.*,1990) indicates that both acutely and chronically infected patients would be at risk. Two of our patients were HBV DNA dot blot negative and one was positive, all three were anti-HBe positive chronic carriers. Therefore Cases 2 and 3 might be predicted to have a lower risk of infection than Case 1.

The rate of reinfection was (Samuel *et al.*,1991) significantly lower in HBV DNA dot blot negative, anti-HBe positive cirrhotic patients (25% at one year) than in the corresponding dot blot positive, HBeAg positive group (82% at one year).

The withdrawal of immunosuppressive therapy has been associated with increased liver damage by HBV (Lau *et al.*,1989, Flowers *et al.*,1990). This may contribute to the distinctive, rapid form of liver disease (fibrosing cholestatic hepatitis) that has been specifically associated with graft infection (Davies *et al.*,1991). A high level of HBcAg

has been associated with this clinical course indicating that damage may be related to a high antigen load (Harrison *et al.*,1993). A cytotoxic T-cell (CTL) response to HBcAg has been demonstrated and proposed as a mechanism for hepatocellular damage (Ishikawa *et al.*,1993).

Coinfection with HDV may confer some protection against graft loss due to HBV infection. In a study by O'Grady *et al.*,(1992) patients who were both HBV and HDV positive had a higher rate of survival. There was an increase in HBV replication following transplant in all patients but this was lower in HDV positive patients. Coinfection with HDV has previously been associated with lower levels of HBV DNA in acutely and chronically infected patients (Krogsgaard *et al.*,1988). This may also apply to transplant patients who become reinfected. In our study the patients were anti-HDV negative prior to transplantation.

Polyclonal anti-HBs prophylaxis has been shown to decrease the risk of graft infection and increase the rate of survival in patients with and without prior HBV infection (Samuel *et al.*,1989). Although our study describes three patients who all died following transplant survival rates of 58% have been reported (Samuel *et al.*, 1991a, Samuel *et al.*,1991b, Emond *et al.*,(1989)). In our study graft reinfection took place after a relatively short period of treatment in Cases 1 and 3, neither developed a significant proportion of mutant virus and both developed HBsAg within three months. Prolonged immune prophylaxis may therefore be associated with the development of mutations in the surface region.
All of the patients had mutations in the precore region, the significance of these has been discussed elsewhere. In these patients the mutations were present in what appeared to be a rapidly replicating virus. The mutations in this case did not affect the replication competence of the virus and all three were of genotype D.

4.12 The application of sequence analysis to the detection of clustered outbreaks of HBV infection

In this study we have demonstrated that sequence analysis of short regions of the HBV genome can help to define hepatitis B outbreaks. We used part of the surface region, including the *a* determinant and subtype defining regions and the 3' X and precore regions. These areas contain 42 recognised points of nucleotide variation which are associated with different subtypes. However, the regions are sufficiently well conserved

so that variation between transmission events is restricted.

Sequences were directly compared by nucleotide alignment. Where there was variation between samples in the proposed cluster, statistical analysis was applied to determine comparative levels of variation in the outbreak samples and a control group. In our study the outbreak group were significantly less variable than the control group in the surface region and identical in the X/precore region.
This method has since been used successfully in our division to identify a number of transmission events and outbreaks.

The replication cycle of HBV includes a reverse transcriptase step and this is associated with an increased rate of sequence variation (Orito *et al.*,1989). In our study the virus was stable over three generations of transmission events indicating that the level of variation is restricted. The HBV genome is small and most regions encode two translation products using alternative reading frames. Thus mutations in the surface region which are silent may adversely effect the overlapping polymerase gene. The polymerase gene is the largest HBV reading frame, spanning the entire preS/ S region, most of the X region and the 3' end of the C region. Mutations in any of these regions could potentially disrupt replication and in that way limit the sequence diversity of HBV.

HBeAg was only detected in one patient, neither HBeAg nor anti-HBe were detectable in the remaining patients. A premature termination codon was identified in all Cases at precore codon 28 with a second mutation at codon 29. The significance of these mutations has been discussed earlier. All five cases were of genotype D (CCU, codon 15), which would permit replication in the presence of these mutations.

The clinical outcome of the patients was variable. The source of the infection was an asymptomatic carrier with a low HBsAg titre. Two patients developed a fulminant fatal hepatitis (HCW 2 and patient 3). These Cases had little in common other than being female. One was a young, presumably healthy, nurse infected parenterally, the other was an elderly orthopaedic patient. HCW 1 and Patient 2 were both infected parenterally, developed acute symptomatic infections and recovered. Patient 1 received a unit of HBV contaminated blood from the donor and did not develop a documented hepatitis. In this study there was no common factor to account for the disease outcome. However, as we

have sequenced only a relatively small region of the genome and have shown that variation within the group did occur, we cannot discount the possibility that more or less virulent strains of HBV exist.

Chapter 5 Concluding discussion

The HBV DNA dot blot assay has been modified and used in a controlled study to examine the effect of coinfection with HIV on the natural history of HBV. We have shown that HIV positive individuals have higher serum HBV DNA and HBV DNAp levels and are less likely to seroconvert to anti-HBe than HIV negative individuals. Although HBV has been shown to transactivate HIV the rate of progression to HIV-disease was not increased. Indicators of HBV related disease (ALT levels) were lower in HIV positive individuals. This might be predicted, as symptomatic hepatitis is related to the immune response which is decreased in HIV-infected patients.

In agreement with other studies we have found no evidence for an effect of AZT on HBV replication in patients with AIDS. We have demonstrated that HBV can be amplified by PCR from small volumes untreated sera. The limit of sensitivity of this method is 100 fold greater than the proposed infectious dose for HBV. The method was useful where very small volumes of samples were available. The addition of a second nested round of PCR (double nested PCR) can achieve single copy sensitivity.

Where larger volumes of serum were available we have found that Proteinase K and detergent digestion followed by phenol/chloroform extraction provided a suitable template for amplification. This method was time consuming and had the potential for cross contamination during excessive sample manipulation. Where low concentrations of HBV DNA were present substantial losses during the extraction procedure may have reduced the sensitivity of the PCR. We have devised an alternative method of sample preparation which, for sample volumes of less than 100 ul, is equally sensitive and considerably simpler and quicker. The lack of sample manipulation significantly decreased the risk of cross sample contamination.

This method involved boiling the serum sample and was sufficiently sensitive to amplify DNA from a single HBV particle. The sensitivity of this method was not affected by the presence of serum contaminants and did not require the use of detergents or proteinases which inhibit the enzyme *TAQ* polymerase. The specificity of the method allowed the direct analysis of the amplified product by nucleotide sequencing and PMA. The amplification of HBV DNA from single molecules removed the requirement for cloning

to analyse mixtures of viral genotypes. A PMA for codons 1 and 28 of the precore region and codon 145 of the surface region provided a simple and sensitive method of identifying viral subpopulations in such mixtures. This method was limited by the requirement for sequence conservation around a defined point of interest but provided an alternative method to the cloning and screening of large numbers of amplificates. The method was sensitive at levels of 2-3% hence screening of more than 50 clones would be required to improve on the sensitivity of this method. The development of specific sensitive methods for the detection of HBV DNA is important despite the availability of good serological assays for HBV markers. We have detected HBV DNA by PCR in the serum of HIV positive patients who were HBsAg/anti-HBs negative. The conservation of sequences between periods of HBsAg-positivity in patients indicated HBV reactivation. However, no mutations were detected in the surface gene which would explain the absence of detectable HBsAg or anti-HBs in the serum. HBV was not detected between HBV infections in a patient who had been reinfected with a different HBV subtype. Again the amplified HBV DNA did not have mutations in the surface region which have been associated with immune escape. These results demonstrate that unusual serologic patterns are not always associated with HBV genome variation. Patients with impaired immune function for example HIV, may be susceptible to reinfection.

HBV DNA was detected in three out of 103 (2.9%) patients who had anti-HBc present only in their serum. This pattern may represent the loss of anti-HBs over time, which can occur in up to 19% of patients (Zito *et al.*, 1987). Our patients were unselected attenders of theMiddlesexHospital GU clinic undergoing pre-HBV-immunisation screening. None of the DNA positive samples had mutations of any apparent significance. The nature of the study precluded any conclusion as to the natural history of the infection in these patients. All three may have been in the "window period" when HBsAg became undetectable and anti-HBs had not developed alternatively two of the patients were HIV positive. These patients may have been undergoing reactivation of their HBV infection as we have shown in two patients described in section 3.7.

In some patients unusual serological patterns can be partially explained by mutation within the HBV genome. An example of this is the detection of DNA in anti-HBe positive patients by DNA dot blot assay (Lok *et al.*,1984). Tur-Kaspa *et al.*,(1984) described an association between liver disease and the detection of HBV DNA by dot blot in HBeAg

negative patients. A high prevalence (82%) was also noted in HBeAg negative Portuguese patients with active liver disease (Monjardino *et al.*,1991). However, whilst the detection of high levels of HBV DNA was not restricted to patients with active liver disease, it does appear to be associated with the Mediterranean and possibly other area where HBV is endemic.

In a study of chronic HBV carriers attending the Middlesex Hospital hepatitis clinic HBV DNA was detectable by dot blot at low levels which were close to the negative cut-off value in seven of these HBeAg negative patients. Two of these patients would have been positive if the blots had been analysed by eye only. This relatively low frequency contrasts with those described above. This may indicate low sensitivity of our assay. However, controlled experiments showed that this was not the case. In contrast to the above studies our patients were generally asymptomatic. The low prevalence of this pattern in the UK means that patients are unlikely to have been infected with precore mutant strain. Also, most of our patients would have been infected as adults by sexual transmission and therefore infected relatively recently compared to carriers from endemic regions infected perinatally or in early childhood.

HBV DNA was amplified from the majority of our HBeAg negative carriers by PCR with. We have demonstrated that anti-HBe positive carriers are likely to remain infectious although at lower levels than described for Mediterranean carriers. This may be relevant given current guidelines for invasive procedures carried out by anti-HBe positive carrier health care workers.

The detection of mutations within the precore region which prevented the translation of HBeAg offered an explanation for the continuation of viral replication in the absence of HBeAg (Carman *et al.*,1989).

Profound phenotypic effects due to single non-silent nucleotide mutations have been described for other viruses and for the human genome. These are generally amino acid substitutions rather than nonsense mutations. The attenuation of the Sabin type 3 oral polio vaccine is attributed to single point mutations; one in the 5' non-coding region and the other in the VP3 coat protein. These mutations confer a low-virulence phenotype on the virus, their reversion being associated with neurovirulence (Almond *et al.*,1987).

Resistance to drugs such as Zidovudine in HIV have also been attributed to nucleotide variation in the reverse transcriptase gene. Mutations in the human genome associated with β-thalassaemia phenotype have also been noted (Kobayashi *et al.*,1987). One of the original papers describing the development of PCR was based on the detection of a single point mutation by restriction digestion which was applied to the diagnosis of sickle cell anaemia.

Molecular studies have revealed that the HBeAg is not required for replication but that its absence may be associated with a more severe form of HBV disease. In our studies we have shown that precore mutant virus is transmissible. Patients and their sexual partners who were the source of their infection had common viral nucleotide sequences which included mutations at codons 28 and 29 of the precore region. The PMA revealed that wild type virus, if present at all in the source of the infection, comprised less than 2.5% of the total viral load and remained at undetectable levels in the infected patient.

The mutant and wild type virus was detected equally in patients with acute uncomplicated or fulminant hepatitis. Original studies from Greece, Israel and Japan showed an association between the mutant virus and more severe HBV related disease (Carman *et al.*,1989, Liang *et al.*,1991, Omata *et al.*,1991). More recently the mutation has been shown to be ubiquitous in Chinese patients (Li *et al.*,1993) and unrelated to HBV disease in that population. We have shown that precore mutant HBV is not always associated with severe HBV disease even in Mediterranean patients and is found equally in chronically and acutely infected patients. Moreover, we present evidence that the mutant virus is rarely present in our asymptomatic UK HBV carriers. Evidence from France (Feray *et al.*,1993) and America (Laskus *et al.*,1992) has shown that precore mutant virus is rare in these countries and, when present is not associated with a fulminant hepatitis.

We have shown that the mutant virus is transmitted perinatally and may be associated with a fulminant outcome. However, transmission of the wild type virus from an anti-HBe positive mother may also result in a fulminant hepatitis. This correlates with evidence presented by Raimondo *et al.*,(1993) who also noted perinatal transmission of precore mutant virus.

The HBeAg has been proposed as a tolerogen which would allow the establishment of a

chronic HBV infection (Millich *et al.*,1991). The absence of HBeAg in the mothers described in our study might explain the high level hepatocellular damage in the infants who have not been exposed to HBeAg.

From our studies and others there appear to be two patterns of infection. The first applies to patients initially infected with precore mutant virus who are more likely to undergo an acute infection in the absence of HBeAg. The second applies to carriers who develop the mutation over time. HBeAg may predispose some infected individuals, for example children and the immunocompromised to develop a chronic HBV infection. In cases where the mutant virus develops over time the clinical outcome may vary. In our study the Greek carriers remained asymptomatic, in other studies (Nauomov *et al.*,1992) there was an association between CAH and the mutant virus in carriers.

Bonino *et al.*,(1993) have proposed a model of chronic HBV infection in which there are three pathogenic phases. Loss of HBeAg mediated tolerance; virus specific inflammatory reaction and then a non-virus specific inflammatory reaction. The excess secretion of HBeAg may induce virus tolerance that is related to both the immune competence of the individual and to the genetic heterogeneity of the host. In patients with reduced immunity eg infants the precore mutant virus may be associate with immune clearance of the virus whereas the transmission of wildtype virus from an HBeAg positive mother is associated with chronic carriage.

