

HBV pre-C/C variation; geographical and functional aspects

by

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**A thesis presented for the degree
of Doctor of Philosophy**

in

**The Faculty of Science
at the
University of Glasgow**

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March 1997

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Acknowledgements

Firstly, I would like to thank Professors J.H. Subak-Sharpe, B. Clements and D. McGeoch for giving me the opportunity to work in the Institute of Virology.

I am particularly grateful to my supervisor Dr Bill Carman for his (unbelievable at times) enthusiasm and help throughout the project and in the writing of this thesis. Whether I shall ever forgive him for introducing me to sequencing is another matter..!

I am indebted to the members of lab 109, both past and present, for their continual help and technical assistance throughout the project, and for making the lab such an enjoyable place to work. In particular, I wish to thank my good friend Dr. Lesley Wallace for always being around when I needed help and for her valuable comments on this thesis.

I would like to thank Dr. Howard Marsden and the members of his laboratory for making my 'stay' most enjoyable and, of course, for all their help and expertise with the synthesis of my peptides. In particular I would like to thank Gillian McVey for introducing me to the 'tea break'!

I would also like to thank the staff of the washroom, media and store who are fundamental to the smooth running of the department.

I greatly appreciate the contribution of the following collaborators: Dr H.J. Schlicht (University of Ulm, Germany) for undertaking the cloning, expression and analysis of U₁₈₅₆ in vaccinia virus (Chapter 5). Dr. E.C. Holmes (University of Oxford, U.K.) who carried out the phylogenetic analysis (Chapter 5). Dr. Mark Thursz (St Mary's, Paddington, London) for carrying out part of the statistical analyses (Chapter 6). Professor Hadziyannis (Hippokration Hospital, Athens, Greece) for provision of

patient samples (Chapter 6) and analysis of the my peptides in T and B cell studies (Chapter 7) and for continuing with this work.

I would like to thank my mum and dad for their continual support - not just financially - throughout my time at University. I am extremely grateful both to them and David's parents, Peter and Eileen Walsh, for their help with babysitting, without which I do not think I would ever have finished writing this thesis.

Finally, thanks to my husband, David, for his encouragement, endless help and for still being my friend despite having to read this thesis, not forgetting his financial support (are we quits now?) and to my son, Michael, who just makes life that extra bit special.

The author was the recipient of a Glaxo scholarship. All work presented, unless otherwise stated, is by the author,s own efforts.

Winifred Boner

March 1997

Acknowledgement to Professor Hadziyannis

In Chapter 7, where *Athens* follows a study number, this indicates that the results presented in these studies were obtained from experiments carried out in Athens and these experiments were undertaken by Professor Hadziyannis's laboratory.

Summary

Infection with hepatitis B virus (HBV) can lead to a spectrum of disease manifestations, from asymptomatic acute hepatitis to fulminant hepatitis. Why such a spectrum should occur is not clear but the immune response to HBV has often been implicated. Normally, after seroconversion to anti-HBe, HBV DNA levels fall and disease activity is minimal. However, a group of individuals from the Far East and the Mediterranean were identified as having continued exacerbations of disease activity even after seroconversion to anti-HBe.

This thesis examines variation in HBcAg from sequential serum samples of these individuals, and analyses the distribution of amino acid substitutions in relation to B- and Thelper cell epitopes.

In the first study, the distribution of HBcAg amino acid substitutions was compared in Chinese patients with different pre-core (pre-C) variants. One variant has a serine at amino acid (aa) 15 of pre-C, and the other is the common A₁₈₉₆ which introduces a stop codon at aa 28. The serine15 strain is shown here to produce antigenically normal amounts of HBeAg. Both variants have been shown to be mutually exclusive, probably due to sequence requirements for encapsidation, and are separate lineages. Sequential sequencing of HBcAg showed that in those individuals with A₁₈₉₆ there are significantly more aa substitutions, and that these are associated with Th and B-cell epitopes.

In the second study, sequential samples were examined from individuals from the Mediterranean who either seroconverted to anti-HBe or who were continually anti-HBe, the results showed that selection of A₁₈₉₆ is temporally associated with aa substitutions in HBcAg. In seroconverters went into remission, aa substitutions occurred in the Th epitope from aa 50 to 69 ($p=0.00045$), but in anti-HBe positive patients with ongoing

disease these occurred in B-cell epitopes ($p=0.0007$ for aa 74 to 83). Analysis of the timing of these substitutions showed that they are not associated with multiple flares of hepatic activity.

Comparison of the results of the two studies shows that the distribution of HBcAg substitutions differs in the Chinese and the Mediterranean groups.

Other mechanisms —
An overlapping peptide series, covering the entire HBcAg, was constructed to test the hypothesis that the observed variants which arose during infection represented immune escape; either humoral, Thelper cell escape or both. Several patients failed to react to their homologous peptide in lymphoproliferation assays suggesting that the lack of response is not due to variation in Thelper cell epitopes. The results from the lymphoproliferation studies defined the Thelper epitope - previously described at aa 1 to 20 - as lying between aa 14 and 23. Antibody binding and T-cell studies indicated that branched chain peptides work in both systems, and that they are more reactive in ELISA when compared to monomeric peptides. Several of the patients studied in the B-cell assay reacted to peptides spanning aa 1 to 43, possibly indicating a putative B-cell epitope in this region.

Abbreviations

A	adenine
aa	amino acid
ALT	alanine transaminase
anti-HBc	antibodies to HBcAg
anti-HBe	antibodies to HBeAg
anti-HBs	antibodies to HBsAg
anti-X	antibodies to HBxAg
APS	Ammonium persulfate
AST	aspartate aminotransferase
bp	base pairs
BCP	basal core promoter
BSA	Bovine serum albumin
C	cytosine
C-	carboxyl-terminus
Ch	Hong Kong Chinese
cm	centimetre
cpm	counts per minute
CTL	cytotoxic T lymphocytes
DHBV	duck hepatitis B virus
DMF	dimethylformamide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ϵ	epsilon, the encapsidation signal
<i>E.coli</i>	<i>Escherichia coli</i>
ELISA	enzyme-linked immunosorbant assay
enh I	enhancer I
enh II	enhancer II
ER	endoplasmic reticulum

FCS	Foetal calf serum
G	guanine
HEPES	N-2 hydroethylpiperazine-N'-2-ethane sulphonic acid
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBV	Hepatitis B virus
HBxAg	Hepatitis B x antigen
HCC	Hepatocellular carcinoma
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HOBt	1-hydroxybenotriazole
H ₂ O	molecular biology grade water
HPLC	high pressure liquid chromatography
IFN- γ	gamma interferon
Ig	immunoglobulin
IL-2	interleukin-2
IPTG	Isopropylthiogalactoside
Kb	kilobase
kD`	kilo dalton
LB	Luria-Bertani medium
M	moles
mA	milliamps
Mab	monoclonal antibody
MAP	multiple antigenic peptide
mg	milligrams
MHC	major histocompatibility complex
min	minutes
ml	millilitres
mm	millimetres
mM	millimolar
mmol	millimoles
MOI	multiplicity of infection

mRNA	messenger ribonucleic acid
N-	amino-terminus
ng	nanograms
NH ₃	ammonia
NK	natural killer cells
nm	nanometers
OD	optical density
ORF	open reading frame
P	polymerase
PBMCs	peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
%	percentage
pre-C	pre-core
PrA	proteinase A
PyBOP	benzotriazolyl-oxy-tris[pyrrolidino]-phosphonium-hexafluorophosphate
RIA	radioimmunoassay
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
SI	stimulation index
T	thymine
TAA	t-amyl alcohol
TCR	T-cell receptor
TEMED	N,N,N',N'-tetramethylethylenediamine
TFA	trifluoroacetic acid
Th	T helper lymphocyte
TIPS	triisopropylsilane
TNF	tumour necrosis factor
tRNA	transfer RNA

U	units
U	uracil
μl	microlitres
UV	ultraviolet
v/v	volume for volume
WHV	woodchuck hepatitis virus
w/v	weight for volume
XRE	X responsive elements

Chapter 1

Introduction

1. **Epidemiology and geographic distribution of hepatitis B virus**

Human hepatitis B virus (HBV) infection constitutes a major global health problem, with an estimated 300 million infective carriers world-wide (Takano *et al*, 1995). Geographic patterns of HBV prevalence are known to vary greatly world-wide with the incidence of HBV infection ranging from <10% in areas of low endemicity to 30-80% in areas of intermediate and high endemicity; of the latter, prevalence is greatest in South East Asia and sub-Saharan Africa. Occurrence of chronic HBV carriers also follows this pattern with <1% and between 2-15% of the adult population of these areas being chronically infected.

In the areas of low endemicity, infection occurs principally in adults and is transmitted predominantly via sexual contact and intravenous-drug abuse, whereas in areas of intermediate and high endemicity transmission occurs primarily via either the maternal-infant route or through close contact in early life. Host factors appear to play a role in the route of transmission. In sub-Saharan Africa, transmission is mostly in childhood (Skinhoj, 1979), whereas in South East Asia transmission is primarily from mother to baby (Stevens *et al*, 1979). This is probably related to the high rate of HBeAg positivity in chronic carriers in this latter group. It is unclear why this group fail to clear HBeAg. As HBeAg positivity correlates with infectivity, this predisposes these individuals to transmitting to their children. In contrast to an adult-acquired infection, where <5% will develop chronic hepatitis, if a child is infected perinatally then the risk of the infection leading to

chronicity is 95%. This incongruity is probably partially due to induction of tolerance to HBeAg *in utero* (discussed in Chapter 1, section 6.4). Although chronicity is a minority outcome in the adult, persistent infection occupies a central role in the biology of HBV as most of the mortality is a consequence of this outcome.

It appears that the only source or reservoir of HBV for human infections is humanity itself. No animal reservoir is known. HBV has been experimentally transmitted to chimpanzees (Maynard *et al*, 1971) and with less effect to African green monkeys, gibbons and rhesus monkeys. However, there is no evidence that non-human primates are important sources of human infections.

2. Outcome of infection

2.1 Clinical features and course of infection

The incubation period of acute hepatitis can range from 4 to 28 weeks: in most cases the interval is between 8 to 16 weeks. Symptoms include: headache, malaise, anorexia, nausea, jaundice, and moderate fever (temperatures of 37.5-39°C) and usually last for 2-10 days. Increased levels of the liver enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), are good indicators of infection.

During acute infection, hepatitis B surface antigen (HBsAg) levels eventually fall and antibodies to HBsAg (anti-HBs) are produced. On complete recovery these antibodies maintain immunity in the individual to further infection by HBV. Acute HBV infection is normally self-limiting but in rare cases (~1% of the total number of cases), the disease can become aggressive causing severe damage to the liver; this is known as fulminant hepatitis and is often fatal. The mortality in such cases is very high, with one in two cases dying within 10 days of the onset of symptoms.

If HBsAg is still detectable in the serum after 6 months, the patient is then classified as a chronic hepatitis B carrier. Most cases of chronic hepatitis B virus infection have a preceding history of symptomatic acute hepatitis B.

In HBV carriers a state of low-level viraemia usually develops after elimination of hepatitis B e antigen (HBeAg) from the blood, while HBsAg persists to circulate. This is thought of as the 'healthy' carrier state (or chronic persistent hepatitis). However, in some individuals symptoms persist - known as chronic active hepatitis.

Despite hepatitis B core antigen (HBcAg) being an internal component of HBV, antibodies to HBcAg (anti-HBc) are produced by virtually 100% of HBV infected patients very early in infection, and therefore their appearance is of no prognostic value.

In contrast, antibodies to HBeAg (anti-HBe) may not develop or appear at various times after anti-HBc and are frequently correlated with viral clearance. ALT levels are often raised at the time of seroconversion to anti-HBe, indicating liver cell damage with no apparent cell damage thereafter.

If an individual fails to seroconvert to anti-HBe, virus titres remain high and extensive liver damage usually results. Thus, seroconversion to anti-HBe is a crucial step in virus elimination.

2.2 Clinical features and course of infection of anti-HBe-positive Mediterranean and Chinese cases

An interesting group of patients with HBV DNA present in their serum, measurable by dot-blot hybridisation, yet who were anti-HBe positive, was discovered in southern Europe and in the Far East (Hadziyannis *et al*,

1983; Chu *et al*, 1985). This group of patients were unusual in that disease progressed - sometimes rapidly - despite the presence of anti-HBe. Interest was then focused onto the pre-core (pre-C) gene, as this is the region involved in synthesis of HBeAg, leading to the discovery of a variant of HBV that was unable to produce HBeAg (Carman *et al*, 1989, Brunetto *et al*, 1989a) - the HBeAg-minus strain. Findings by Brunetto *et al* (1991), suggested that the HBeAg producing strain of HBV caused a milder form of hepatitis than that seen in those with HBeAg-minus strain.

The major routes of transmission in Southern Europe and the Far East are different. Transmission in the Far East is primarily vertical (mother-to-baby, at or around the time of birth). This is due to the prolonged duration of HBeAg in these patients. Whereas HBV in Southern Europe is acquired via horizontal transmission early in childhood. Why females in the Far East remain HBeAg positive into their child-bearing years is not clear but this does account for the different transmission routes between these geographical areas.

2.3 Hepatocellular carcinoma (HCC)

HCC has a world-wide distribution and, numerically, is one of the major cancers in the world. The association between HCC and HBV infection is strong (Beasley, 1982). The percentage of chronic carriers is higher among patients with HCC than among the rest of the population (Szmunes, 1978). There is at least a 100-fold increased risk of developing HCC if you are a chronic carrier (Beasley *et al*, 1991).

The high incidence of HCC in woodchucks (*Marmota monax*) residing in the Philadelphia Zoo, led to the identification of the woodchuck hepatitis virus (WHV) (Summers *et al*, 1978). As HCC has yet to be described in the chimpanzee, the woodchuck is currently used to study the molecular mechanisms of HCC development. Animals chronically infected with

hepadnaviruses, e.g. woodchucks, develop hepatomas (Chen *et al*, 1986). Integrated viral DNA has been found in tumours (Zhou *et al*, 1987) although the mechanism of hepatocarcinogenesis is far from clear.

3. Taxonomy and classification of HBV

A genetic classification of HBV strains was originally performed by Okamoto *et al* (1988) on 18 HBV whole genome clones. This identified four genotypes, designated A to D, based on nucleotide divergences of 8% or more between the strains. Norder *et al* (1992) extended this study to 32 HBV strains, this time only examining the surface (S) gene sequence. The results obtained confirmed the original classification, showing that classifying HBV genomes on the basis of the S gene alone is feasible. Their results also showed a higher divergence between genotypes and serotypes than had been previously reported, resulting in the designation of two new genotypes E and F.

3.1 Subtypes

HBsAg variation leads to stable subtypes, defined by monospecific polyclonal antibodies. Antigen reactivities that are found on all recognised HBsAg isolates are known as determinant *a*. Le Bouvier *et al* (1972), and Bancroft *et al* (1972), described the mutually exclusive determinants *d* or *y* and *w* or *r* respectively, enabling the distinction of four major HBsAg subtypes, *adw*, *adr*, *ayw* and *ayr*. Determinant *d* has a lysine at amino acid 122, *y* an arginine (Peterson *et al*, 1984). Determinant *w* has a lysine at amino acid 160 and *r* an arginine (Okamoto *et al*, 1988).

Additional serological specificities have allowed the identification of four serotypes of *ayw* and two of *adw*. Thus, there are eight defined subtypes, *ayw1*, *ayw2*, *ayw3*, *ayw4*, *ayr*, *adw2*, *adw4* and *adr*. The *q* determinant was originally found on all HBsAg subtypes except *adw4*. Subsequently,

lack of *q* was also demonstrated in some *adr* subtypes. *adr* strains can therefore be defined as either *adrq*⁺ or *adrq*⁻. Strains specifying *adw* are found in groups A, B, C and F, and those specifying *ayw* in groups A, B, D and E (Norder *et al*, 1992). Strains specifying *r* have so far only been found in group C.

The world-wide molecular epidemiology of HBV, based on S-gene sequences, has shown that E and F genotypes originated in aboriginal populations of West Africa and the New World respectively. Genotype A is found in North-western Europe and sub Saharan Africa, genotypes B and C are confined to original populations of the Far East, while genotype D is widespread; however it is the predominant genotype found in the Mediterranean and the Near East (Magnius and Norder, 1995).

4. Structure and molecular biology of HBV life cycle

4.1 Structure

Electron microscopy of partially purified preparations of HBV from human serum revealed three types of particles:

1. 43nm double-shelled particles - termed 'Dane particles' - are now known to be the intact virion.
2. 20nm spheres, usually present in 10³-10⁶ excess over virions
3. 20nm diameter filaments, varying in length. (Robinson and Lutwick, 1976; Dane *et al*, 1970)

All three display HBsAg on their surface. Dane particles contain the L, M and S proteins in approximately equimolar amounts, whereas the 20nm particles are mainly composed of the S protein, a variable amount of M protein and, in limited quantity, host-derived lipids. In addition, the filaments also contain some L protein. The L and M protein are thought to mediate binding and entry of the virus into hepatocytes (discussed below

in Chapter 1, section 4.4). Only Dane particles contain nucleic acid; therefore, only this type of particle is infectious. The filaments and spheres are produced in vast quantities during infection. The reason for this is still unclear: one possibility is that these 'dummy' particles serve to adsorb neutralising anti-HBs.

HBsAg surrounds the nucleocapsid, which consists of a 21Kd phosphoprotein, HBcAg. Virion cores are known to contain the viral DNA polymerase, which is covalently bound to the HBV DNA, and a protein kinase. Figure 1.1 shows a schematic model of HBV.

4.2 Genomic organisation

HBV belongs to the Hepadnaviridae family. The genome was first isolated in 1974 (Robinson and Lutwick, 1976) and at 3.2 kilobases (Kb), it is the smallest DNA virus known to infect man .

The circular genome displays two remarkable asymmetries that sets it apart from all other viral genomes. The first is a length asymmetry in the two strands: one DNA strand is unit length, while its complement is variably less than this. The full length strand is complementary to the viral messenger RNAs (mRNAs) and is thus designated to be of minus polarity. Hence, the shorter complementary strand is termed the plus strand. The position of the 5' end of the plus strand is fixed but the 3' end is variable. The genome is therefore only partially double stranded (Delius *et al*, 1983), possessing a single-stranded gap region from 20-80% of unit length. Circularity is maintained by a 5' cohesive terminus of 224 base pairs (bp) (Sattler and Robinson, 1979).