The loss of virus tolerance is associated with the destruction of infected hepatocytes by HLA class 1-restricted cytotoxic T-cells. This response is directed to a region common to the HBcAg and HBeAg. Thus the expression of HBeAg on the cell surface introduces a positive selection pressure for a virus unable to secrete HBeAg. It has been shown that mutations in the core region, which also effect HBeAg may increase the severity of HBV related disease. Mutations in a segment of the core gene spanning amino acids 84 to 101 were found 15 of 20 patients with advanced liver disease but not in 10 healthy HBV carriers. As this region is a major immunological target of cytotoxic T lymphocytes these mutations probably arose because of immune pressure but they may also have contributed to the liver damage by stimulating a greater immune response (Ehata *et al.*,1992).

The absence of the precore mutant virus in some populations may be related to the presence of sequences within the precore region which are geographically restricted. In our studies the precore mutation has been found only in patients with genotype D (CCU precore codon 15). This concurs with evidence that genotype A viruses are unable to replicate in the presence of a mutation at precore codon 28. This association was common to all groups studied in this thesis. In our studies we have shown that the precore mutation is not always associated with more severe HBV related disease in either chronically or acutely infected individuals.

Genotype analysis of patients may help to identify those at highest risk of developing HBeAg negative HBV related disease. This may also be useful when identifying infants of HBeAg negative mothers who require immunisation where this is not available to all infants regardless of the mother's HBeAg/anti-HBe status. Furthermore it may identify HBeAg negative healthcare workers who may be at risk of transmitting HBV to their patients through invasive procedures.

We have shown that the precore mutation does co-exist with mutations in the *A* determinant of the surface gene. A mutation in the surface region at codon 145 was only detected in patients receiving HBIg prophylaxis. A mutation at codon 143 was found in one HBV carrier. The mutation at codon 145 prevented detection of the HBsAg with a monoclonal antibody based assay. However, a mutation at codon 143 did not have a similar effect. This indicates that the mutation at codon 145 has a specific effect on the conformation of HBsAg and that mutations at other codons in the A determinant are not always associated with decreased in antigenicity.

These studies have emphasised the importance of using polyclonal assays or mixed monoclonal assays for the detection of HBsAg. Also the implication for vaccine development must also be taken into account. Whilst this may not be relevant in areas with a low prevalence of infection the inclusion of variants may be required in HBV endemic regions, both in mixed monoclonal antibody HBsAg assays and also in genetically engineered vaccines.

In the final study we present a model for the detection of clustered outbreaks of infection. This is applicable to all samples from which HBV DNA can be amplified and has

considerable advantages over serological methods of analysis. In this study all of the samples had mutations in the precore region at codons 28 and 29. The severity of disease varied throughout the group however, all of the patients who became infected suffered an acute infection.

The methods developed in this thesis may be used to continue investigations into the relationship between HBV genome variation and pathogenicity.

The detection and analysis of hepatitis B virus genome variation and its use in clinical studies

Appendices

Appendix 1

Composition of reagents

HBV DNA polymerase assay

30% sucrose solution	0.01 M	Tris HCl pH 7.4
	0.15 M	NaCl
	0.001 M	EDTA
	1.0 mg/ml	Bovine serum albumin
	0.1%	2-Mercaptoethanol
	30%	Sucrose
Nucleotide mix E	0.200 M	Tris/HCl pH 7.4
	0.008 M	$MgCl_2$
	0.12 M	NH_4 Cl
	0.5 mM	dATP in 50mM Tris/HCl
	0.5 mM	dCTP in 50mM Tris/HCl
	0.5 mM	dCTP in 50mM Tris/HCl
	1.5 ml	sterile H_2O
	0.5 mM	^3H TTP (40-60 ci/μmol (5-7.5μM
TN Buffer	0.01 M	Tris HCl pH 7.4
	0.15 M	NaCl
TN/NP40/2MET	50 ml	TN Buffer
	1%	NP40
	0.1%	2ME
TNE buffer	0.04 M	Tris HCl
	0.3 M	NaCl pH 8.0
	0.02 M	EDTA

TE Buffer	10 mM	Tris HCl pH 7.5
	1 mM	EDTA

HBV DNA dot blot assay

1 x SSC solution	0.15 M	NaCl
	0.015 M	Na citrate

Oligolabelling buffer

Solution O	1.25 M	Tris HCl pH 8.0
	0.125 M	$MgCl_2$

Solution A	1ml	Solution 0
	18 ul	2ME
	0.0005 M	dATP
	0.0005 M	dTTP
	0.0005 M	dGTP

Each triphosphate previously dissolved in 3 mM Tris HCl 2 mM EDTA pH 7.0 to 0.1M

Solution B HEPES titrated to pH 6.6 with 4 M NaOH

Solution C Hexadeoxyribonucleotides in 1 X TE Buffer 9000 units/ml.

Final buffer for labelling A:B:C mixed in the ratio 100:250:150.

TAE buffer	1.6 M	Tris Base
	0.8 M	Na acetate $3H_20$
	40 mM	EDTA-NaH_20 pH 7.2

1 x Dernhardt's solution	0.02%	Ficoll
	0.02%	Polyvinylpyrolidone
	0.02%	BSA (pentax fraction V)
SSPE 20 x	3 M	NaCl
	0.2 M	$NaH_2 PO_4 H_2O$
	0.02 M	EDTA to 7.4 with NaOH
Phosphate buffered saline	137 mM	NaCl
	27 mM	KCl
	10 mM	Phosphate buffer
Prehybridising solution A	0.2 ml	100 x Denhardt's solution
	0.1 mg/ml	2.0 ml Calf thymus DNA(1mg/ml)
	0.5%	1.0 ml SDS 10%
	3.3 x	6.0 ml SSC x 20
	10.8 ml	sterile H_20

Hybridising solution A

10%	8 ml Dextran sulphate (25%)
0.4 x	4 ml SSC x 2 (filter sterile)
0.5%	1.0 ml SDS (10%)
0.1 mg/ml	2.0ml calf thymus DNA (1.0 mg / ml)
4.6 ml	H_2O (sterile)
340ng	400 μl labelled probe

Prehybridising buffer B

50%	Formamide
4 x	SSPE
1%	SDS
0.5%	Blotto

Hybridising buffer B	As for prehybridising buffer B containing 340ng ^{32}P dCTP labelled DNA probe in 400ul of TE pH7.5

Post hybridisation washing buffers

Washing buffer 1	0.01 mg/ml	Calf thymus DNA
	1 x	Denhardts solution
	6 x	SSC (20 X)
	0.5%	SDS
	Distilled H_2O End volume 1l	
Washing buffer 2	1 x	Denhardts solution
	1 x	SSC (20 x)
	0.5%	SDS
	Distilled H_2O End volume 1l	
Washing buffer 3	0.1 x	SSC (20 x)
	0.5%	SDS
	Distilled H_2O End volume 400ml	

Phosphate buffered saline	137 mM	NaCl
	27 mM	KCl
	10 mM	Phosphate buffer
TNE buffer	10 mM	Tris HCl pH 7.5
	10 mM	NaCl
	1 mM	EDTA
TBE buffer	20 x	1 M Tris Base
	1 M	Boric Acid
	2.0 mM	EDTA NaH_2O

Buffering solutions for use in DNA digestion

Restriction enzyme		
BSTE 11	50 mM	Tris HCl (pH 8.)
	10 mM	$MgCl_2$
	50 mM	NaCl
ECO RI	50 mM	Tris HCl pH 8.0
	10 mM	$MgCl_2$
	100 mM	NaCl
Hind III	50 mM	Tris HCl pH 8.0
	10 mM	$MgCl_2$
	50 mM	NaCl

Culture Media

L Agar :	Bacto tryptone	10 g
1 litre	Bacto yeast extrac	5 g
	Nacl	10 g 0.17 M
	Agar	15 g/litre
	pH 7.2	
L Broth	Bacto typtrone	10 G
	Bacto yeast extract	5 g
	Nacl	5 g 0.085 M
	pH 7.2	

PCR Reagents

10 x reaction buffer	500 mM	K Cl
	100 mM	TrisHCl pH 8.3 (RT)
	15 mM	$MgCl_2$
	0.1%	Gelatin
1 x TBE buffer	0.089 M	Tris Borate
	0.089 M	Boric Acid
	0.002 M	EDTA
6 X loading buffer	0.25%	Bromophenol blue
	0.25%	Xylene cyanol
	30%	Glycerol in H_2O
HBV DNA Extraction Buffer	60 mM	Tris/Hcl pH 7.6
	1 %	SDS
	12 mM	EDTA
	27 %	Sucrose
	2.5 mg/ml	Proteinase K

Sequencing reagents

All reagents copied from USB kit unless otherwise stated.

Annealing Reaction	4 µl	DNA
	2 µl	Primer (100 ng/ sample)
	2 µl	Sequenase buffer
	2 µl	dH_2O

Sequencing Reaction

Add to Annealing reaction	1 µl	0.1M DTT
	0.5 µl	alpha 35-S dATP (Amersham, 1000 Ci/mM,.
	1 µl	Labelling mix, diluted 1:15 in dH2O
	1 µl	Sequenase (2 u, diluted from stock 1:6 in ice-cold TE)
	0.5 µl	dH_2O

Termination Reaction

USB Termination mixes	2µl	10% w.r.t DMSO
	3.5 µl	Sequencing reaction
	4 µl	Stop Solution.

Labelling Mix	7.5 mM	dCTP (0.75µl 100 mM dCTP in 10 mls)
	7.5 mM	7-dGTP(7.5µl 1 mM 7-dGTP in 10 mls)
	15 mM	dTTP (1.5µl 100 mM dTTP in 10 mls)

Termination Mixes

dNTP mixture:	160 mM	dATP (16µl 100 mM dATP in 10 mls)
	160 mM	dTTP (16 " dTTP " " ")
	80 mM	dCTP (8 " dCTP " " ")
	40 mM	dGTP (4 " dGTP " " ")

Modified termination mixtures

'A' termination mix:	8 mM ddATP (2.5 mls dNTP mix + 4 µl 5mM ddATP)
'T' " " " :	8 mM ddTTP (2.5 µls dNTP mix + 4 µl 5mM ddTTP)
'G' termination mix:	4 mM ddGTP (2.5 mls dNTP mix + 2 µl 5mM ddGTP)
'C' " " " :	4 mM ddCTP (2.5 mls dNTP mix + 2 µl 5mM ddCTP)

Sequencing gel-loading buffer	98%	Deionised formamide
	10 mM	EDTA (pH 8.0)
	0.025%	Xylene cyanol FF
	0.025%	Bromophenol blue

Polyacrylamaide sequencing gel

Wedge gel, 150 mls

final volume	75 g	Urea,
	10 ml	Sanger TBE (pH9.2)
	20 ml	Acrylamide Mix (40%)
	62 ml	dH2O
	150 mg	$(NH_4)2SO_4$
	15µl	TEMED

Point mutation asssay reagents

Capture reagents	25 µg/ml	Streptavidin
Coating buffer	25 µg/ml	Streptavidin
	100 mM	Tris-HCl pH 7.6
	0.1%	Sodium azide
Blocking buffer	1%	BSA in phosphate buffered saline
	0.1%	Sodium azide

Washing buffer/
PCR product diluent buffer

(TTA)	10 mM	Tris-HCL pH 7.6
	0.05%	Tween 20
PMA diluent	40 mM	Tris-HCl pH 7.6
	20 mM	$MgCl_2$
	50 mM	NaCl

Anneal mix	0.025μl	oligonucleotide probe diluted in 25 μl PMA diluent
Labelling Mix	40 mM	Tris/HCl
	16.6 μM	dithiothreitol
	0.15 units	Klenow DNA polymerase
	0.16 µM	^{35}S dNTP, (1,000 Ci/mmol)
	0.016%	BSA

Appendix 2

Standard protocols, not developed as part of this thesis.

Assays for the detection of HBsAg

I. A commercially available, amplified enzyme immunoassay (Wellcozyme, Murex diagnostics Ltd). This is a competitive assay in which HBsAg is captured on to a mouse monoclonal antibody coated microtitre plate in the presence of a competitor antibody conjugated to alkaline phosphatase. The bound antibody was visualised with the substrate nicotinamide adenine dinucleotide phosphate. HBsAg present in the serum sample reduced the intensity of this signal. The sensitivity of this assay was approximately 1 ng/ml of serum.

II. HBsAg was quantified by titration in a reverse passive haemagglutination assay (Hepatitis B surface antigen HA screening kit. Murex diagnostics Ltd). HBsAg present in the serum caused agglutination of turkey red cells that have been coated in anti-HBs globulin. Serial dilutions of the test sample in phosphate buffered saline with 5% Normal horse serum and 2% normal rabbit serum were then assayed for agglutination.

III. The subtype of HBsAg was determined by a radioimmunoassay. The test sample was incubated in a microtitre plate coated with either anti-*d* or anti-*y* immunoglobulin. The ratio of binding of the sample to the immunoglobulins was compared and the subtype assessed.

IV. The specificity of the HBsAg assays were confirmed by neutralisation of the test sample with anti-HBs-positive immune horse serum.

A radioimmunoassay for the detection of anti-HBs

Serum anti-HBs was captured on to an HBsAg coated solid phase which was purified from the serum of two HBsAg carriers of subtype *ad* and *ay*. Uncomplexed HBsAg on the solid phase was then detected with ^{125}I labelled commercially available anti-HBs (Sorin Biomedica).

Hepatitis B core antigen detection (Neurath *et al.*,(1982).