The second asymmetry occurs at the 5' termini: minus strand DNA contains protein in covalent linkage at this position (Gerlich and Robinson, 1980), whereas the plus strand contains an unattached 5'

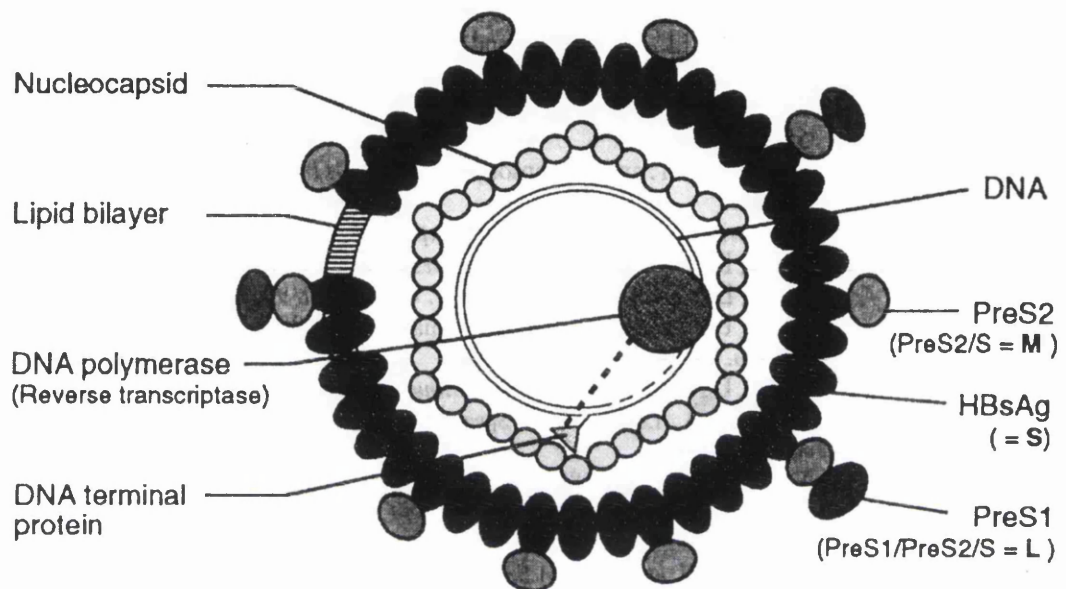


Figure 1.1 Schematic cross-sectional representation of the structural organisation of the hepatitis B virion. The infectious HBV virion consists of an outer envelope of host-cell-derived membrane, in which the products of the pre-S2/S ORF are embedded. The surface proteins have a common S domain, to which the pre-S2, or pre-S1 and pre-S2, domains are optionally added, forming the middle (M) and large (L) proteins respectively. The inner capsid is formed from HBcAg and encloses the partially double-stranded, circular DNA genome and the P protein, which is covalently linked to the (-)-strand DNA.

oligoribonucleotide. These asymmetries result from the novel replicative mechanism of the genome (discussed in section 4.8). Despite its compact size, HBV DNA encodes all the proteins it requires, with all the regulatory elements residing within the proteins.

4.3 Genes and their protein products

HBV encodes four major open reading frames (ORF): the surface, the polymerase, the pre-C/C and the X (Figure 1.2). Two further putative ORFs have been described and are termed ORF 5 and ORF 6. *In vitro* translational experiments have looked for the presence of mRNAs for both these ORFs without success Chang *et al* (1994).

4.3.1 Surface gene

The surface gene contains three translational start codons (AUG) and one common stop codon (UAA), giving rise to three ORFs: pre-S1, pre-S2 and S. These ORFs are translated as the following products: the large protein (pre-S1, pre-S2 and S) of 39 kilodaltons (kD) (unglycosylated) and 42kD (glycosylated), the middle protein (pre-S2 and S) of 33 and 36kD and the small protein (S alone equivalent to HBsAg) of 24 and 27kD.

HBsAg is the most abundant polypeptide in all three HBV-associated particles, whereas middle protein is a minor component in all three. The large protein is more prevalent than the middle protein in virions and filaments, but less prevalent in HBs spheres. It appears that the proportion of large protein determines the morphology of the HBs particles (Marquardt *et al*, 1987), while the ratio of middle protein to HBsAg does not significantly alter morphology.

Functional roles of the pre-S proteins in the viral life cycle have yet to be clearly defined. Pre-S1 probably plays an important role in the entry of the virus into hepatocytes (Pontisso *et al*, 1989).

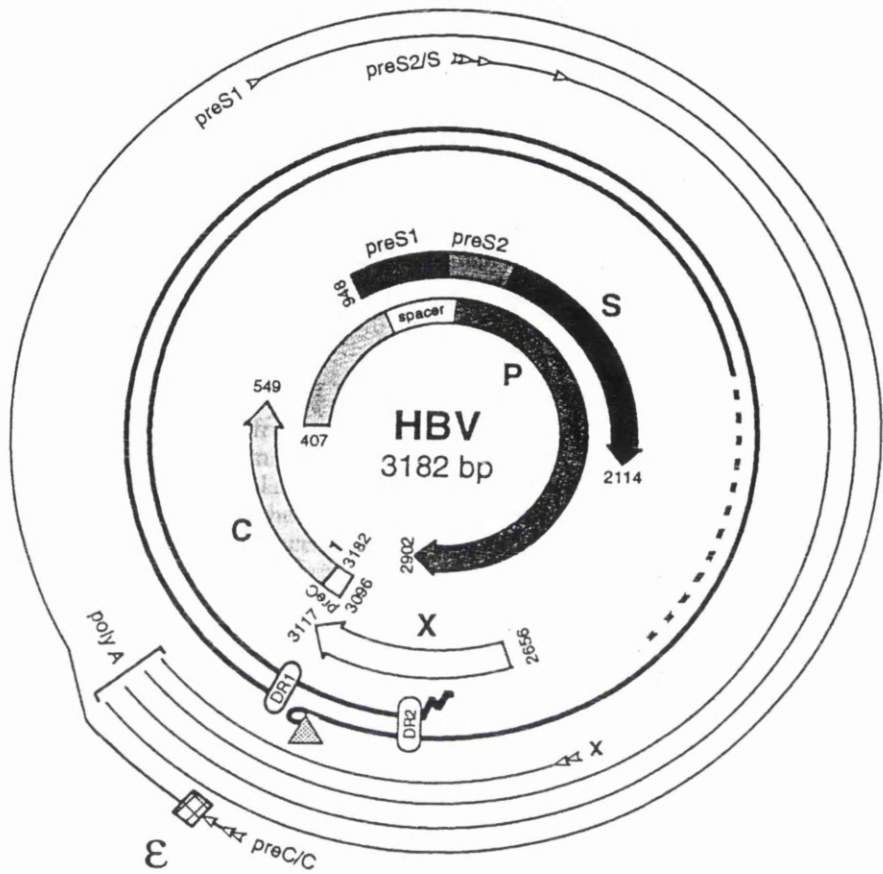


Figure 1.2 Genomic organisation of hepatitis B virus. The outer thin lines represent the different classes of transcripts, the bold line represents the DNA genome. The four major ORFs are indicated in the centre.

4.3.2 Core gene

Like the S gene, the C gene is preceded by an in-frame ORF termed the pre-C region. Translation from the pre-C AUG produces a protein (pre-C protein) with a molecular mass of 24kD, and which has the entire HBcAg sequence plus a 29 amino acid residue amino-terminal (N-) extension. The first 19 amino acids of the pre-C protein form a signal peptide sequence which directs the pre-C protein to the endoplasmic reticulum (ER), where this region is cleaved; resulting in a 22kD protein that is translocated to the lumen of the ER (Garcia *et al*, 1988).

The carboxy-terminus (C-) has a strongly basic domain that in most HBV isolates contains 16 arginine residues, in clusters, in a stretch of 34 amino acids. This domain undergoes further proteolysis, in the golgi, at amino acid 149 in the carboxyl terminus of HBcAg (Wang *et al*, 1991), resulting in the production of HBeAg (Ou *et al*, 1988). This cleavage event probably involves a cellular, aspartyl-like protease (Jean-Jean *et al*, 1989, Nassal *et al*, 1989). HBeAg is then secreted in a monomeric form into the blood (Wasenauer *et al*, 1992).

The mechanism which leads to the production and secretion of HBeAg is identical for two other hepadnaviruses; duck hepatitis virus (Schlicht *et al*, 1987) and woodchuck hepatitis virus (Carrier *et al*, 1994).

The role of the arginine-rich domain in the generation of HBeAg is still unclear. HBe-like proteins, from which the arginine-rich domain has been removed but not the signal peptide, have been found in the sera of HBV carriers (Takahashi *et al*, 1992); this indicates that the signal peptide is not the only requirement for secretion of HBeAg. Work by Bruss and Gerlich (1988) based on *in vitro* translation experiments, suggests that the arginine-rich domain could act as a translocation stop signal. This domain, therefore, would have to be removed in the ER before HBeAg could be secreted into serum. However, this has not been supported by later work

as it has been shown that removal of the arginine-rich domain takes place in the golgi and not the ER (Wang *et al*, 1991, Carlier *et al*, 1995).