Hepatitis B Dane particles were pelleted by centrifugation through a sucrose cushion as

described in section 2.2. The pellet was resuspended in 50 μl of Tris saline (TN) / non ionic detergent (NP40) / 2-mercapto-ethanol (2-ME), (TN / NP40 / 2 ME buffer (Appendix 1)) buffer. Sodium thiocyanate (NaSCN, 350 μl, 3 M) was added to each sample and the samples incubated with a 6 mm polystyrene bead (Precision Plastic Ball Co, Chicago) overnight at room temperature. The beads were washed five times in distilled H_2O. Bound core antigen was detected by incubation of the bead for 1 hour at 45 °C with ^{125}I labelled anti-HBc and 300 μl of 50 μg/ml bovine serum albumin (Fraction V) in 0.14M NaCl/0.01M Tris HCl, pH7.2. The beads were washed five times in distilled water and analysed for the presence of ^{125}I anti-HBc in a NEN 1600 series gamma counter.

Assays for the detection of anti-HBc

I. A Passive Haemagglutination test kit for the detection of anti-HBc. Modified from Corecell™ (The Green Cross Corporation)

This assay is based on the capture of anti-HBc by HBcAg coated cells. The captured antibody linked the cells together so that its presence can be visualised. A modification of the standard protocol was used (Dr Mark Johnson, PHLS Colindale). 50 μl of phosphate buffered saline were added to each well of a V well microtitre plate and 1 µl of the sample; the control samples provided in the kit were used at a 1:10 dilution in PBS.

The test cells were reconstituted in 5 mls of PBS for storage. Stored cells were diluted 1/10 in PBS immediately before use. One drop (approximately 25 μl) of cells was added to each sample. The plate was incubated at room temperature for 10 minutes and then spun in a Titertek microplate holder for 2 minutes at 1000 rpm. The plates were carefully placed on a 70^0 slope for 10 minutes and then read. Anti-HBc positive samples remained as a tight button of linked cells, anti-HBc negative samples showed as a streak. Positive and negative control cells were included on each plate.

II. Radioimmunoassay for anti-HBc IgG fraction

A recombinant HBcAg was used to capture serum anti-HBc on to a solid phase. The inhibition of anti-HBc IgG labelled with ^{125}I binding to the solid phase was then measured.

III. Radioimmunoassay for anti-HBc IgM fraction

IgM antibody within the test sample was captured onto a solid phase coated with anti-

human IgM. Cloned HBcAg, was used above, was then incubated simultaneously with ^{125}I labelled anti-HBc to allow detection of anti-HBc IgM.

A radioimmunoassay for the simultaneous detection of HBeAg and anti-HBe

In this assay the solid phase was coated with a mixture of gamma-globulin from two anti-HBe murine ascitic fluids produced against two monoclonal antibodies. Donor supplied HBeAg was added simultaneously with the test sample to the solid phase.

Following an overnight incubation an ^{125}I labelled immunoglobulin fraction from an ascitic fluid produced against an alternative monoclonal antibody was added to the solid phase. A decreased signal in comparison with the anti-HBe positive control serum sample indicated the presence of anti-HBe in the test serum. An increased signal in comparison with the HBeAg positive control serum indicated the presence of HBeAg in the test serum.

Commercial assays for the detection of delta and hepatitis A serological markers

Commercial enzyme immunoassays for the detection of total anti-delta, anti-delta IgM, delta antigen, total anti-hepatitis A and anti-hepatitis A IgM were performed according to the manufacturers instructions (Murex Diagnostics Ltd).

HBV cDNA Probe preparation

Lambda HBV 1150 Phage Preparation

The lambda strain 1150 containing the HBV insert was amplified in *E.Coli* strain QR 48 bacteria from which DNA was purified for use as an HBV cDNA probe.

E. Coli QR48 were inoculated onto a 30 ml Luria Agar plate and incubated at 37°C overnight. One colony from this plate was inoculated into 10 mls of Luria Broth (L broth, Appendix 1) and incubated, with shaking, overnight at 37°C. The overnight culture was diluted 1/50 in 50 ml of L Broth, supplemented with 5 ml 10mM $MgSO_4$. The cells were incubated at 37°C with constant shaking and the optical density (OD) measured every 30 minutes. When the appropriate level of bacterial growth had taken place (i.e. the OD 650 reached 0.40-0.6, bacterial density being 2-3 x 10^8 cells/ml) the phage stock was titrated in phage buffer. Five and a half mililitres of phage stock was added to 500 ml of L broth give a multiplicity of infection of between 1 and 10. Growth was allowed

to continue at 37 °C until the OD 650 had reached a peak and then fallen to less than 0.7 O.D.units. The culture was lysed by the addition of 1 ml of chloroform and 20 g of NaCl to the culture and incubated on ice for 1 hour. Fifty grams of solid polyethylene glycol (PEG 6000) were added at this point to a final concentration of 10%. The bacterial suspension was divided into 2 x 250 ml aliquots and pelleted by centrifugation at 10,000 r.p.m. (11,000g) for 10 minutes. The supernatant was removed and the pellet washed gently with 10 ml of phage buffer (Appendix 1). The pellet was resuspended in 5 ml of phage buffer. The two aliquots were combined at this stage. The phage were purified by centrifugation through a Caesium chloride step gradient at 22K RPM at 20°C for 1½-2 hours. The phage band was isolated and further purified by centrifugation through 41.5% $CsCl_2$ solution at 35,000 rpm for 24 hours at +4°C. The phage were dialysed against Tris EDTA buffer for 1 hour at +4°C.

The phage DNA was extracted with phenol. The resultant DNA preparation was dialysed (Sambrook *et al.*,1989) in TE buffer overnight. Restriction enzyme analysis with Hind III and BamH1 confirmed the identity of the DNA preparation.

Transformation of *E. Coli* (HB 101) with lambda HBV 1150 DNA (Lederberg and Cohen.,1974).

E. Coli (HB101) from a stored stab culture were streaked onto a Luria-Bertani (LB) Agar plate and incubated overnight at 37°C. A colony was picked off with a sterile wire loop and inoculated into a 1.0 ml aliquot of LB medium for overnight incubation at 37°C. The overnight culture was diluted 1:50 into LB medium and allowed to grow, with agitation, in an incubator at 37°C until the optical density (650 nm) reached 0.55 - 0.65. The cell culture was then divided and 25 ml of the culture chilled on ice for 25 minutes. The cells were pelleted by centrifugation at 4000 g for 5 minutes at 4°C, the supernatant discarded and the cell pellet resuspended in 12.5 ml of ice cold 0.1M $MgCl_2$. The cells were immediately repelleted and resuspended in 1.5 ml of ice cold 0.1M $CaCl_2$ and then held on ice until use. The phage DNA used for transfection was diluted to a final concentration of 0.1 μg/ml in TE buffer (pH 7.5). This was further diluted in 1 x SSC, 0.1M $CaCl_2$ so that 1.0 ng of DNA was added to 0.2 ml of competent cells.

The bacteria and phage DNA were held on ice for 30 minutes, the bacteria were heat

shocked at 42°C for 2 minutes and immediately returned to ice for 30 minutes. Finally the bacteria were suspended in 2.5 mls of top agar (0.7%) supplemented with 0.01 M $MgSO_4$, which had been maintained at 60°C , and poured onto LB Agar plates. The plates, were incubated overnight at 37°C and the plaques present counted to give an indication of the efficiency of transfection. The number of plaques present increases with a greater efficiency of transfection.

Plasmid preparation (pHBV 130)

E. Coli bacteria (HB101) containing plasmid (pHBV 130) were stored in a stab culture at room temperature. Cells were inoculated onto a LB Agar plate (Appendix 1) supplemented with 25 μg/ml of tetracycline and then incubated overnight at 37°C. From this plate one colony was cultured in LB medium with tetracycline (25 μg/ml) overnight. The bacteria were pelleted by centrifugation and resuspended in 3.5 ml of ice-cold lysis buffer (25mM TrisHCl pH 8.0, 10mM EDTA, 15% sucrose). Eight mg of lysozyme in 0.5 ml of the above buffer were added to the cells and the preparation held on ice for 30 minutes. Eight mls of fresh 0.2 M NaOH, 1% SDS were added, the preparation was mixed gently by inversion and held on ice for a further 10 minutes. Five ml of 3M NaAc pH 5.2 were added, mixed as before and the solution returned to ice for a further 10 minutes.

The cellular and bacterial debris were removed by centrifugation at 20,000 rpm (11000 g) for 10 minutes at 5°C in a Beckman SW27 rotor. The supernatant was transferred to a new tube and 20 μl of 10 mg/ml RNase added. The plasmid DNA was purified by phenol:chloroform:Isoamylalcohol(P:C:I) extraction (Appendix 2) and concentrated by ethanol precipitation (Appendix 2). The DNA was resuspended in 1 ml of sterile TE buffer pH 8.0. The concentration and purity of the preparation was determined by optical densitometry (Appendix 2). The final yield of this preparation was 340 ng/μl.

Transformation of NEM 259 cells by pHBV 130 DNA (Lederberg and cohen 1974)

Transformation was achieved using the same process to produce competent cells as described for transfection with lambda DNA. One nanogram and 2 ng of pHBV130 DNA were inoculated into bacteria (NEN 259) in two separate experiments. The bacteria were grown overnight in selective medium (LB Agar supplemented with tetracycline (0.25

ug/ml). The cells and DNA were then plated out as described for transfection. Only those cells containing the plasmid which carries a gene making it resistant to the antibiotic teracyclene could grow on this medium; individual colonies were picked for plasmid purification.

Restriction endonuclease digestion of DNA

Digestion of DNA was carried out according to the manufacturers instructions (BRL). The following were added to a sterile microcentrifuge tube; 10 μg of purified DNA to be digested; 15 units of Hind III restriction buffer in 1.5 μl of stock solution, 2 μl of 10 x restriction buffer and distilled H_2O to a final volume of 20 μl. The digestion was incubated for 90 minutes at 37°C in a heating block. Where a second digestion was required, a further restriction enzyme was added to the digestion mix after the initial incubation. The salt concentration of the restriction buffer was altered as necessary.

The digested DNA was separated by gel electrophoresis on a 1% agarose gel. Ten micrograms of HBV DNA in plasmid pBR322 was digested with Bst EII as described above to produce the 3.2 Kb fragment of HBV specific DNA. The agarose gel containing this fragment was cut out and stored at -20°C for oligolabelling.

Southern transfer of DNA (Southern E,1975)

The specificity of the PCR product was confirmed by Southern blotting. The PCR product was separated from unreacted nucleotides and primers by electrophoresis in an agarose gel. The DNA was visualised with ethidium bromide (0.5 μg/ml) as prviously described. The DNA was denatured in the gel by immersing the gel in 0.2M NaOH/0.6M NaCl for 30 minutes at room temperature with gentle agitation. The gel was then washed in three changes of 10 x SSC (Appendix 1) for one hour. A nylon membrane (Gene Screen^{TM}) was cut slightly larger than the gel and soaked in 10 x SSC for 20 minutes. Whatman 3mm blotting paper was cut to the size of the glass support plate. Two wicks of filter paper were then cut to reach the bottom of the buffer reservoir and cross the glass support plate. The wicks were thoroughly soaked and placed on to the support plate, trapped air bubbles were forced out by rolling the paper with a 10 ml glass pipette. The gel was then placed, face-down, on the paper and air bubbles rolled out. The presoaked membrane was then placed on the gel,air bubbles removed, and a 1" layer of filter papers placed on top. Finally a glass plate and a 0.5 kg weight covered the apparatus. The SSC

was drawn up through the gel by capillary action, eluting the denatured DNA, which bound to the Gene Screen. The reservoir contained 1 litre of buffer (10 x SSC) and was left overnight at room temperature. The wick and base were covered with clingfilm to prevent evaporation of the buffer reservoir. The following morning the weight, top glass plate, tissue and blotting paper were removed, the clingfilm carefully peeled away the gel was lifted with the Gene Screen in place and turned over so that the position of the Screen on the gel could be marked using a 2B pencil. The Gene Screen was floated in 0.4M NaOH for 3 minutes to help binding of the DNA to the membrane and then washed in 2 x SSC for 5 minutes to remove any residual agarose from the surface. The filter was baked for 2 hours at 80°C. Prehybridisation, hybridisation and post hybridisation washing were carried out as previously described. The probe was prepared by random primed oligolabelling.

The purification of nucleic acids with phenol/chloroform

Chloroform was mixed with isoamyl alcohol in a 24:1 ratio and added to an equal volume of phenol. The phenol had been pre-equilibrated with 0.1M TrisHCl pH 8.0. The phenol/ chloroform/iso-amyl alcohol 25:24:1 (P:C:1) mixture was added to an equal volume of DNA sample in a sealable tube. The solutions were then mixed thoroughly and depending on the volumes being used the aqueous and organic phases were separated by centrifugation at 1600 g for 5 minutes or for 15 seconds in an Eppendorf centrifuge (13000 RPM) (sample vol < 0.5 ml). The upper aqueous layer was removed with a wide bore pipette and transferred to a fresh tube. This procedure was repeated once more with P:C:I. and once with C:I. only.

The Concentration of DNA with Ethanol

The sample volume was measured and 2 volumes of ice cold ethanol added to it, the concentration of salt was raised by the addition of 0.1 vol of 3M sodium acetate pH 5.2 to 0.3 M and all of the solutions mixed well by vortexing. The solutions were then incubated at -20 °C for 30-60 minutes. The DNA was pelleted by centrifugation at 0 °C 12,000 g or in an eppendorf centrifuge for 10 minutes if the total volume was < 1 ml. The supernatant was discarded and the tubes drained. The DNA was then either dried in a nitrogen gas stream or excess liquid was left to evaporate at R.T. The pellet was resuspended in an appropriate volume of TE buffer to give a final concentration of 1 ug/ul.

Scanning densitometry of autoradiographs

The densitometer used was a CAMAG TLC PHTLC scanner model 76500 and the integrator was an Haitachi chromo-integrator model D-2000.