Experiments have shown that the C-terminus is crucial for HBeAg production, as cells infected with a recombinant virus truncated by 34 amino acids at the C-terminus secrete about 20 times less HBeAg than those infected with a virus containing the entire pre-C region (Schlicht and Wasenauer, 1991). A recent paper, by Carlier *et al* (1995) confirmed these findings and showed that a C-terminus deletion of 60 amino acids completely abrogated HBeAg secretion. They indicated that the low secretion efficiencies of C-terminally truncated pre-C proteins are due to a slowing down in the intracellular transport of the HBeAg precursor. A similar situation has been reported for the precursor of the *Saccharomyces cerevisiae* proteinase A (PrA), a vacuolar hydrolase, which contains a prodomain proteolytically removed when PrA is about to reach its final destination. When the prodomain of PrA is deleted, a two- to four-fold delay in delivery to the vacuole is observed (Klionsky *et al*, 1988). Thus, the arginine-rich domain could also act as a prodomain and may contribute to the overall conformation of the pre-C protein which may be required for its efficient intracellular transit. When the pre-C sequence is replaced by the signal sequence of influenza virus haemagglutinin, the arginine-rich domain is not required for efficient secretion. However, in this case the HBe-like protein has a dimeric structure (Schlicht and Wasenauer, 1991). Such a finding is consistent with the understanding that the monomeric structure of HBeAg is determined by an intermolecular disulphide bond between a cysteine (Cys) in the pre-C region Cys⁷ (numbering from the methionine of HBcAg) and Cys⁶¹ (Wasenauer *et al*, 1993, Nassal and Rieger, 1993). An alternative hypothesis is that some sequences within the C-terminus interact with a cellular protein, acting as a chaperone, in the secretion process. Deletions in this area would therefore result in abrogation of these interactions, explaining the observed slow intracellular transport of the truncated forms of the pre-C

proteins. Such sequences could be in the region valine (amino acid 124) to arginine (amino acid 133) which have been reported to be at the surface of the pre-C protein (Sallberg *et al*, 1993).

Although the primary amino acid sequence of this protein is nearly identical to the HBcAg sequence, HBeAg does not form core particles and, unlike HBcAg, is secreted from the infected cells. The prevention of particle formation can be attributed to a cysteine residue at amino acid 23 of pre-C.

The true function of HBeAg has yet to be elucidated; one theory proposes that HBeAg acts to induce T-cell tolerance to nucleocapsid antigens coexpressed with the major histocompatibility complex at the hepatocyte surface (discussed below in Chapter 1, section 5.4). As HBeAg is able to cross the placenta, such a mechanism could explain the high incidence of chronic disease in the newborn (Thomas *et al*, 1988). Low molecular weight proteins can induce tolerance. This is particularly true in cases where the immune system is exposed to such a protein at an early stage of development. This mechanism for tolerisation has been demonstrated in a transgenic mouse model for HBeAg (Milich *et al*, 1990), and may explain why infants infected at birth continue to exhibit viremia and HBe antigenemia for many years. Thus, tolerisation to HBeAg in the newborn would favour the passage of the virus from generation to generation. A second hypothesis as to why >90% of children infected at birth become chronic carriers is that the humoral response to the core proteins - particularly HBeAg - is capable of inhibiting the cytotoxic T-cell response and thus leads to destruction of infected hepatocytes leading to a protracted course of infection (Mondelli *et al*, 1982, Pignatelli *et al*, 1987). Thus, the passive transfer of antibody from the mother would inhibit the induction of specific cytotoxic T cells by the child. Guidotti *et al* (1996) suggest a role for the pre-C protein in the virus life cycle. Their experiments showed that over expression of pre-C protein eliminated

nucleocapsid particles from the cytoplasm of hepatocytes (in mice) and abolished HBV replication, but it did not however affect the abundance of pregenomic RNA. This suggests, therefore, that excess pre-C protein may inhibit viral replication by destabilising the nucleocapsid particles and in this way prevent HBV replication. Lambert *et al* (1993) have previously demonstrated that the pre-C protein can exert a dominant negative effect on HBV replication *in vitro*.

Translation from the second AUG produces the core protein (HBcAg) which spontaneously assembles to form the virus nucleocapsid. HBcAg is synthesised in the cytoplasm of the infected cell. HBcAg is a 185 (genotype A) or 183 (all other genotypes) amino acid polypeptide with a molecular mass of 21.5kD. The HBcAg of woodchuck and ground squirrel hepatitis viruses are similar in size and amino acid sequences, and yet the duck hepatitis B virus is considerably larger at 262 amino acids and shares very little sequence homology. These differences between mammalian and avian HBcAgs are more than superficial. The former species participates in forming mixed capsids whereas duck HBcAg is excluded, implying that the molecular interactions that drive capsid assembly are not conserved between the mammalian and the avian hepadnaviruses.

The C-terminus of HBcAg encodes for an extremely basic arginine-rich segment (often termed the protamine domain), comprising the final 36 amino acids (amino acid 150 to 185, genotype A) or 34 amino acids (amino acid 150 to 183, other genotypes). This region is highly protease-sensitive, confers nucleic acid binding capacity and is required for encapsidation (Nassal, 1992). The first 149 amino acids of HBcAg form a hydrophobic domain that shows an intrinsic resistance to proteolysis (Seifer and Standring, 1994): the first approximately 144 amino acids constitute a minimal assembly domain that is responsible for directing the

assembly of core proteins into both dimers and capsids (section 4.9) (Figure 1.3).

No crystallographic structure has been obtained for HBcAg. Monoclonal antibody (MAb) and site-specific protease studies have resulted in a model for the structure of HBcAg (Figure 1.4) (Seifer and Standring, 1994). Only a small area, between amino acids 145 to 153, is susceptible to proteases and antibodies in assembled core particles. This protease-sensitive region falls between the two functional domains of HBcAg, leading to the hypothesis that it acts as a hinge region similar to that seen in other multidomain proteins such as immunoglobulins (Davies and Metzger, 1983). A hinge would permit the flexibility required in conformational changes that occur during core particle assembly and RNA packaging.

HBcAg is involved in packaging the viral mRNA and the viral DNA polymerase and it assembles into core particles (Discussed in Chapter 1, section 4.8). A protein kinase, of probable cellular origin, is also packaged.

4.3.3 X gene

The smallest ORF encodes the X protein, of 154 amino acids, which has a molecular mass of 17kD. It shares nucleotide sequences with both the polymerase gene and the pre-C region and contains several regulatory signals critical to the replicative cycle (discussed in section 4.6). The role in the biology and pathology of HBV has yet to be elucidated. It is produced during the viral life cycle, as shown by the induction of anti-X antibodies during infection (Moriarty *et al*, 1985, Stemler *et al*, 1990). However, the HBx protein has never been reliably demonstrated in, or isolated from infected liver tissue (Henkler and Koshy, 1996). This is probably due to the lack of reliable antibodies to HBxAg. Studies that

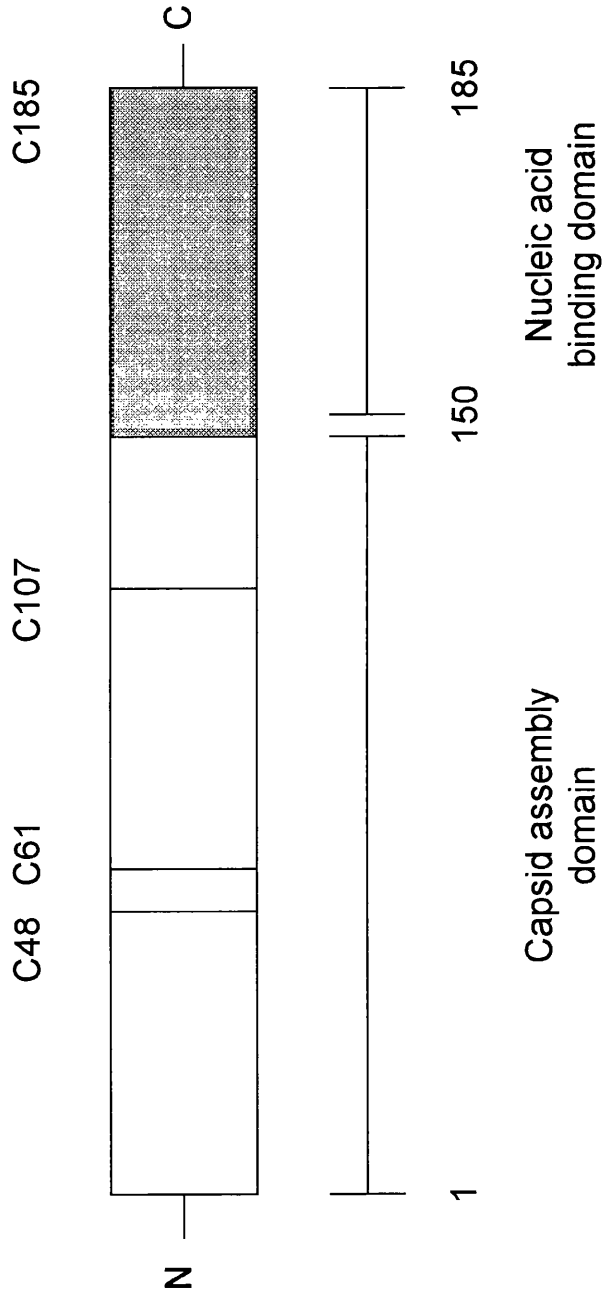


Figure 1.3 Schematic structure of the hepatitis B virus HBcAg. Amino-terminus (N) is shown at the left and the carboxyl-terminus (C) at the right. The unshaded region depicts the hydrophobic assembly domain (aa 1-149). The Arg-rich nucleic acid binding region (protamine-like region, aa 150-185) is shown shaded. The vertical bars represent the four Cys residues (adapted from Seifer & Standing, 1995)

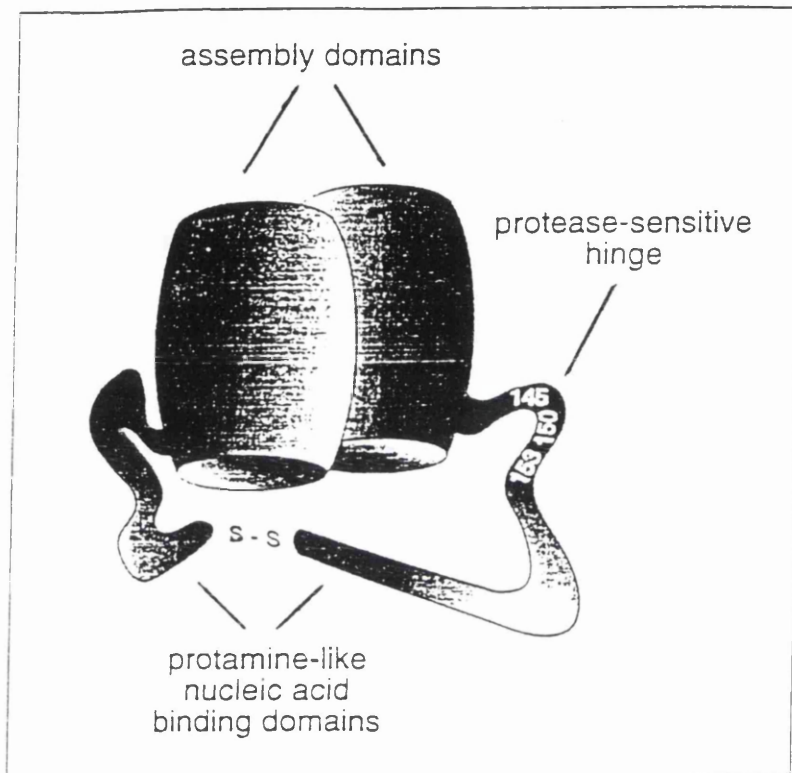


Figure 1.4 Model of the hepatitis B virus core protein dimer. Each 21.5kD monomer subunit is shown with a globular minimal capsid assembly domain (barrel) and a protamine-like nucleic acid binding domain (arm). The putative protease-sensitive hinge region links these two domains (Seifer and Standing, 1995).

have shown the expression of HBxAg in chronic liver disease utilised antibodies that cross-reacted with cellular proteins.

HBxAg does not appear to be required for HBV replication or gene expression *in vitro* (Blum *et al*, 1992) and there is no gene encoding a corresponding protein in the duck hepatitis B virus (DHBV) (Mandart *et al*, 1994). However, data from deletion studies in the woodchuck hepatitis model suggests that an X minus mutant is not infectious *in vivo* (Chen *et al*, 1993, Zoulim *et al*, 1994), indicating that HBxAg is essential for replication *in vivo*. There are reports of various biochemical activities of HBxAg, such as protein kinase (Wu *et al*, 1990), dinucleotide kinase (Shaul, 1991) or protease inhibitor (Arii *et al*, 1992), but they have yet to be unequivocally shown to be functional *in vivo*. It is known that HBxAg can activate the transcription of HBV genes, other viral sequences and a variety of cellular genes (Kekule *et al*, 1993, Seto *et al*. 1988, Cologrove *et al*, 1989). The mechanism of HBxAg transcriptional activation is still a contentious point. Currently there are two models: the first is that HBxAg activates transcription directly via associations with DNA-binding proteins. This model has been supported through experiments artificially targeting HBxAg to DNA, mainly through fusion proteins linking HBxAg to various DNA-binding domains (Seto *et al*, 1990, Unger *et al*, 1990). Qadri *et al* (1995) have reported an interaction between HBxAg and components of the promoter complex, as there was an apparent interaction of HBxAg with the TATA binding protein. These experiments suggest a general mechanism for HBxAg activation.

The second model speculates that HBxAg transactivates by interfering with intracellular signal transduction pathways. Considering the range of elements e.g. c-jun (Twu *et al*, 1993), c-fos (Avantaggiati *et al*, 1993) and NF κ B (Lucito *et al*, 1992) that can be activated by HBxAg and its predominant cytoplasmic location in transfected cells (Henkler *et al*, 1995), this is a plausible idea.

X responsive elements (XRE) for HBxAg have been reported as the following; AP-1- and AP-2-binding sequences in SV40 (Seto *et al*, 1990), NF κ B-like sequence in human immunodeficiency virus (HIV)-long terminal repeat (Siddiqui *et al*, 1989; Twu *et al*, 1989). The 26bp XRE enhancer I (Faktor *et al*, 1990) and HBxAg was found to transactivate a certain gene through a different XRE in each cell line e.g. AP-2-binding sequence responded to HBxAg in CV-1 cells (Seto *et al*, 1990), but not in HepG2 cells (Twu *et al*, 1989). Thus, the efficacy of transactivation of a target gene was found to be dependant on the cell line used (Seto *et al*, 1989). It is likely, therefore, that HBxAg has a repertory of interaction partners, selects an appropriate one and transactivates a specific set of genes in the cell or that HBxAg catalytically modifies a specific set of transcription factors. Maguire *et al* (1991) have shown that HBxAg can bind to CREB or ATF-2, enabling the latter to tightly bind to a CRE-related sequence in the HBV enhancer I. How this relates to the transactivation function of X protein is not clear. A direct interaction of HBxAg with other XRE-binding proteins, such as NFKB or AP-1, has yet to be described.

HBxAg shares amino acid sequence homology with the Kunitz domain of the Kunitz-type serine protease inhibitors, and thus, given that this Kunitz-like domain has been shown to be requisite to the transactivation function of HBxAg there is speculation that HBxAg functions as a serine protease inhibitor in hepatic cells (Arii *et al*, 1992). Serine protease inhibitors inhibit their targets by binding to the active centre of the protease and its surroundings. Digestion analysis, using highly purified serine proteases, and immunoprecipitation experiments have indicated a direct interaction of HBxAg with trypsin TL₂, a major serine protease in hepatic cells (Takada *et al*, 1994).

Deletion studies have indicated that there are two regions which are essential for the transactivation function of HBxAg: amino acids 28 to 62 and amino acids 118 to 142. Both these regions overlap with highly

conserved HBxAg amino acid sequences among hepadnaviruses, at amino acids 58 to 84 and amino acids 98 to 140.

A role in carcinogenesis has been implicated as truncated integrated X gene sequences have been associated with the development of hepatocellular carcinoma (HCC) (Takada and Koike, 1990). HBxAg has the ability to transform normal rodent cells (Seifer *et al*, 1991) and induce HCC in transgenic mice (Kim *et al*, 1991). Despite this basic property of HBxAg, no specific integration site has been identified in human HCC (Tokino *and* Matsubra, 1991). Thus, it is likely that its transactivation/oncogenic function manifests via protein-protein interaction with cellular factor(s). The HBxAg sequences usually found in HCC tumours have 3' deletions, resulting in truncation and fusion with cellular sequences. However, they encode functionally active transactivator proteins (Schluter *et al*, 1994), and transcripts containing HBxAg sequences have been detected in HCCs (Paterlini *et al*, 1995, Wei *et al*, 1995)

Disruption of p53 activity (p53 is the suppressor oncogene protein) has been closely related to the development of many human cancers (Gerwin *et al*, 1992). In virus-transformed cells it has been shown that p53 complexes with viral oncoproteins, such as the adenovirus E1B protein (Sarnow *et al*, 1982) or the Epstein-Barr virus-encoded nuclear antigen, EBNA-5 (Szekely *et al*, 1993); such protein-protein interactions inactivate p53 functions, e.g. sequence-specific DNA binding and gene transactivation. Experiments have shown that HBxAg forms a complex with normal p53 protein (Feitelson *et al*, 1993) and that it can suppress p53-mediated transcriptional activation (Wang *et al*, 1994). Therefore, by altering the activity of p53, HBxAg may be involved in the initial process of HBV-related hepatocarcinogenesis.

The size and number of HBxAgS expressed from the ORF *in vivo* are unknown, but the conservation of its three start codons and the results of mutational studies suggest that the smaller potential proteins are expressed and are transcription activating (Kwee *et al*, 1992).

4.3.4 Polymerase gene

The polymerase gene overlaps all the other genes and encodes for the polymerase protein. Mutational analysis of the polymerase gene and studies on the mechanism of genome replication, indicate that most parts of the gene are indispensable for the virus. Such studies suggest that polymerase has four clearly distinguishable domains (Schlicht *et al*, 1991). From the amino to carboxyl terminus these are: terminal protein, which is linked to the 5' end of the minus strand DNA and serves as a protein primer for reverse transcription of the RNA pregenome into (-) strand DNA; a spacer region, which can be deleted without loss of enzyme activities; DNA polymerase/reverse transcriptase domain and RNaseH domain, which digests the RNA if it is present as hybrids of RNA and DNA. Unlike the ORF P of retroviridae it has no protease or integrase domain but it has a primase domain that is absent in retroviridae.

4.4 Attachment and entry

Virus attachment is one of the crucial steps that determines the host range and organ tropisms of viruses. A serious drawback for the study of the attachment of HBsAg to cell surface proteins is the lack of susceptible cell systems. There are many cell lines that are permissive for HBV replication but are not able to be infected by HBV. An example of this is the human hepatoblastoma cell line HepG2, which cannot be infected but replicates and secretes HBV efficiently after transient or stable introduction of HBV DNA. Bchini *et al* (1990) have claimed to have achieved successful infection in this cell line. Gripon *et al* (1988) have successfully infected primary hepatocytes from adult liver when the cells were treated with

polyethylene glycol. Effective systems are available for some of the animal hepadnaviruses, e.g. duck hepatitis B virus (DHBV) and WHV. Primary hepatocytes from these animals are susceptible to infection by their respective viruses. However, the hepatocytes lose this susceptibility within several days in culture. The reason for the non-susceptibility of long-term hepatocyte cultures is unknown, but it appears to be due to a block in attachment and/or virus entry.