The autoradiograph was attached to white card and placed on the scanner platform. The sample dots on the autoradiograph were lined up with the scanning beam and a test run carried out on the cleanest area of the autorad. This established the background noise level below which signal would not be analysed. The sensitivity of the photomultiplyer was adjusted according to the depth of colour on the autorad and the amplification adjusted accordingly with the potentiometer control. The scan could be controlled manually or automatically by presetting the length of the scan, distribution of samples and number of scans on that autorad. The autorad was scanned at 320 nm. The scan measured the density of colour and size of each sample. The reading was given in microamperes. This was converted to units by the integrator and represented as area under the curve of each peak on the scan.

Appendix 3

Reagent sources

Amersham International PLC	[methyl-^{3}H] Thymidine 5'-triphosphate ammonium salt [40-60ci/mmol] 1 mci/ml
	Deoxycytidine5'-[α-^{32}P] triphosphate, triethyl ammonium salt
BDH chemicals ltd.	Glycerol
	Ethylenediaminetetra-acetic acid 'Analar'
	tri-Sodium citrate
	Sodium dodecyl sulphate
	Potassium chloride
	Dithiothreitol (Cleland's reagent)
	Gelatine Powder
	Boric acid
	Xylene cyanol
Difco chemicals	Bacto tryptone
	Bacto yeast extract
	Agar
	Polyvinyl pyrilodine (p.v.p.)
Dupont NEN (UK)ltd	Deoxyadenosine 5'-(α-thio)triphosphate, [^{35},S]-
	Deoxycytidine 5'-(α-thio)triphosphate, [^{35},S]-
	Deoxyguanosine 5'-(α-thio)triphosphate, [^{35},S]-
	Thymidine 5'-(α-thio)triphosphate, [^{35},S]-
Dynal UK Ltd	Dynabeads M-280 Streptavidin
Fisons	Formamide
	Hepes buffer
Genetic Reearch	Auomatic autoradiograph Processing
Instrumentation ltd	RGD Developer
	RGF Fixer
ICN	Deoxyribonucleic acid from calf thymus (Sodium salt)

Pharmacia	Hexadeoxy ribonumcleotides
	Ficoll
	Restriction enzymes; buffers
Promega	Agarose
	Taq DNA polymerase
	10x Thermophilic Buffer
	25 mM $MgCl_2$
Rathbone chemical Co	Phenol
Sigma chemical Co Ltd	Magnesium chloride (1 M solution) Dextran sulphate
	Bromophenol blue
	Nonidet P-40
	2-mercaptoethanol
	Acetic acid (98%)
	Bovine serum albumim
	Magnesium sulphate
Sigma chemicals	Deoxy cytidine 5' Triphosphate Sodium salt
	Deoxy adenosine 5' Triphosphate Sodium salt
	Deoxy guanosine 5' Triphosphate Sodium salt
	Trizma base
Severn Biotech Ltd	Acrylamide/Bis-acrylamide stck solution 40% w/v-2.105% w/v

All reagents used were of molecular reagent or analar grade

Appendix 4

HBV DNA Probes used for the detection of HBV DNA

HBV 1150 phage. This is a modified strain of lambda phage (1150) initially transfected into E. Coli 259 cells. The phage contains an HBV insert in plasmid pBR322 at an unique Eco RI/Hin D III site. The phage was amplified in E. Coli QR 48 cells.

The plasmid pBR322 continued a single copy of the HBV genome inserted at the first BSTE II site. The plasmid was transfected into E. Coli (strain HB101) cells which are also used for its amplification.

Appendix 5

Publications arising to which this work has contributed

Hawkins AE; Gilson RJC; Beath SV; Boxall EH; Kelly DA; Tedder RS; Weller IVD. Novel application of a point mutation assay: Evidence for the transmission of hepatitis B viruses with precore mutations and their detection in infants with fulminant hepatitis B. J.Med.Virol. 1994; 44:13-21

Hawkins AE; Gilson RJC; Bickerton; Tedder RS; Weller IVD. Conservation of precore and core sequences of hepatitis B virus chronic viral carriers. J.Med.Virol. 1994; 43:5-12

Hawkins AE; Gilson RJC; Gilbert N; Gray JJ; Boer A-D; Wreghitt; Tedder RS; Alexander GJM. Hepatitis B virus surface mutations associated with infection after liver transplantation. J.Hepatol. In Press

Gilson RJ; Hawkins AE; Kelly GK; Gill SK; Weller IV. No effect of zidovudine on hepatitis B virus replication in homosexual men with symptomatic HIV-1 infection: AIDS. 1991; 5(2):217-220

Zuckerman MA, Hawkins AE, Briggs M, Waite J, Balfe P, Thom B, Gilson RJC, Tedder RS. The investigation of hepatitis B virus transmission in an health care setting-application of direct sequence analysis. J.Inf.Dis. In Press

Appendix 6
Tables and figures not included in the main text of the thesis

Table 2.6(i) Sequences, temperatures of dissociation and annealing temperatures of oligonucleotides used as HBV-PCR primers for the precore/core gene

Primer Number[3]	Primer position[1]	Primer Sequence	T_d[2]	Annealing temperature		
				PCR Round		
				1	2	3
C1[4] 188/661/378	1944-1964	CTTGGATCCTATGGGAGTGG	62	45		
C2[5] 189/658	1473-1444	CCATGTTCAGCGCAGGGTCCCCAATCCTCG	96	45		
C3* 190[5]	3182-3212	GCCTCCAAGCTGTGCCTTGGGTGGCTTTG	92		50	50
C4 666	531-516	TTGAGATCTTCTGCGACG	54		50	50
C5 664	1-19	ATGGACATTGACCCTTAT	50	45	45	

Notes: 1 Nucleotide 1 is AUG in the core gene,serotype adyw (Pugh *et al.*,1986). 2 T_d is the temperature at which a perfectly homologous DNAmolecule will be half-dissociated. 3 All primers were designed by A Hawkins from published sequences unless otherwise indicated. 4 Primer sequence taken from Larzul *et al.*, (1988). 5 Primer sequence supplied by Professor K Murray Edinburgh University). * 5' Biotinylated versions of these primers are used to produce a biotinylated PCR productfor sequencing and Point Mutation analysis. C Primers to the core region.

Table 2.6(i)continued **Sequences,temperatures of dissociation and annealing temperatures of oligonucleotides used as HBV-PCR primers for the precore/core gene**

Primer Number[3]	HBV nucleotide position[1]	Primer Sequence	T_d[2]	Annealing temperature		
				PCR Round		
				1	2	3
PC1* 439	2951-2970	TCTTGCCCAAGGTCTTACAT	58	45	50	55
PC2 441	54-35	AAACGAGAGTAACTCCACAG	58		50	55
PC3 440	77-55	GGAAGGAAAGAAGTCAGAAGGCAA	60	45		55
PC4* 373	45-27	TAACTCCACAG(T/A)AGCTCCA	60		50	55
PC5 459	2961-2979	GGTCTTACATAAGAGGAC	52	45		50

Notes

1 Nucleotide 1 is AUG in the core gene,serotype adyw (Pugh *et al.*,1986)

2 T_d is the temperature at which a perfectly homologous DNA molecule will be half-dissociated

3 All primers were designed by A Hawkins from published sequences unless otherwise indicated

* 5' Biotinylated versions of these primers are used to produce a biotinylated PCR product for sequencing and point mutation analysis

PC Primers to the precore region

Table 2.6(ii) Sequences, temperatures of dissociation and annealing temperatures of oligonucleotides used as HBV-PCR primers in the surface gene

Primer Number[3]	HBV nucleotide position[1]	Primer Sequence	T_d[2]	Annealing temperature	
				PCR Round	
				1	2
S1[6] 376[6]	1740-1765	CTCATCTTCTTGTTGGTTCTTCTGGA	74	45	
S2[6] 377[6]	1991-1965	ACTGAGCCAGGAGAAACGGACTGAGGC	88	45	
S3[6]* 374[6]	1767-1796	TATCAAGGAATTCTGCCCGTTTGTCCTCTA	56		55
S4[6]* 375[6]	1966-1935	CCACTCCCATAGGAATTCTGCGAAAGCCCA	64		55

Notes

1 Nucleotide 1 is AUG in the core gene,serotype adyw (Pugh *et al.*,1986)

2 T_d is the temperature at which a perfectly homologous DNA molecule will be half-dissociated

3 All primers were designed by A Hawkins from published sequences unless otherwise indicated

6 Primer sequences from Dr Sheila Grace (Murex Diagnostics Ltd)

* 5' Biotinylated versions of these primers are used to produce a biotinylated PCR product for sequencing and Point Mutation analysis

S Primers to the surface region

Table 2.6(ii)continued Sequences, temperatures of dissociation and annealing temperatures of oligonucleotides used as HBV-PCR primers in the surface gene

Primer Number[3]	HBV nucleotide position[1]	Primer Sequence	T_d[2]	Annealing temperature	
				PCR Round	
				1	2
S5[4] 185	914-933	GGGTCACCATATTCTTGGGA	58	45	55
S6[4] 186	2157-2136	TTAGGGTTTAAATGTATACCC	56	45	55
S7 187	2063-2051	CTCAAGCTTCATCATCCATATA	58		55
S8 163	2073-2054	CCCAATACCACATCATCCA	56		55
P1[7]	1964-2008	GCCTCAGTCCGTTTCTCTTGGCTCAGTTTACTAGTGCCATTTGT			

Notes

1 Nucleotide 1 is AUG in the core gene,serotype adyw (Pugh *et al.*,1986).

2 T_d is the temperature at which a perfectly homologous DNA molecule will be half-dissociated.

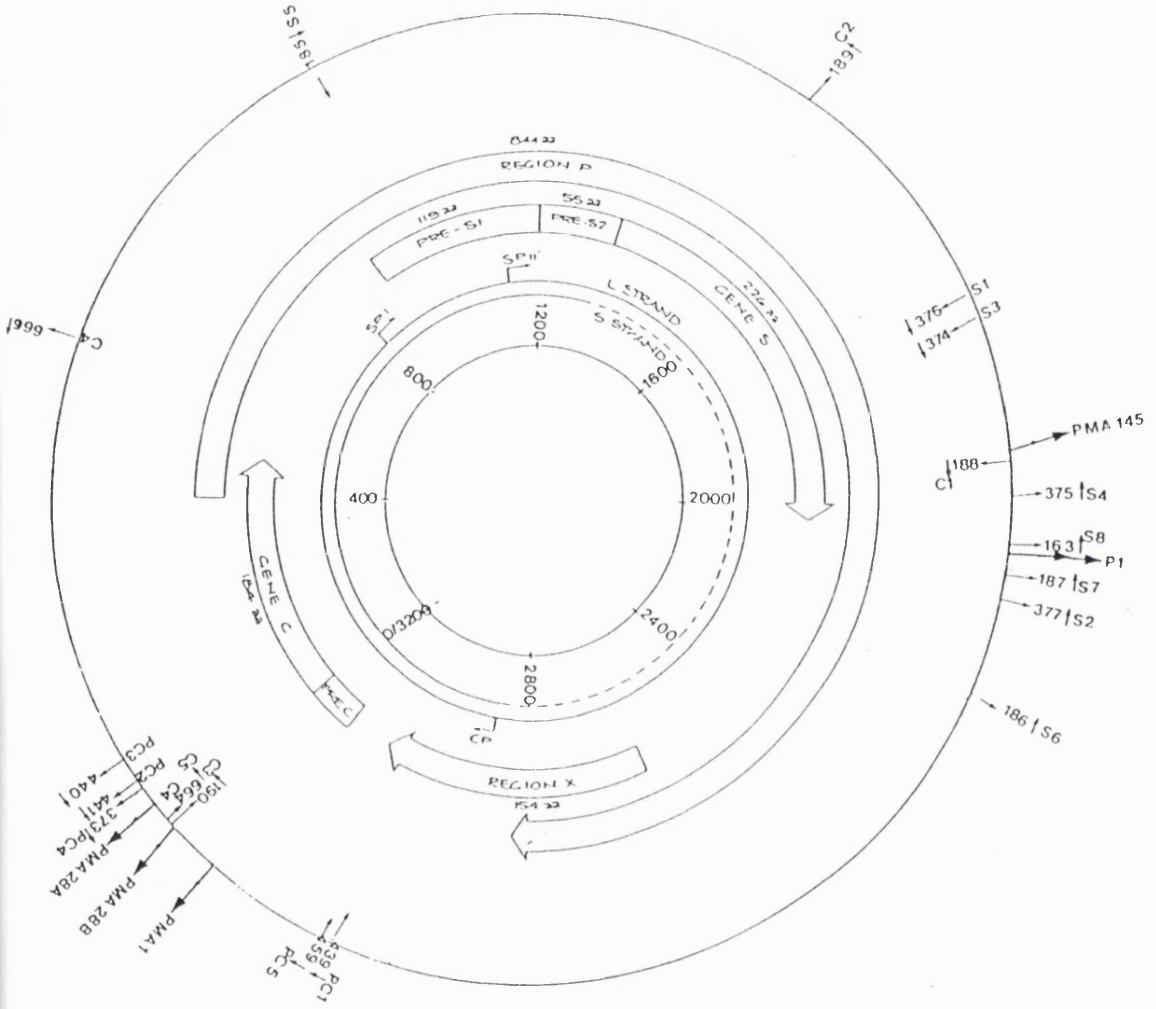
3 All primers were designed by A Hawkins from published sequences unless otherwise indicated.

4 Primer sequence taken from Larzul *et al.*, (1988).

7 oligonucleotide probe for the surface gene.

S Primers to the surface region.