The region, amino acids 21 to 47 of pre-S1 has been shown to be involved in attachment to the cell surface. The binding can be blocked by antibodies to the pre-S1 sequence and competed for, by the peptide sequence itself (Neurath *et al*, 1986), and MAbs to this region can block attachment to HepG2 cells and infectivity for primary human hepatocyte cultures (Petit *et al*, 1991 and 1992). However, although amino acids 21 to 47 may be essential for the infection process, their attachment to the cell surface is obviously not sufficient for infectivity, otherwise HepG2 cells would be more susceptible to infection by HBV. A human liver protein that specifically binds to this region of pre-S1 has yet to be identified (Petit *et al*, 1992; Dash *et al*, 1992)

It has been shown that middle surface protein binds to monomeric or polymerised human serum albumin via its pre-S2 region (Dash *et al*, 1991; Krone *et al*, 1990), and polymerised human serum albumin binds to hepatocytes. Hertogs *et al* (1993) have identified human liver annexin V as a specific binding protein of small HBsAg. The same group (Hertogs *et al*, 1994), in a later study, described the spontaneous development of anti-idiotypic antibodies in rabbits immunised with human liver annexin V, which were able to compete with annexin V for the binding to small HBsAg, thus indicating a ligand-receptor relationship between annexin V and HBsAg. In a recent study De Bruin *et al* (1995) demonstrated the specific binding and internalisation of small HBsAg, conjugated to colloidal gold particles, to human hepatocytes. The binding was inhibited

by either anti-annexin V or anti-idiotypic (anti-HBsAg) antibodies, indicating a direct role for annexin V in binding and uptake of HBV envelope proteins.

There are two ways in which animal viruses can enter a cell: by fusion of the viral envelope with the cell membrane, and thereby causing the release the of nucleocapsid into the cytoplasm, or by receptor-mediated endocytosis. The mechanism of HBV entry into hepatocytes is still unknown.

In DHBV it was originally reported that entry could be blocked by substances that inhibit acidification of endosomes, thus implicating a receptor-mediated endocytosis mode of entry (Offensperger *et al*, 1991), but later data suggested that low pH was not involved in the entry mechanism (Rigg and Schaller, 1992).

4.5 Uncoating and transport of DNA to nucleus

Even less is known about the steps involved in capsid transport and genome release. In DHBV, it is known that 24 hours after infection, viral DNA is present in the nucleus and has been converted to covalently closed circular molecules (Tuttleman *et al*, 1986) but it is not known if it is the core particles or free DNA that is transported to the nucleus.

4.6 Transcription and regulatory elements

Transcription of double-stranded DNA into RNA is a highly regulated process that controls the expression of all genes in a timely and structurally ordered manner. All genomes, therefore, contain not only genes that encode protein sequences, but also regulatory elements that bind cellular or viral proteins which in turn act as positive or negative transcription factors.

There are two types of elements that contribute to transcription.

1. *Promoters*. These are necessary for the binding of essential transcription factors, bringing the RNA polymerase into a position where it begins to transcribe from one DNA strand at a defined site.
2. *Enhancers*. These are segments of DNA that enhance transcription initiation at a given promoter, but are not required to be at a defined position next to the promoter or even in the same orientation as the promoter.

Promoters are, therefore, clearly a defined part of a gene, whereas enhancers may act on the expression of several genes. To date, four promoters and two enhancers have been described for HBV (Siddiqui, 1991, Schaller and Fischer, 1991)

Promoters: The promoters direct initiation of RNA synthesis upstream of the HBc/e gene, the large HBs gene, the middle and small HBs genes and the HBx gene (Figure 1.2). Only the S promoter 1 (Sp1), which directs the large HBs gene, has a typical TATA box, and only the mRNA encoding large HBs protein has a sharply defined 5' end. TATA-less promoters usually have multiple initiation sites, as is the case for the mRNAs of HBc/e, middle and small HBs and HBx.

There are four classes of HBV transcripts. These are; the pre-C/C mRNAs (3.5kb), the pre-S1/S2 mRNAs (2.4 and 2.1kb), the S mRNAs (2.1kb) and the X mRNAs (0.9kb). The transcripts are initiated sequentially on the circular DNA genome.

Although the two 3.5kb transcripts - the pregenomic and the pre-C - are very similar, both terminating at the same polyadenylation site while the pre-C transcript initiates approximately 30 nucleotides upstream of the start site of the pregenomic RNA, they have very different functions. Pre-C RNA encodes the pre-C protein, which is subsequently processed into

HBeAg. The pregenomic RNA has two roles: it can code for HBcAg and polymerase protein, and it can be packaged into viral capsids with the polymerase protein, and serve as the template for replication of the viral genome. The basal core promoter (BCP) precisely directs initiation of both the pre-C and pregenomic RNAs (Yuh *et al*, 1992). Instead of the standard TATA element, a stretch of 15 nucleotides containing four-TA sequences, functions as both an initiator and a TATA element for the pre-C and pregenomic RNAs (Chen *et al*, 1995).

All the transcripts are capped, unspliced and end after a common polyadenylation signal. There are two polyadenylation sites present on the transcripts but the first is ignored. This selective poly (A)-site usage is thought to be due to a non-perfect poly (A)-signal consensus sequence, activating upstream elements, and a minimal distance from the promoter is also important (Cherrington *et al*, 1992).

Enhancers: Two regions of the HBV genome act as enhancers; enhancer I (enh I) and enhancer II (enh II) and can enhance the activity of all the HBV promoters. Enh I has been mapped to nucleotide positions 970 to 1250, between the surface and X ORFs. It has been suggested that this element is responsible for liver-specific gene expression since transcriptional activation by this enhancer is greater in some cultured hepatoma cells than in nonhepatic cells (Shaul *et al*, 1985).

Enh II lies immediately upstream from the coding region of the core ORF, at nucleotide positions 1616 to 1785. The activity of enh II is highly liver-specific, functioning only in highly differentiated human hepatoma cell lines. In addition, enh II activity varies in different hepatoma cell lines, implying that the differentiation state of the cell line used regulates the enhancer.

4.7 Translation

The pregenome serves as template for both core and polymerase translation. HBcAg is synthesised from the first cistron in an unprocessed form and polymerase from the second. Theoretically, this should lead to inefficient translation of polymerase and, unlike retroviruses, hepadnaviruses do not produce core and polymerase as a fusion protein. Although the two genes overlap, polymerase is produced separately from core (Schlicht *et al*, 1989)

4.8 Encapsidation and replication

HBV utilises a novel replication strategy for a DNA virus. Three components are required: the RNA pregenome, the polymerase protein and the core protein. Biosynthesis of these proteins is closely coupled to replication since the pregenome serves as their mRNA .

Unlike the retroviruses, HBV does not require integration into the host genome, and thus polymerase does not have an integrase domain. Instead, it harbours the terminal protein domain which is intimately involved in replication (Radziwill *et al*, 1990). While other reverse transcriptases require proteolytic cleavages for maturation into active enzymes, there is no evidence of such processing in polymerase (Bartenschlager and Schaller, 1992).

Once sufficient quantities of HBcAg, polymerase and the RNA pregenome have accumulated in the cytoplasm, core particles are formed (discussed in 4.10), thus providing the necessary machinery for replication. Hepadnaviruses have developed a unique strategy for the encapsidation of the correct RNA and polymerase from the pool of cellular nucleic acids and proteins available. Polymerase itself is directly involved in incorporation of the correct pregenomic RNA into nucleocapsids: this

requires none of its enzymatic properties but does require the entire primary sequence. Furthermore, it preferentially acts on its own mRNA, probably as it is being synthesised (Bartenschlager *et al*, 1990 and Hirsch *et al*, 1990).

A region of 85 nucleotides near the 5' end of the pregenomic RNA (this is within the pre-C/C region) is the *cis*-acting element for encapsidation, termed epsilon (ϵ); ϵ is essential for packaging and replication of HBV (Junker-Niepmann *et al*, 1990). A similar region has been identified in DHBV but deletion analyses have shown that additional sequences close to the centre of the pregenomic RNA are also required for RNA encapsidation (Calvert and Summers, 1994). The encapsidation signal contains several nested inverted repeats with the capacity to form a bulged stem-loop structure (Figure 1.5) (Knaus and Nassal, 1993, Pollack and Ganem, 1993). Studies by Tong *et al* (1992 and 1993) have shown that both sequence and base-pairing within ϵ are important. Of the possible eight stop codons that can arise in ϵ by a single nucleotide change, only A₁₈₉₆ (codon 28) has been found in patients. Tong *et al* (1992) constructed the other seven and tested their replication capacities. Two stop codons (at codon 23 and 26) failed to replicate and the remaining five (12,13,14,18 and 21) did not replicate as competently as A₁₈₉₆. The replication competence of the three stop codons (12, 13 and 14) in stem I suggests that this region of ϵ can tolerate small sequence changes. This is also true of the bulge area, as the mutated codon 18 was still replication competent. However, mutations occurring in the loop area (codon 23) and stem II (codons 21 and 26) had a greater effect on replication. When codons 23 and 26 were mutated, no replication was evident and although replication did occur when codon 21 was mutated, the efficiency was very much decreased when compared to the other mutants. Therefore, it is evident that disruptions to the sequence within this region can interfere with encapsidation.

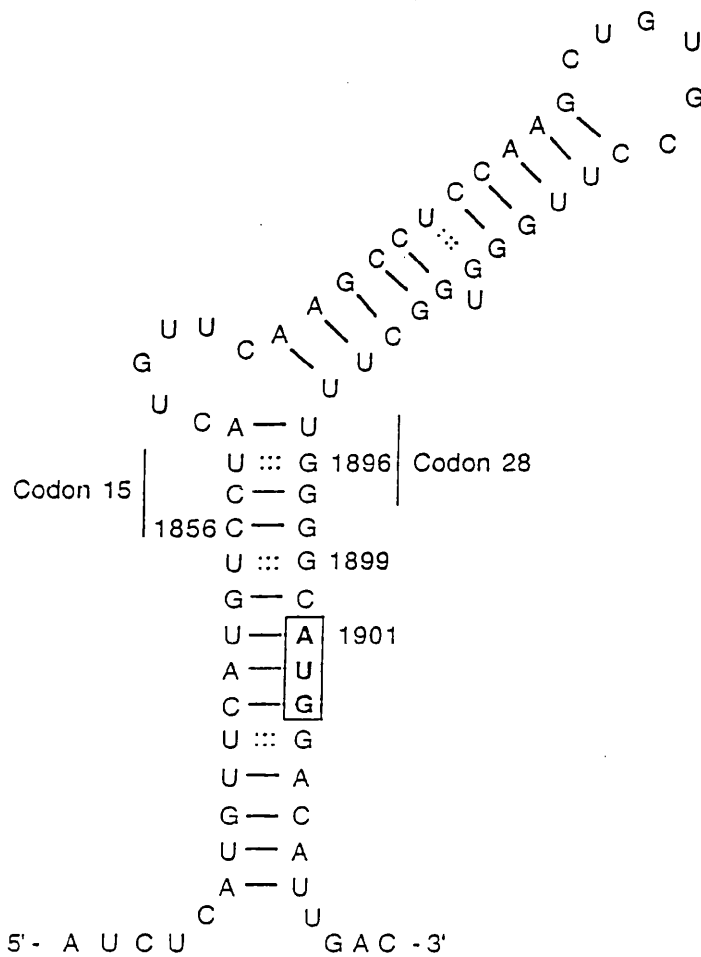


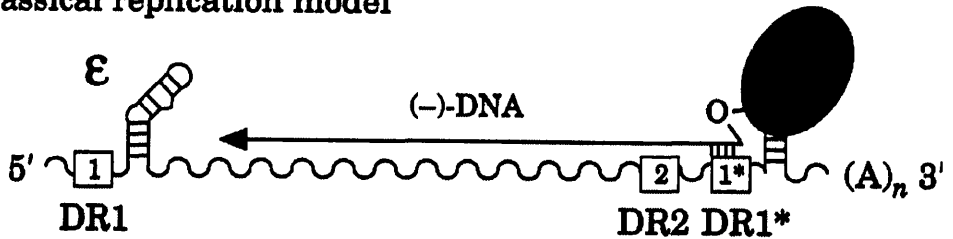
Figure 1.5 Predicted secondary structure of the hepatitis B virus encapsidation signal. The start codon (AUG) of HBcAg is highlighted in bold and boxed. The positions of codons 15 and 28, of pre-C, are indicated (kindly supplied by D. Williams).

Mutational analyses suggest that, in addition to the overall structure, the sequence of the loop and the first two nucleotides in the bulge are important for productive interaction with polymerase (Reiger and Nassal, 1995). Polymerase binds directly to ϵ . The other four nucleotides, when mutated, do not significantly influence encapsidation (Laskus *et al*, 1994; Lok *et al*, 1994) but reverse transcription is affected (Knaus and Nassal 1993; Fallows and Goff, 1995)

A model for HBV replication is given in Figure 1.6 (Nassal and Schaller, 1996) The classical model assumed (-) DNA synthesis was a continuous process starting *de novo* from within the 3'-proximal direct repeat (3'-DR1*) proceeding continuously to the 5'-end of the RNA template (Seeger and Maragos, 1991), where immediately on translation from the full length mRNA acting as the pregenome, polymerase binds to ϵ on the same RNA. The revised model proposes a discontinuous mechanism whereby a short DNA primer, covalently linked to polymerase, is translocated to DR1*. This model is similar to the replication mechanism observed for retroviruses. However, in HBV it is ϵ , rather than a host-derived tRNA, that positions the reverse transcriptase over the origin of replication. Owing to the terminal redundancy, DR1 and ϵ are present twice on the pregenome. Reiger and Nassal (1996) have demonstrated that replication origin function is confined to the 5'- ϵ while primer translocation occurs only to the 3'-DR1*. The authors hypothesise that, *in vivo*, the two ends of the pregenome are held spatially close by some kind of circularisation mechanism. The molecular basis for this spatial arrangement is still unclear.

Binding of polymerase to ϵ suppresses core translation, deprives the RNA of ribosomes and triggers the addition of HBcAg dimers, thus stabilising the complex. Further HBcAg dimers are attracted to the RNA via their nucleic acid binding domains, thus completing the capsid shell. This

Classical replication model



Revised replication model

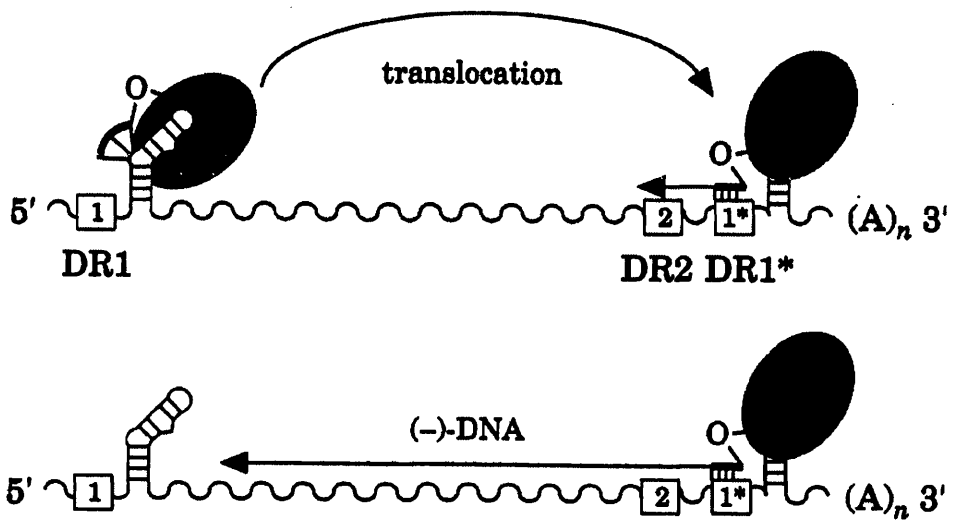


Figure 1.6 Classical and revised models for initiation of reverse transcription in hepatitis B virus. The classical model assumed (-) DNA synthesis to be a continuous process starting within the 3'-DR1*. The revised model proposes that protein bound to ϵ copies part of the ϵ bulge and translocates to the 3'-DR1* and the primer extends from there (Nassal and Schaller, 1996).

mechanism ensures the efficient co-encapsidation of the pregenome and polymerase, and prevents indiscriminate reverse transcription of cellular RNAs by polymerase while also explaining the low number (probably one) of polymerase molecules per particle (Bartenschlager and Schaller, 1992).

The next step in the process is the conversion of the pregenome into the partially double-stranded, circular DNA genome (Summers and Mason, 1982). Polymerase binds to a recognition signal on the RNA pregenome between DR2 and DR1, priming DNA synthesis via its terminal protein, domain and pulls the RNA through its active centre, looping out the newly synthesised minus (-) strand DNA. Concurrently, the RNaseH activity of polymerase degrades the RNA template to the last 15 to 18 nucleotides (Loeb *et al*, 1991). This short oligoribonucleotide serves as a primer for the second (+) DNA strand (Lien *et al*, 1986); it is transferred to DR2 at the 3' end of (-) strand DNA, via their matching 11 nucleotide sequence, thus circularising the genome. Elongation from this point leads to the characteristic partially double-stranded, circular HBV genome (Figure 1.7).

4.9 Assembly and release

Though very little is understood about the molecular interactions that underlie the various steps of virion assembly, it is almost certain that specific core amino acids are closely involved in drawing together the viral polymerase and RNA pregenome into the nucleocapsid, while different residues are probably involved in envelopment of the finished core particle by HBsAg.