Figure 2.6(i) Cartoon illustration of the relative positions of PCR primers



Notes

PC, precore primer. C, core primer. S,surface primer.
PMA, Oligonucleotide probe/primer for use in the PMA
→ 5′ to 3′ orientation of the primer
↑ Position of the primer

Table 2.6(iii) Primer combinations and thermocycling conditions for the amplification of the hepatitis B precore region

Sense primer	Anti-sense primer	PCR product size (base pairs)	PCR round 1,2 or 3	Strand denaturation (95°C) Time (minutes)	Primer annealing Temperature (°C)	Primer annealing Time (minutes)	Strand elongation (72 °C) Time (minutes)
C1 188	C2 189	2734	1	1.5	45	2	2.5
PC1 439	C4 666	792	2	1.2	50	1.5	1.5
PC1 439	PC4 441	306	2/3	1.2	50/55	1	1.5
C1 188	PC3 440	1338	1	1.5	45	2	2.5
PC5 459	PC 2 441	296	3	1.2	50	1.5	2

Notes

PC Primers to the precore region.

C Primers to the core region.

Table 2.6(iv) Primer concentration and thermocycling conditions for the amplification of the hepatitis B core region

Sense primer	Anti-sense primer	PCR product size (base pairs)	PCR round 1,2 or 3	Strand denaturation (95°C) Time (minutes)	Primer annealing Temperature (°C)	Primer annealing Time (minutes)	Strand elongation (72 °C) Time (minutes)
C1 188	C2 189	2734	1	1.5	45	2	3.5
PC1 439	C4 666	792	2	1.2	50	1.5	2
C3 190	C4 666	561	3	1.2	50	1.5	2
C1 190	C2 189	1768	1	1.5	45	2	3.5
C5 664	C4 666	531	2	1.5	50	1.5	2

Notes

PC Primers to the precore region.

C Primers to the core region.

Table 2.6(v) Primer combinations and thermocycling conditions for the amplification of the hepatitis B surface gene

Sense primer	Anti-sense primer	PCR product size (base pairs)	PCR round 1,2 or 3	Strand denaturation (95°C) Time (minutes)	Primer annealing Temperature (°C)	Primer annealing Time (minutes)	Strand elongation (72 °C) Time (minutes)
S1 376	S2 377	251	1	1.5	45	2	3.5
S3 374	S4 375	170	2	1.2	55	1	1.2
S5 185	S6 186	1243	1	2	45	2	3.5
C1 188	S7 187	128	2	1.2	55	1	1.2
S1 376	S8 163	255	1	1.5	45	2	2.5
S3 374	S4 375	195	2	1.2	55	1	1.2

Notes

S Primers to the surface region.

C Primers to the core region.

Table 3.1(i) A comparison between random primed labelling and nick translation with ^{32}P dCTP of DNA for use as a nucleic acid probe.

Method of labelling	Nick translation	Random primed oligolabelling
Percentage incorporation of ^{32}P dCTP	15	35
Sensitivity under typical assay conditions*	1.0 pg	0.1 pg

Notes
*Hybridisation, post hybridisation washing and autoradiography were carried out as described in sections 2.3.7-2.4

Table 3.1(ii) Results of densitometric analysis by area under the curve and height of the peak for autoradiographs from serum samples treated with 5% NP40, 1.5% 2 ME.

HBV DNA polymerase (CPM)	Mean of duplicate samples area under curve (units)	Mean of duplicate samples height of curve (units)
10	120894	23132
10	181092	34613
292	614734	59917
354	800279	84696
235	911968	81695
476	768925	83994
513	869546	73961
205	620509	71228
72	765701	81658
200	418718	57943
345	896647	85712

Notes

Correlation coefficient of HBV DNA polymerase values with area under curve, r = 0.858

Correlation coefficient of HBV DNA polymerase values with height of curve, r = 0.823

Correlation coefficient of area under curve with height of curve, r = 0.966

Table 3.1(iii) Results of densitometric analysis, by area under the curve of 24 hr and 72 hr exposure autoradiographs from serum samples treated with 5% NP40, 1.5% 2ME.

HBV DNA polymerase CPM	Mean result of duplicate sample dots 24 hr autoradiograph (units)	Mean result of duplicate sample dots 72 hr autoradiograph (units)
10	138488	177999
10	12779	24721
292	749396	811213
354	742583	854438
235	856737	936751
476	696372	887081
513	680596	803498
205	599741	638141
72	700425	788445
200	631075	645081
345	974347	1024780

Notes

Correlation of DNA polymerase with mean result of sample dots, 24 hr exposure r = 0.828

Correlation of DNA polymerase with mean result of sample dots, 72 hr exposure r = 0.718

Table 3.1(iv) A comparison of 24 hour autoradiograph results for samples treated with NP40 and 2 ME between two hybridisation assays carried out under identical conditions. Confirmation of inter-assay variation.

HBV DNA polymerase (CPM)	Mean of duplicate samples (area under curve) Run 1 (units)	Mean of duplicate samples (area under curve) Run 2 (units)
10	120894	132994
10	181092	61573
292	614734	811213
354	800219	854438
235	911968	936751
476	768925	887081
513	869546	803498
205	620509	633191
72	765701	788445
200	418713	643081
345	896647	1023280

Notes

Correlation coefficient of results from Run 1 with run 2,

$r = 0.972$ ($p =< 0.001$)

Table 3.2(i) The detection of HBV DNA in a selected group of serum samples by PCR and Southern hybridisation.

Sample number	HBsAg	HBeAg	anti-HBe	anti-HBc	PCR
1	+	-	-	+	-
2-6	+	+	-	+	+
7,8,	+	-	+	+	-
9	+	-	+	+	+
10	+	-	+	+	-
11-15	-	-	-	+	-
16-18 Negative control	-	-	-	-	-
19-20 Positive control (serum)	+	+		+	+
21 Positive control (pHBV 130)					+

Notes

- No PCR product visible after ethidium bromide staining and agarose gel electrophoresis

+ PCR product clearly visible after ethidium bromide staining and agarose gel electrophoresis

Table 3.2(ii) Double nested PCR amplification of HBV DNA from 1 μl serum samples

Sample	HBeAg/ anti-HBe	Precore PCR		Core PCR	
		Round 2	Round 3	Round 2	Round 3
1	HBeAg	-	+	-	+
2	HBeAg	+	+	+	+
3	HBeAg	-	+	+	+
4	HBeAg	+	+	+	+
5	HBeAg	+	+	+	+
6	anti-HBe	+	+	-	+
7	anti-HBe	-	+	-	-
8	anti-HBe	-	-	-	-
9	anti-HBe	-	-	-	+
10	anti-HBe	-	-	-	-

Notes

- No PCR product visible after ethidium bromide staining and agarose gel electrophoresis
+ PCR product clearly visible after ethidium bromide staining and agarose gel electrophoresis

Table 3.2(iii) Results of a Magnesium Chloride titration for core primers

Sample number	HBV DNA concentration (copies)	$MgCl_2$ concentration (mM)	PCR result
1-5	0	0	-
6	0	5.0	-
7	1000	0	-
8	1000	1.0	-
9	1000	1.5	+
10	1000	2.0	+
11	1000	3.0	+
12	1000	5.0	+
13-18	1	0	-

Notes

- No PCR product visible after ethidium bromide staining and agarose gel electrophoresis

+ PCR product clearly visible after ethidium bromide staining and agarose gel electrophoresis

Table 3.2(iv) An analysis of the effect of TE buffer on the effective magnesium chloride concentration in a PCR

Sample copies of HBV DNA	Volume of TE added / 100μl reaction	2.0 mM $MgCl_2$	3.0 mM $MgCl_2$	4.5 mM $MgCl_2$	6.0 mM $MgCl_2$	7.5 mM $MgCl_2$	10 mM $MgCl^2$
10	0	+	+	+	+/-	-	-
1000	0	+	+	+	+	+	-
10	24 μl	-	-	-	+	+	+
1000	24 μl	+	+	+	+	+	+
0	0	-	-	-	NT	NT	NT
0	24 μl	NT	-	-	-	NT	NT

Notes

- No PCR product visible after ethidium bromide staining and agarose gel electrophoresis
+/- Minimal PCR product visible after ethidium bromide staining and agarose gel electrophoresis
+ PCR product clearly visible after ethidium bromide staining and agarose gel electrophoresisNotes

Table 3.2(vi) A summary of results for the comparison of sample extraction buffers and conditions

Sample	Buffer 1*	Buffer 2*	Buffer 3*	Buffer 4*	HBV Dot blot result	Heat treated sample
1	+	-	+	-	-	+
2	+	+/-	+	-	-	+
3	+	+	-	-	-	+
4	+	+	-	+	-	+
5	+	+	+	-	+	+
+ control	+	+	+	+	-	+
- control	-	-	-	-	-	-

Notes

* for buffer components see table 3.2(v)

\- No PCR product visible after ethidium bromide staining and agarose gel electrophoresis

+/- Minimal PCR product visible after ethidium bromide staining and agarose gel electrophoresis

\+ PCR product clearly visible after ethidium bromide staining and agarose gel electrophoresis

Table 3.2(vii) **A comparison of sample treatment methods**

Sample number	Sample pretreatment method				
	PEG precipitation of serum		RF absorption	None	None
	100 μl	500 μl	25μl	25μl	100 μl
	PCR result				
1	-	+/-	+	+	-
2	+/-	+	++	-	++
3	++	++	+	++	+
4	++	++	+	+	++
5	++	-	++	-	++
negative control	-	-	-	-	-

Notes

- No PCR product visible after ethidium bromide staining and agarose gel electrophoresis
+/- Minimal PCR product visible after ethidium bromide staining and agarose gel electrophoresis
+ PCR product clearly visible after ethidium bromide staining and agarose gel electrophoresis
++ High concentration of PCR product after ethidium bromide staining and agarose gel electrophoresis

Table 3.2(viii) **A comparison of PCR sensitivity on processed and unprocessed serum**

Sample dilution	Unprocessed Serum PCR result		Purified HBV DNA PCR result		Lysed serum PCR result	
	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2
1000,000	-	++	+	+	+	+
100,000	-	++	+	+	+	+
10,000	-	++	+	+*	-	+
1000	-	+	+	+	-	+
100	-	+	-	+	-	+
10	-	+	-	+	-	-
1	-	+	-	-	-	-
0.1	-	-	-	-	-	-
0.01	-	-	-	-	-	-

Notes

*HBV was not amplified from this sample in this experiment but was amplified after repetition of the nested round of amplification.

- \- No PCR product visible after ethidium bromide staining and agarose gel electrophoresis
- +/- Minimal PCR product visible after ethidium bromide staining and agarose gel electrophoresis
- \+ PCR product clearly visible after ethidium bromide staining and agarose gel electrophoresis
- ++ High concentration of PCR product after ethidium bromide staining and agarose gel electrophoresis

Table 3.2(ix) A comparison of PCR sensitivity for an HBV positive control diluted in non-haemalysed, freshly prepared serum.

Sample dilution	1 μl serum PCR result		20 μl serum PCR result		20 μl heat treated serum PCR result	
	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2
100,000	-	+	-	+	+	+
10,000	-	+	-	+	+	+
1000	-	+	-	+	+	+
100	-	+/-	-	+	-	+
10	-	-	-	+	-	+
1	-	-	-	-	-	+
0.1	-	-	-	-	-	-
0.01	-	-	-	-	-	-
Negative control serum	-	-	-	-	-	-

Notes

- No PCR product visible after ethidium bromide staining and agarose gel electrophoresis
+/- Minimal PCR product visible after ethidium bromide staining and agarose gel electrophoresis
+ PCR product clearly visible after ethidium bromide staining and agarose gel electrophoresis

Table 3.2(x) A comparison of the sensitivity of PCR on positive control HBV diluted in unfiltered, particulate serum.

Sample dilution	1 μl serum PCR result		20 μl serum HBV DNA PCR result		20 μl heat treated serum PCR result	
	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2
100,000	-	+	-	+	+	+
10,000	-	+	-	+	+	+
1000	-	+	-	+	+	+
100	-	+	-	+	-	+
10	-	+	-	+	-	+
1	-	-	-	-	-	+
0.1	-	-	-	-	-	-
0.01	-	-	-	-	-	-
Negative control serum	-	-	-	-	-	-

Notes

- No PCR product visible after ethidium bromide staining and agarose gel electrophoresis
+ PCR product clearly visible after ethidium bromide staining and agarose gel electrophoresis

Table 3.2(xi) A comparison of PCR sensitivity for an HBV positive control diluted in haemolysed serum.

Sample dilution	1 μl serum PCR result		20 μl serum PCR result		20 μl heat treated serum PCR result	
	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2
1000,000	-	+	-	+	-	+
100,000	-	+	-	+	-	+
10,000	-	+	-	+	-	+
1000	-	+	-	+	-	+
100	-	+	-	-	-	+
10	-	-	-	-	-	+
1	-	-	-	-	-	+
0.1	-	-	-	-	-	+
0.01	-	-	-	-	-	-
Negative control serum	-	-	-	-	-	-

Notes

- No PCR product visible after ethidium bromide staining and agarose gel electrophoresis
+ PCR product clearly visible after ethidium bromide staining and agarose gel electrophoresis

Table 3.5(i) HBV DNA probe titrated against unlabelled probe DNA for sample standardization

HBV DNA pg	Mean of duplicate samples (area under curve) units
1000	2885806
500	2023455
250	2084515
100	1720029
10	653386
5	440671
1	912474*
0.1	312120
0	42793
2.6 ng/ml Positive control sample 2	376396
<0.1 pg/ml	64446

Notes
*Non-specific hot spot hybridisation

HBV DNA polymerase values, counts per minute (CPM)

HBV DNA values, area under curve as measured by scanning densitometry (units).

HBV Cut-off values were calculated as the Mean of the negative control sera ± 3 SD

HBV DNA polymerase cut-off = 1 unit

HBV DNA cut-off = 0.26 ng/ml

Table 3.5(iv) Detailed results of a cross sectional survey of HBV DNA and HBV DNA polymerase in serum from HBV carriers

HBeAg/ anti-HBe	HBV DNA polymerase CPM	Standardised value units	HBV DNA units	HBV DNA ng	HBV DNA units	HBV DNA ng	Mean HBV DNA ng
eAg	414	20	674931	2.2			
eAg	675	94	1665766	5.40			
eAg	256	12	579675	1.91			
eAg	88	4.2	165910	0.54			
eAg	114	3.9	238123	0.78			
eAg	14	0.67	221385	0.72	4152	9.8×10^{-3}	0.36
eAg	169	8.1	478716	1.40			
eAg	293	14.2	523883	1.72			
eAg	168	8.13	560982	1.85			
eAg	349	17	648990	2.14			

Table 3.5(iv)continued Detailed results of a cross sectional survey of HBV DNA and HBV DNA polymerase in serum from HBV carriers

HBeAg/ anti-HBe	HBV DNA polymerase CPM	Standardised value units	HBV DNA units	HBV DNA ng	HBV DNA units	HBV DNA ng	Mean HBV DNA ng
eAg	273	9.5	499666	1.64			
eAg	119	4	357172	1.18			
eAg	399	13.6	873969	2.88			
eAg	393	13.5	628942	2.07			
eAb	12	0.58	19518	0.06			
eAb	8	0.22	66463	0.17			
eAb	9	0.23	104137	0.53	101348	0.40	0.47
eAb	72	3.3	17109	0.06			
eAg	152	6.1	777487	4.03			
eAg	436	14.9	890429	4.65			
eAg	37	1.79	221216	1.15	73734	0.28	0.72

Table 3.5(iv)continued Detailed results of a cross sectional survey of HBV DNA and HBV DNA polymerase in serum from HBV carriers

HBeAg/ anti-HBe	HBV DNA polymerase CPM	Standardised value units	HBV DNA units	HBV DNA ng	HBV DNA units	HBV DNA ng	Mean HBV DNA ng
eAg	242	7.3	776239	4.02			
eAg	130	3.9	308984	1.67			
eAg	580	17.5	713999	3.70			
N	12	0.41	36823	0.19			
N	10	0.28	175601	0.91	26957	0.10	0.50
eAb	8	0.24	107736	0.55	20978	0.079	0.32
eAb	9	0.26	109236	0.56	13423	0.058	0.31
eAb	551	12	226958	1.18			
eAb	23	0.91	38099	0.28	39767	0.15	0.17
eAg	113	5.8	368408	1.66			

Table 3.5(iv)continued Detailed results of a cross sectional survey of HBV DNA and HBV DNA polymerase in serum from HBV carriers

HBeAg/ anti-HBe	HBV DNA polymerase CPM	Standardised value units	HBV DNA units	HBV DNA ng	HBV DNA units	HBV DNA ng	Mean HBV DNA ng
eAg	507	16.3	801621	3.60			
eAg	393	13.5	649771	2.92			
eAg	472	24	912466	4.10			
eAg	61	3.1	393830	1.77			
eAg	1620	81	1193314	5.36			
eAg	422	21	587587	2.64			
eAg	675	34	917126	4.13			
eAg	903	44	932981	4.20			
eAg	15	0.75	238615	1.07			
eAg	329	11.3	816173	3.66			
eAg	265	16.1	465023	2.09			

Table 3.5(iv)continued Detailed results of a cross sectional survey of HBV DNA and HBV DNA polymerase in serum from HBV carriers

HBeAg/ anti-HBe	HBV DNA polymerase CPM	Standardised value units	HBV DNA units	HBV DNA ng	HBV DNA units	HBV DNA ng	Mean HBV DNA ng
eAg	417	21	676808	3.04			
eAb	11	0.44	17567	0.05	35259	0.13	0.09
eAb	21	0.32	240934	1.08			
N	32	1.1	380413	1.71			
eAb	14	0.65					
N	19	0.96	14572	0.21	97070	0.25	0.22
N	10	0.3	21821	0.48	37822	0.141	0.31
eAb	20	1.0	19104	0.26	13644	0.054	0.11
N	18	0.95	14388	0.19			
N	17	0.9	12214	0.22	91708	0.15	0.18

Table 3.5(iv)continued Detailed results of a cross sectional survey of HBV DNA and HBV DNA polymerase in serum from HBV carriers

HBeAg/ anti-HBe	HBV DNA polymerase CPM	Standardised value units	HBV DNA units	HBV DNA ng	HBV DNA units	HBV DNA ng	Mean HBV DNA ng
N	78	0.83	99451	0.40	20976	0.07	
eAg	95	4.5	485373	1.92	370927	1.39	0.66
eAg	737	35.6	975353	3.87			
Ag	367	18.4	806976	3.20			
eAg	9	0.44	257147	1.02	52248	0.19	0.19
eAg	215	10.4	606384	2.41			
eAg	1199	38	363984	1.49			
eAg	159	5.4	367637	1.46	1901872	2.89	2.2
eAg	397	13.6	457377	1.85	430399	1.61	1.73
eAg	330	16.7	567045	2.25			

Table 3.5(iv)continued Detailed results of a cross sectional survey of HBV DNA and HBV DNA polymerase in serum from HBV carriers

HBeAg/ anti-HBe	HBV DNA polymerase CPM	Standardised value units	HBV DNA units	HBV DNA ng	HBV DNA units	HBV DNA ng	Mean HBV DNA ng
eAg	201	6.1	425559	1.69			
eAg	53	1.6	186761	0.7			
eAg	198	9.2	448557	1.09			
eAg	50	1.4	342987	1.3	161568	0.61	0.69
eAg	750	22.6	479792	0.98			
eAb	8	0.24	56184	0.22			
eAg	148	4.4	180096	0.71			

Table 3.5(iv)continued Detailed results of a cross sectional survey of HBV DNA and HBV DNA polymerase in serum from HBV carriers

HBeAg/ anti-HBe	HBV DNA polymerase CPM	Standardised value units	HBV DNA units	HBV DNA ng	HBV DNA units	HBV DNA ng	Mean HBV DNA ng
∘Negative control samples 1							SD 92651
2							SD106057
3							SD55993
∘Positive control samples 1							SD182985
2							SD99952
3							SD359991

Notes

* DNA polymerase cut off value = 0.75 units, ∘ Mean Value of each assay run in the study

Column 1 patients serological status for hepatitis B e antigen (eAg) or the antibody to that antigen (eAb)

Column 2 DNA polymerase value of sample CPM. Column 4 Densitometric analysis of the autoradiograph expressed in units.

Column 5 As for 4 expressed in ng/ml of serum. Column 6 As for 4 Repeat test of the sample

Column 7 As for 5. Column 8 As for 4 Test repeated a third time. Column 9 As for 5

Column 10 Mean value of all tests carried out (if repeated) expressed as ng/ml of serum

HBV DNAp HBV DNA polymerase assay, proportion of incorporated ^{3}H expressed as counts per minute Standardised value = $\frac{\text{Sample value}}{\text{Mean negative control value.}}$

Table 3.9(iii) A comparison of viral concentration in partners of patients developing a fulminant or acute hepatitis, measured by limiting dilution.

		Sample dilution						
Outcome Case ID	Sample ID	0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
fulminant 306 BD	306	+	+	+	+	-	-	-
fulminant 418 BD	418	+	+	+	-	-	-	-
fulminant 489 BD	2946	+	+	-	-	-	-	-
fulminant 791 BD	9880	+	-	-	-	-	-	-
fulminant 799 BD	10025	+	-	-	-	-	-	-
fulminant 803 BD	10206	+	+	+	-	-	-	-
acute 809 BD	10400	+	-	-	-	-	-	-
acute 824 BD	10500	+	+	+	+	-	-	-
acute 895 BD	12088	+	+	-	-	-	-	-
acute 905 BD	12380	+	+	+	-	-	-	-
acute 685 BD	7640	+	-	-	-	-	-	-
acute 680 BD	7591	+	+	+	+	-	-	-

Table 3.9(vi) Comparison of sequences around the A determinant of the surface gene between samples from patients with fulminant hepatitis and their sexual partners

Case ID	Sample ID	Codon	Consensus sequence	Patient sequence	Partner sequence	Partner	Sample ID
382	382(1)	113	TCA	TCT	TCT	306 BD	306 BD(1)
		131	ATC	ACC	ACC		
1434	1434(1)	111	CCA	CCG	CCG	418 BD	418 BD
		113	TCA	TCG	TCG		
		117	AGC	AGG	AGC		
1574	2500	131	ATC	ACC		489 BD	2946[2]
	2533	120	CCC	CTA			
		127	CCT	TCT			
		131	ATC	ACC			
2342	9748	113	TCA	TCT	TCT	791 BD	9880
		118	ACG	GCG	ACG		
		131	ATC	ACC	ACC		
	9795	113	TCA	AGT			
		131	ATC	ACC			
2373	10174	NS					
2400	10184[3]	113[3]	ATC		ACC	803 BD	10206

Notes 1. Samples homolgous to pHBV 130 or published subtypic variations. Samples from which HBV DNA was not amplified were 382 and 1434(1')2. 3. Samples amplified as single molecules.

Table 3.9(vii) **Comparison of sequences in the A determinant of the surface gene between patients with acute , asymptomatic hepatitis and their sexual partners**

Case ID	Sample ID	Codon	Consensus sequence	Patient sequence	Partner sequence	Partner ID	Sample ID
2423	10324	113	TCA	TCT	TCT		
		118	ACG	GCG	GCG	809BD	10400
2401	10183	111	CCA	CGA	AGG	824BD	10500
		112	GGA	TCT	TCT		
2587	11869	113	TCT	TCA	TCA	895BD	12088
		114	TCA	TTA	TCA		
		126	ACT	ACA	ACT		
		135	CCC[4]	TCC/CCC	CCC		
2626	12171[1]					905BD	12380[1]
1898	5627[1]	118	ACG	ACG	GCG	685BD	7640
1628	3018[1]	0[1]				680BD	7591[1]

Notes

1 Samples homolgous to pHBV 130 or published subtypic variations

2 Samples from which HBV DNA was not amplified

4 A mixed sequence was detected at this point

consensus seuqence pHBV 130

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Appendix 7

Amino acid and Nucleotide sequence analyses of samples from individual studies included in the thesis

Figure 3.7(i)	Sequence analysis around the *a* determinant of the HBV surface gene amplified from the serum of Cases 1, 2 and 3
Figure 3.7(ii)	Sequence analysis of the HBV X-C region amplified from the serum of Cases 1, 2 and 3
Figure 3.8(i)	Amino acid sequences of samples analysed from amino acid 1 in the HBV precore region to amino acid 1 in the core region
Figure 3.8(ii)	Amino acid sequences of samples analysed in the X region from codon 129 to codon 154
Figure 3.8(iii)	Amino acid sequence from samples analysed from amino acid 18 to 73 of the core region
Figure 3.9(i)	Sequences from the HBV X gene (amino acids 106-amino acid 154)
Figure 3.9(ii)	Sequencing of the HBV precore region from patients with fulminant hepatitis B and their sexual partners
Figure 3.9(iii)	Sequencing of the HBV X region (amino acid 106-amino acid 154) from individual molecules in patients with fulminant hepatitis and their sexual partners
Figure 3.9(iv)	Sequencing of the HBV precore region from individual molecules in patients with fulminant hepatitis and their sexual partners
Figure 3.9(v)	Sequencing of the HBV X region (amino acid 106- amino acid 154) in patients with acute hepatitis B and their sexual partners
Figure 3.9(vi)	Sequencing of the HBV precore region in patients with acute hepatitis B and their sexual partners
Figure 3.10(i)	Amino acid sequences of samples analysed from precore amino acids 1 to 29
Figure 3.10(ii)	Amino acid sequences in the X region from position 122 to 154 determinant of samples included in the blinded study
Figure 3.12(iii)	Nucleotide sequence analysis of the HBV surface gene *a* determinant of samples included in the blinded study
Figure 3.12(iv)	Nucleotide sequence analysis of the HBV surface gene *a* determinant amplified HBV DNA from subtype *ayw* control samples
Figure 3.12(v)	Sequence analysis of amplified HBV DNA:3' region of X,the entire precore region and the 5'core regions

Figure 3.7(i) Sequence analysis around the ^{a} determinant of the HBV surface gene amplfied from the serum of Cases 1,2 and 3

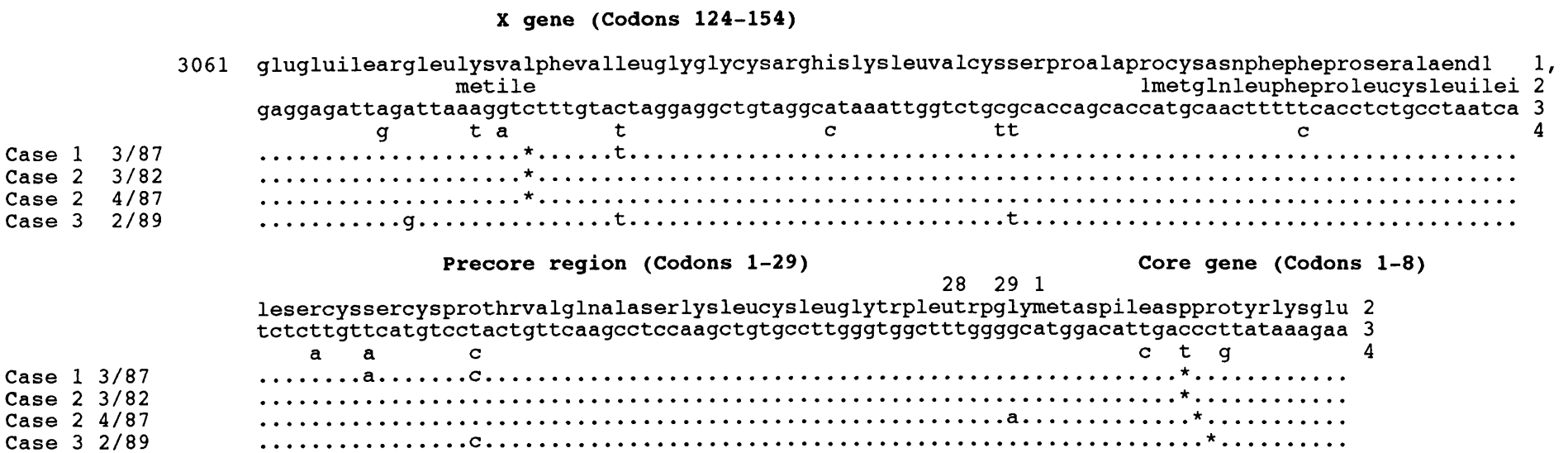
```
                    leuleuproglyserserthrthrserthrglyprocysargthrcysthrthrproalaglnglyilesermetpheprosercyscyscysthrlyspros 1
                              thrthr                        lys         ilethr         asn                                 2
                                                            gln                        thr
                 1809 ctaattccaggatcatcaaccaccagcacgggaccctgcagaacctgcacgactcctgctcaaggaatctctatgtatccctcctgttgctgtacaaaacctt 3
                      c        a ca    a     t     g  a   cag       t  t a          c a     a  t      a          c      a 4
                                 t     t                                           c               t
Case 1  3/87          .............t............gt......a..................a...t........c.............................c.......
Case 2  3/82          .....................................a..................a............c.............................c.......
Case 2  4/87          .....................................a..................a............c.............................c.......
Case 3 12/87          ............................*......a....a.............................c........t......a..................a
Case 3  2/89          .............*.a....a.....t........a....a.............................c........t......a..................a

                     eraspglyasncysthrcysileproileprosersertrpalapheglylyspheleutrpleut 1
                                                                alaarg                  2
                     cggatggaaactgcacctgtattcccatcccatcatcctgggctttcggaaaattcctatgggagt 3 1962
                         c     t     t                 g             c  g  a            4
Case 1  3/87          ....c.....t.....................................................
Case 2  3/82          ..........t.............................t.........................
Case 2  4/87          ..........t.............................t.........................
Case 3 12/87          ....c.....t.........................g.............c.....a..........
Case 3  2/89          ....c.....t.........................g.............c.....a..........
```

Notes

1 Consensus amino acid sequence 2 Recognised amino acid variants 3 Consensus nucleotide sequence : Subtype *adyw* 4 Recognised nucleotide variants * End of nucleotide sequence analysis

Figure 3.7(ii) Sequence analysis of the HBV X-C region amplfied from the serum of Cases 1,2 and 3



Notes

1 Consensus amino acid sequence: X region 2 Consensus amino acid sequence: precore/core region

3 Consensus nucleotide sequence : Subtype *adyw* 4 Recognised nucleotide variants * End of nucleotide sequence analysis

Figure 3.8(ii) Amino acid sequences of samples analysed from amino acid 1 in the precore region to amino acid 1 in the core region

Sample (HBeAg/anti-HBe status)	Amino acid sequence
pHBV 130	MetGlnLeuPheHisLeuCysLeuIleIleSerCysSerCysProThrValGlnAlaSerLysLeuCysLeuGlyTrpLeuTrpGly
	(Pro) (Thr) (c)
1 (eAg)	. . . . . t . . . . .
26 (eAg)	
6 (eAg)	. . . . . c . . . . .
34 (neither Ag/Ab)	
37 (eAb)	
15 (eAg)	. . . . . Thr. . . . . Asn. . . . .
33 (eAb) A	Leu. . . . .
33 B	Met. . . . .
38 (eAb)	. . . . . Thr. t . . . . . X .
All remaining samples	. . . . . Thr. c . . . . .

Notes

The consensus sequence (pHBV 130) shown in Figures 3.8(ii), (iii) and (iv) is subtype adyw[Pugh *et al.*,1986]

Letters in italics indicate silent nucleotide changes

X indicates a stop codon.

.indicates sequences identical to the consensus sequence.

( ) indicates published nucleotide substitutions or amino acid variation between subtypes.

Figure 3.8(iii) Amino acid sequences of samples analysed in the X region from codon129 to codon154.

Sample number (HBeAg/anti-HBe status)	Amino acid sequence
pHV130	^{129} LysValPheValLeuGlyGlyCysArgHisLysLeuValCysAlaProAlaProCysAsnPhePheProSerAlaXXX^{154}
	(MetIle) (Ser)
1 (eAg)	. . . c . . . .
18 (eAg)	
11 (Ag)	MetIle. . . . .
14 (eAg)	. . . . Thr. . . . .
23 (eAg)	
25 (eAg)	. . Tyr. . . . .
33 (eAb)	. . . . c . . . .

Figure 3.8(iv) Amino acid sequence from samples analysed from amino acid 18 to 73 of the core region

Sample number (HBeAg/anti-HBe status)	Amino acid sequence
pHBV130	^{18}PheLeuProSerAspPhePheProSerValArgAspLeuLeuAspThrAlaAlaAlaLeuTyrArgAspAlaLeuGlu
	(Ile) (Ser) (Glu)
1 (eAg)	. . . . . g a c . a . t . . . . . Glu. . .
17 (eAg)	. . . . . t . . . . .
21 (eAg)	. . . . .
22 (eAg)	. . . . .

	SerProGluHisCysSerProHisHisThrAlaLeuArgGlnAlaIleLeuCysTrpGlyAspLeuMetAsnLeuAlaThrTrpValGly^{73}
	(Thr)
. . . . . t . . . . . a a t . . . c a	.1
. . . . .	.17
. . . . . c . . .	.21
. . AspAsp.	.22

Figure 3.9(i) Sequences from the HBV X gene from patients with fulminant hepatitis and their sexual partners

Sample Number	Amino acid sequence
pHBV130	ThrAspLeuGluAlaTyrPheLysAspCysLeuPheLysAspTrpGluGluLeuGlyGluGluIleArgLeuLysValPheValLeuGlyGly a
	Val MetIle b
	accgaccttgaggcatacttcaaagactgtttgtttaaagactgggaggagttgggggaggagattagattaaaggtctttgtactaggaggc c
	c g g c g t a t d
382.1	.....g.....Thr.....Pro..g...Met.....
2500	.....MetIle.....
2533	.....MetIle.....
9748	.....g.....c.....MetIle.....
9795	NOT SEQUENCED
10013[1]	
10174	.....g.....
10184[1]	
10202[1]	
306	.....g.....Thr.....Pro..g...MetIle.....
418BD	..a.c...g....t.a.....
2946	.....MetIle.....
9880	.....g.....
10025	.....g...../Met/Ile.....t.....
10206	.....g.....Ile.....

Notes

1 See Figure 3.9(iii) for sequences of amplificates from single molecules
* Incomplete sequence
a Consensus amino acid sequence (pHBV 130)
b Recognised amino acid variants
c Consensus nucleotide sequence (pHBV 130)
d Recognised nucleotide variants

Figure 3.9(i)(continued)

```
          CysArgHisLysLeuValCysAlaProAlaProCysAsnPhePheThrSerAlaXXX a
                               Ser                         Pro      b
          tgtaggcataaattggtctgcgcaccagcaccatgcaacttttcacctctgcctaa c
                               tt                          c        d
382       ..........................................Ser.............
382.IY    NOT SEQUENCED
1434.1    NOT SEQUENCED
1434.1Y   NOT SEQUENCED
2500      ..................................................................
2533      ..................................................................
9748      ..................................................................
9795      NOT SEQUENCED
10013[1]
10174     ..................................................................
10184[1]
10202[1]

306BD     ..................................................................
418BD*    ..................................................................
2946      ..................................................................
9880      ..................................................................
10025     ............................SerSer..............................
10206     ..................................................................
```

Notes

1 See Figure 3.9(iii)for sequences of amplificates from single molecules
* Incomplete sequence
a Consensus amino acid sequence (pHBV 130)
b Recognised amino acid variants
c Consensus nucleotide sequence (pHBV 130)
d Recognised nucleotide variants

Figure 3.9(ii) **Sequencing of the HBV precore region from patients with fulminant hepatitis B and their sexual partners**

Sample	Amino Acid sequence	
	MetGlnLeuPheProLeuCysLeuIleIleSerCysSerCysProThrValGlnAlaSerLysLeuCysLeuGlyTrpLeuTrpGly	a
	His Thr	b
pHBV130	atgcacctttttccctctgcctaatcatctcatgttcatgtccaactgttcaagcctccaagctgtgccttgggtggctttggggc	c
	a t a c	d
382.1	.....XXXAsp	
382.1Y	PCR negative	
1434.1	PCR negative	
1434.1'	PCR negative	
2500	.....XXXAsp	
2533	.....XXXAsp	
9748	.....XXX...	
9795	NOT SEQUENCED	
10013	.....Phe.....A.....	
10174	.....	
10184[1]		
10202[1]		
306BD	.....XXXAsp	
418BD*	.....	
2946	.....XXXAsp	
9880	.....XXX...	
10025	.....Phe.....A.....	
10206	.....XXXAsp	

Notes

1 See Figure 3.9(iv)for sequences of amplificates from single molecules
* Incomplete sequence
a Consensus amino acid sequence (pHBV 130)
b Recognised amino acid variants
c Consensus nucleotide sequence (pHBV 130)
d Recognised nucleotide variants

Figure 3.9(iii) **Sequencing of the HBV X region (amino acid 106-amino acid 154) from individual molecules in patients with fulminant hepatitis and their sexual partners**

Sample Number	Amino acid sequence
pHBV130	ThrAspLeuGluAlaTyrPheLysAspCysLeuPheLysAspTrpGluGluLeuGlyGluGluIleArgLeuLysValPheValLeuGlyGly a
	Val MetIle b
	accgaccttgaggcatacttcaaagactgtttgtttaaagactgggaggagttgggggaggagattagattaaaggtctttgtactaggaggc c
	c g g c g t a t d
10013.1-5,7	.....c.....
10013.6	.....g.....g.....c.....tgt[2].....
10013.8	.....c.....c..a.....
10013.9	.....g.....
10202.1	.....c.....a.....a.....c.....t.....
10202.2,3,5-9	.....c.....a.....c.....t.....
10184.1-4	.....c.....a.....c.....t.....t.....

Notes

2 Nucleotide insert
a Consensus amino acid sequence (pHBV 130)
b Recognised amino acid variants
c Consensus nucleotide sequence (pHBV 130)
d Recognised nucleotide variants

Figure 3.9(iii)

```
            CysArgHisLysLeuValCysAlaProAlaProCysAsnPhePheThrSerAlaXXX a
                                 Ser                       Pro          b
            tgtaggcataaattggtctgcgcaccagcaccatgcaacttttcacctctgcctaa c
                                 tt                        c            d
10013.1,3-7,
9,10        ..................................................... .....
10013.2     ..............................................caactttt[2].....
10013.8     ..........c....a.....................................  .....
10013.9     ....................................a.....................  .....
10202.1-9   ..................................................... .....
10184.1-4   ..................................................... .....
```

Notes

2 Nucleotide insert
a Consensus amino acid sequence (pHBV 130)
b Recognised amino acid variants
c Consensus nucleotide sequence (pHBV 130)
d Recognised nucleotide variants

Figure 3.9(iv) **Sequencing of the HBV precore region from individual molecules in patients with fulminant hepatitis and their sexual partners**

```
Sample                          Amino Acid sequence
        MetGlnLeuPheProLeuCysLeuIleIleSerCysSerCysProThrValGlnAlaSerLysLeuCysLeuGlyTrpLeuTrpGly a
                     His                          Thr                                          b
pHBV130 atgcacctttttccctctgcctaatcatctcatgttcatgtccaactgttcaagcctccaagctgtgccttgggtggctttggggc c
                      a                       t   a       c                                      d

10013.1,3-5,
7,8,10   ..........................................................................................
10013.2  ............caacttttt[2].....................................................................
10013.6  ............c.............................................................................
10013.9  ..a.........................................................................................
10202.1  .....,.............................a.....................................................a..a.
10202.4,9.............................c.....................................................a..a.
10202.5  ............c.....................................................................a..a.
10202.2,3,
6-8      ..........................................................................................a..a.
10184.1-4.............................c..... .....................
```

Notes

2 9 base pair insert
a Consensus amino acid sequence (pHBV 130)
b Recognised amino acid variants
c Consensus nucleotide sequence (pHBV 130)
d Recognised nucleotide variants

Figure 3.9(v) **Sequencing of the HBV X region (amino acid 106- amino acid 154) in patients with acute hepatitis B and their sexual partners**

Sample Number	Amino acid sequence	
pHBV130	ThrAspLeuGluAlaTyrPheLysAspCysLeuPheLysAspTrpGluGluLeuGlyGluGluIleArgLeuLysValPheValLeuGlyGly	a
	Val MetIle	b
	accgaccttgaggcatacttcaaagactgtttgtttaaagactgggaggagttgggggaggagattagattaaaggtctttgtactaggaggc	c
	c g g c g t a t	d
10324	NOT SEQUENCED	
10183	NOT SEQUENCED	
11869	.....	
12171	.....Lys.....	
5627	.....Leu.....MetIle.....	
3018	.....Leu.....MetIle.....	
10400	.....MetIle.....	
10500	.....	
12088	NOT SEQUENCED	
12380	.....Leu.....MetIle.....	
7640	.....Leu.....MetIle.....	
7591	.....Leu.....MetIle.....	

Notes

a Consensus amino acid sequence (pHBV 130)
b Recognised amino acid variants
c Consensus nucleotide sequence (pHBV 130)
d Recognised nucleotide variants

Figure 3.9(v) (continued)

```
                CysArgHisLysLeuValCysAlaProAlaProCysAsnPhePheThrSerAlaXXX a
                                  Ser                     Pro          b
                tgtaggcataaattggtctgcgcaccagcaccatgcaacttttcacctctgcctag c
                                 tt                      c             d
10324           NOT SEQUENCED
10183           NOT SEQUENCED
11869           ..........................................................
12171           ..........................................................
5627            ..........................................................
3018            ..........................................................
10400           ..........................................................
10500           ..........................................................
12380           ..........................................................
7640            ..........................................................
7591            ..........................................................
```

Notes

a Consensus amino acid sequence (pHBV 130)
b Recognised amino acid variants
c Consensus nucleotide sequence (pHBV 130)
d Recognised nucleotide variants

Figure 3.9(vi) Sequencing of the HBV precore region in patients with acute hepatitis B and their seual partners

Sample	Amino Acid sequence	
	MetGlnLeuPheProLeuCysLeuIleIleSerCysSerCysProThrValGlnAlaSerLysLeuCysLeuGlyTrpLeuTrpGly	a
	His Thr	b
pHBV130	atgcacctttttcccctctgcctaatcatctcttgttcatgtcctactgttcaagcctccaagctgtgccttgggtggctttggggc	c
	a a a c	d
10324	NOT SEQUENCED	
10183	NOT SEQUENCED	
11869	.....XXX...	
12171	.....XXXAsp	
5627	.....	
3018	.....XXX/Asp	
10400	.....XXX...	
10500	.....XXX/Asp	
12088	NOT SEQUENCED	
12380	.....	
7640	.....XXX...	
7591	.....XXX...	

Notes

a Consensus amino acid sequence (pHBV 130)
b Recognised amino acid variants
c Consensus nucleotide sequence (pHBV 130)
d Recognised nucleotide variants

Figure 3.10(i) Amino acid sequences of samples analysed from precore amino acids 1 to 29

Sample	Amino acid sequence
pHBV 130[1]	MetGlnLeuPheHisLeuCysLeuIleIleSerCysSerCysProThrValGlnAlaSerLysLeuCysLeuGlyTrpLeuTrpGly
2a	
2b	. . . . . X Asp
2c	
3	. . . . .
4	. . . . . [X][2]
HCW	. . . . . X .

Notes

1 The consensus sequence shown in Figures 3.10(i) and 3.10(ii) is subtype *adyw* [Pugh *et al.*,1986]

2 A second band representing a minor population with a mutation at this position was detected on the sequencing film. The remainder of the sequence was homogeneous.

X Stop codon

. Sequence identical to the consensus sequence

( )Published amino acid variation between subtypes

Figure 3.10(ii) Amino acid sequences in the X region from position 122 to 154

Sample number[1]		Amino acid sequence
pHBV130	122	LeuGlyGluGluIleArgLeuMetIlePheValLeuGlyGlyCysArgHisLysLeuValCysAla
		(LysVal) (Ser)
2a		
2b		. . . . . g[2] . .
2c		
4		. . . . Met. . . . .

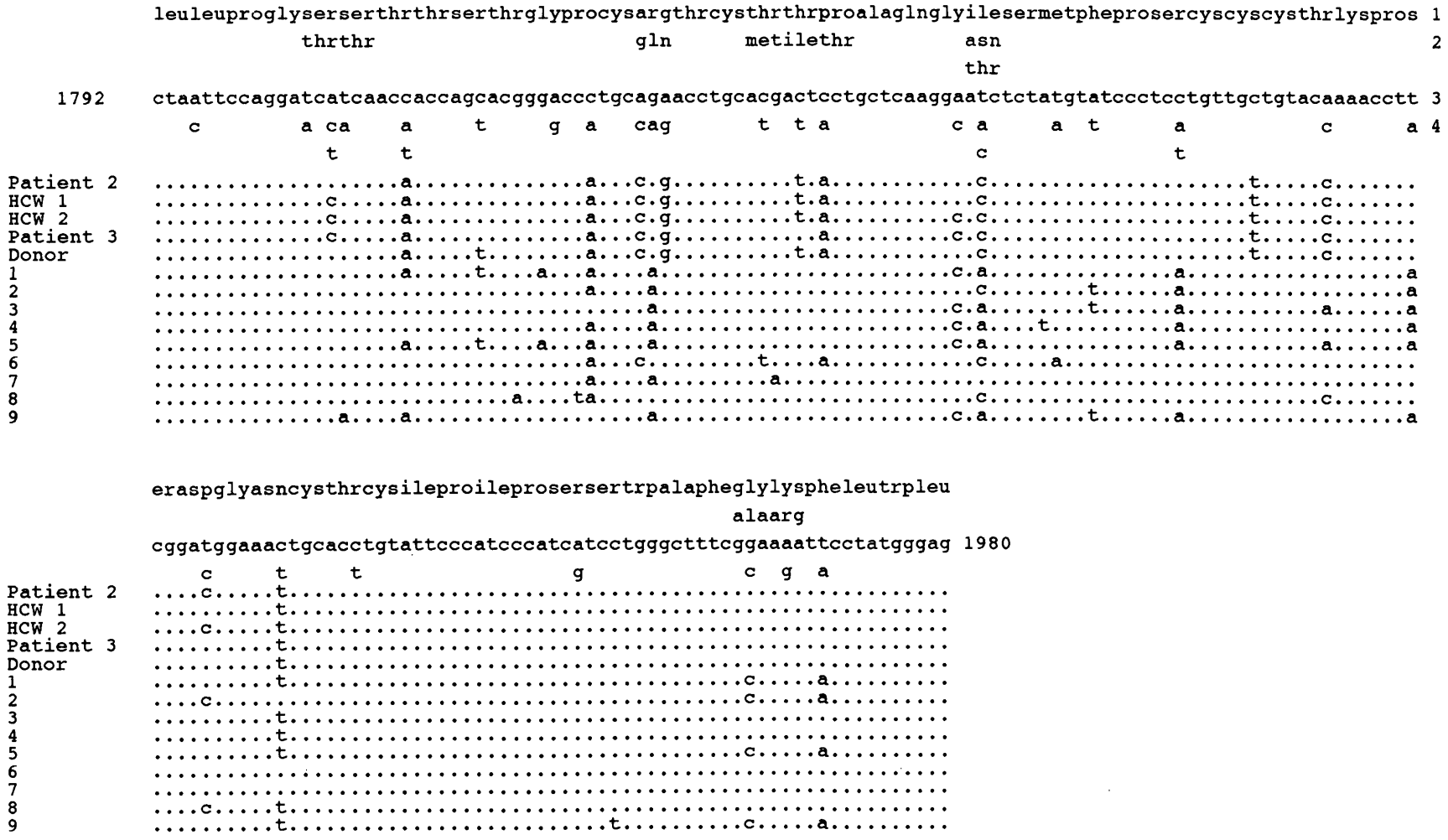
pHBV130	144	ProAlaProCysAsnPhePheHisSerAlaXXX
		(Pro)
2a		
2b		. . . . .
2c		
4		. . . . .

Notes

1 Sample from Case 3 not shown, but identical to consensus sequence
In all cases the infant and maternal samples were identical

2 Silent base change

Figure 3.12(iii) Nucleotide sequence analysis of the HBV DNA surface gene[a] determinant of samples included in the blinded study



Notes 1 Consensus amino acid sequence. 2 Recognised amino acid variants. 3 Consensus nucleotide sequence : Subtype adyw. 4 Recognised nucleotide variants

Figure 3.12(iv) Nucleotide sequence analysis of of the HBV surface gene ^{a} determinant from subtype *ayw* control samples

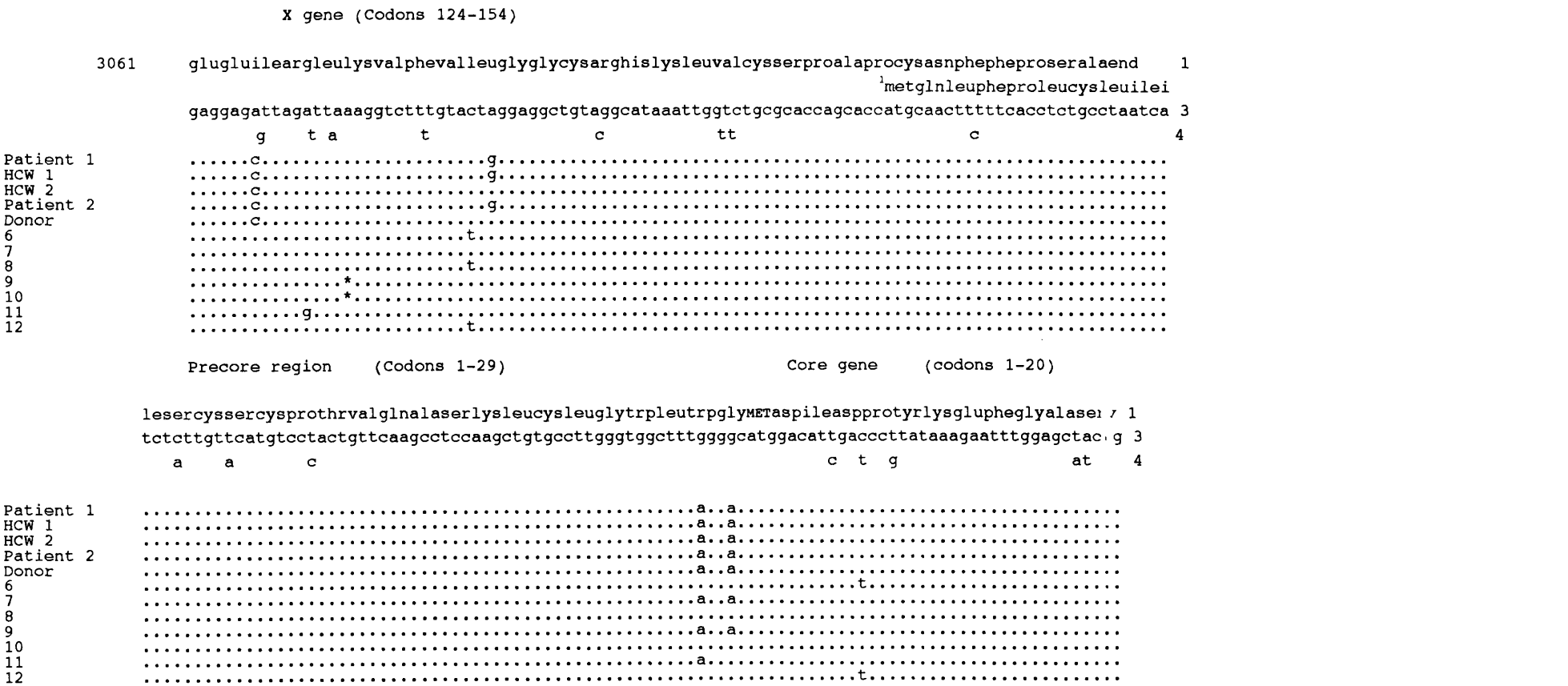
```
                leuleuproglyserserthrthrserthrglyprocysargthrcysthrthrproalaglnglyilesermetpheprosercyscyscysthrlyspros 1
                          thrthr                    gln      metilethr        asn                                    2
                                                    lys                       thr
        1972    ctaattccaggatcatcaaccaccagcacgggaccctgcagaacctgcacgactcctgctcaaggaatctctatgtatccctcctgttgctgtacaaaaccttt 3
                  c       a ca    a     t     g  a  cag      t  t a         c a     a  t     a          c      a 4
                            t     t                                        c             t
Patient 1       ..................a.............a...c.g.........t.a...........c.....................t.....c.......
HCW 1           .............c.....a.............a...c.g.........t.a...........c.....................t.....c.......
HCW 2           .............c.....a.............a...c.g.........t.a.........c.c.....................t.....c.......
Patient 3       .............c.....a.............a...c.g...........a.........c.c.....................t.....c.......
Donor           ..................a.....t........a...c.g.........t.a...........c.....................t.....c.......
6               .............t...................a.................................c.....................c.......
7               .............t...................a.................................c.....................
8               .............t...................a.................................c.....................c.......
9               .............t...................a.................................c.....................c.......
10              .............t............g.......a.................a............c.....................c.......
11              .............ta.........t............c.............................c........t......a..................
12              .............t.............a......a.................................c.....................c.......

                eraspglyasncysthrcysileproileprosersertrpalapheglylyspheleutrpleu
                                                                        alaarg
                cggatggaaactgcacctgtattcccatcccatcatcctgggctttcggaaaattcctatgggag 1960
                    c     t     t                 g             c  g  a

Patient 1       ....c.....t.....................................................
HCW 1           .........t.....................................................
HCW 2           ....c.....t.....................................................
Patient 3       .........t.....................................................
Donor           .........t.....................................................
6               ....c.....t.........................t...........................
7               ....c.....t.........................t...........................
8               ....c.....t.........................t...........................
9               ....c.....t.....................................................
10              ....c.....t.....a.............................a.................
11              ....c.....t.....t...................a...........................
12              .........t.........................t...........................
```

Notes 1 Consensus amino acid sequence. 2 Recognised amino acid variants. 3 Consensus nucleotide sequence : Subtype adyw. 4 Recognised nucleotide variants

Figure 3.12(v) Sequence analysis of amplified HBV DNA:3' region of X,the entire precore region and the 5'core regions.



Notes 1 Consensus amino acid sequence. 3 Consensus nucleotide sequence : Subtype adyw. 4 Recognised nucleotide variants

Samples 6-12 are control samples of the same subtype *(ayw)*