The ability to assemble into particles resides in the first 144 amino acids of HBcAg (Birnbaum and Nassal, 1990; Nassal, 1992) (Figure 1.3). Deletion beyond residue 139 completely abrogates capsid assembly. As detailed earlier, although a considerable amount of work has been carried

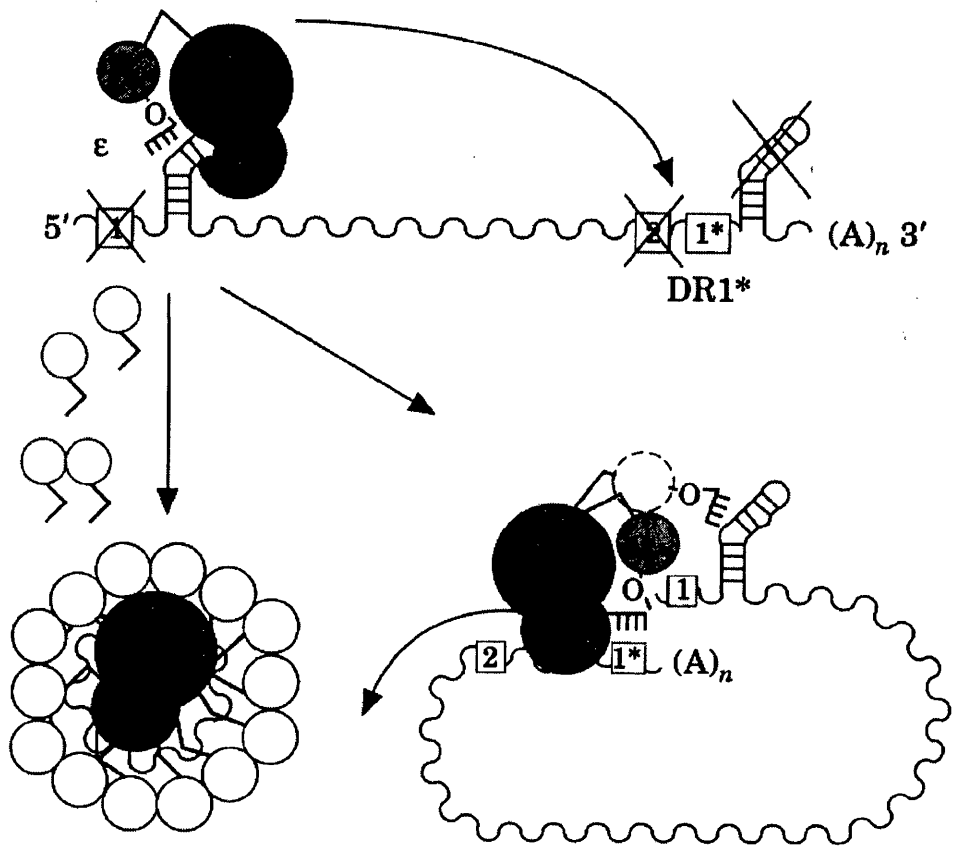


Figure 1.7 Dual role of P-ε interaction in the hepatitis B virus replication cycle. The binding of polymerase (P) protein, translated from the pregenome, to 5'-ε triggers core protein dimers to initiate capsid assembly, and also initiates reverse transcription (Nassal and Schaller, 1996).

out on the effect of carboxy (C)-terminal deletions, considerably less has been investigated on the effect of amino (N)-terminal deletions. Chang *et al* (1994) have shown that HBcAgS lacking the first 28 or 37 amino acids are incapable of participating in HBcAg oligomerization. Studies involving the introduction of foreign sequences into the HBcAg (usually for engineering highly immunogenic, multivalent capsids for vaccine purposes), have shown that there are three regions not essential for particle formation: the immediate N-terminal, around amino acid 80 and at the C-terminus at either amino acid 144 or 156 (Schodel *et al*, 1992; Clarke *et al*, 1990; Francis *et al*, 1990).

Although the protamine region is dispensable for capsid assembly, this does not exclude it from playing a role in the assembly process. There are 17 arginine residues within the 36 amino acids of the protamine region and they are clustered into four repeat sequences (amino acids 150 to 156, 157 to 163, 164 to 171 and 172 to 179). Studies involving deletions of these four regions have shown that they are closely involved in the interactions between HBcAg and RNA and DNA (Hatton *et al*, 1992), with the arginine residues of repeat I primarily responsible for RNA binding (Hatton *et al*, 1992). The motif SPRR is commonly found in DNA binding proteins and is found in repeats II, III and IV. Replication takes place in the capsid, and as the carboxy-terminal has been shown to be required for HBV replication (Nassal, 1992; Beames and Lanford, 1993), a role for the DNA binding motifs in facilitating replication can be envisaged.

It appears that a critical concentration of HBcAg is necessary for assembly. First of all, dimers of HBcAg are formed (Zhou and Standing, 1992), which then assemble at 0.8 μ M concentration (Seifer *et al*, 1993) to isometric particles of T3 symmetry i.e. 180 HBc subunits form one particle (Birnbaum, 1990). This process appears to be facilitated by RNA (Seifer *et al*, 1993), as ablation of HBcAg RNA binding motifs drastically increases the initial dimer concentration required for capsid formation.

Antibody binding studies have shown that the protamine region faces the interior of the core particle, supporting a role in the binding of RNA and drawing it into the nascent capsid (Seifer and Standring, 1994).

HBcAg has four cysteine residues at amino acids 48, 61, 107 and 185 (Figure 1.3); mutational analysis has shown that these residues serve to stabilise the core particles via disulphide bonds as capsids formed from cysteine-minus HBcAg, (under oxidising conditions), are far less stable than their cysteine containing counterparts (Zhou and Standring, 1992), with homologous Cys 61-Cys 61 and Cys 185-Cys 185 the key disulphide bonds stabilising the assembled HBcAg (Nassal, 1992; Zhou and Standring, 1992). Formation of disulphide bonds between HBcAg are not required for capsid assembly (Zhou and Standring, 1992).

5. Immune response to HBV, focusing on HBcAg

The immune mechanisms responsible for viral clearance and liver cell damage in HBV infection are not well understood. HBV has not been shown to be cytopathic, and the histopathology and clinical symptoms observed in HBV infection are believed to be the result of elimination of infected hepatocytes by the immune response.

It is generally understood that HBV is completely cleared by the immune system during acute viral hepatitis B infection. Rehermann *et al* (1996) have recently demonstrated that traces of HBV can still be detected in the blood several years (even decades) after clinical recovery, thus maintaining the CTL response and apparently creating a negative feedback loop that keeps the virus under control.

Despite HBcAg and HBeAg sharing a large proportion of their amino acid sequence - approximately 149 amino acids - they are immunologically quite different. HBcAg behaves in both a T-cell dependent and

independent manner, whereas, HBeAg only acts in a T-cell dependent manner and is a relatively poor B-cell immunogen (Bichko *et al*, 1993).

5.1 Humoral immune responses

There is a protective immune response directed mainly against the surface proteins of HBV, with the a determinant (amino acids 124 to 137) exhibiting the major antigenic effect. Early studies showed that anti-HBc and anti-HBe do not act as virus neutralising antibodies. When monoclonal antibodies to these proteins are injected into chimpanzees they do not prevent infection, whereas infection is prevented with monoclonal antibody to HBsAg (Iwarson *et al*, 1985); similarly, babies born to mothers with high levels of anti-HBc have these antibodies passively transferred, yet still develop chronic infection. It is surprising, therefore, that there is documented evidence that immunisation of chimpanzees with HBcAg, either purified from liver or produced from bacteria, can lead to protection (in a population of animals). This is probably due to HBcAg acting as a T-cell immunogen, as HBcAg efficiently induces HBcAg-specific Thelper (Th) cells in mice. Anti-HBe also appears to be protective, suggesting a role for HBc/eAg in protecting against HBV re-infection.

Hepatocytes, infected with HBV, have been shown to express nucleocapsid antigens on the cell surface which is essential for antibody directed cellular cytotoxicity or complement mediated lysis by anti-HBc or anti-HBe antibodies (Schlicht and Schaller, 1989). An increase in catabolism of complement factors C1q and C3 have also been observed (Potter *et al*, 1989; Thomas *et al*, 1979).

Reports have indicated that X induces antibody responses (Moriarty *et al*, 1985; Stemler *et al*, 1990). Antibodies to polymerase have been detected in patients with acute and chronic disease (Weimer *et al*, 1989).

By using recombinant HBcAg and HBeAg as well as denatured HBcAg, MAbs recognising HBcAg were obtained and immunodominant regions identified. The major B-cell epitope in HBcAg is located between amino acids 73 and 89 (Colucci *et al*, 1988; Salfeld *et al*, 1989; Sallberg *et al*, 1991). This epitope is believed to be part of a larger conformational epitope (Ferns and Tedder, 1986). The peptide used by Colucci *et al* (1988), although it encompassed this epitope, had a much reduced binding affinity compared with that of HBcAg particles indicating that it is part of a discontinuous or conformational epitope. Colucci *et al* (1988) also described an epitope spanning amino acids 107 to 118. Two B-cell epitopes have been described in HBeAg: a linear epitope, e1, and a probable discontinuous epitope, e2, located between amino acids 130 and 138 (Salfeld *et al*, 1989). Bichko *et al* (1993) analysed the humoral immune response to denatured HBcAg and identified a highly antigenic region between amino acids 134 and 140, a similar location to the e2 epitope described by Salfeld *et al* (1989). A second region, from amino acids 134 to 154, was also antigenic.

Machida *et al* (1989) characterised an epitope in the arginine-rich carboxyl-terminus of HBcAg, between amino acids 150 and 159. It is of interest that variants often appear in this region (Chapter 1, section 6.2, Figure 1.8)

Pushko *et al* (1994) investigated the exposed and masked regions of HBcAg after assembly of the nucleocapsid. Two areas were found to be exposed: between amino acids 78 to 83 and 127 to 133. Despite both regions being exposed on the core particle, only the region between amino acids 78 and 83 is within the immunodominant region recognised by anti-HBc, whereas anti-HBe recognises both these areas: amino acids 76 to 89 and 127 to 133 (Salfeld *et al*, 1989; Sallberg *et al*, 1991 and 1993)

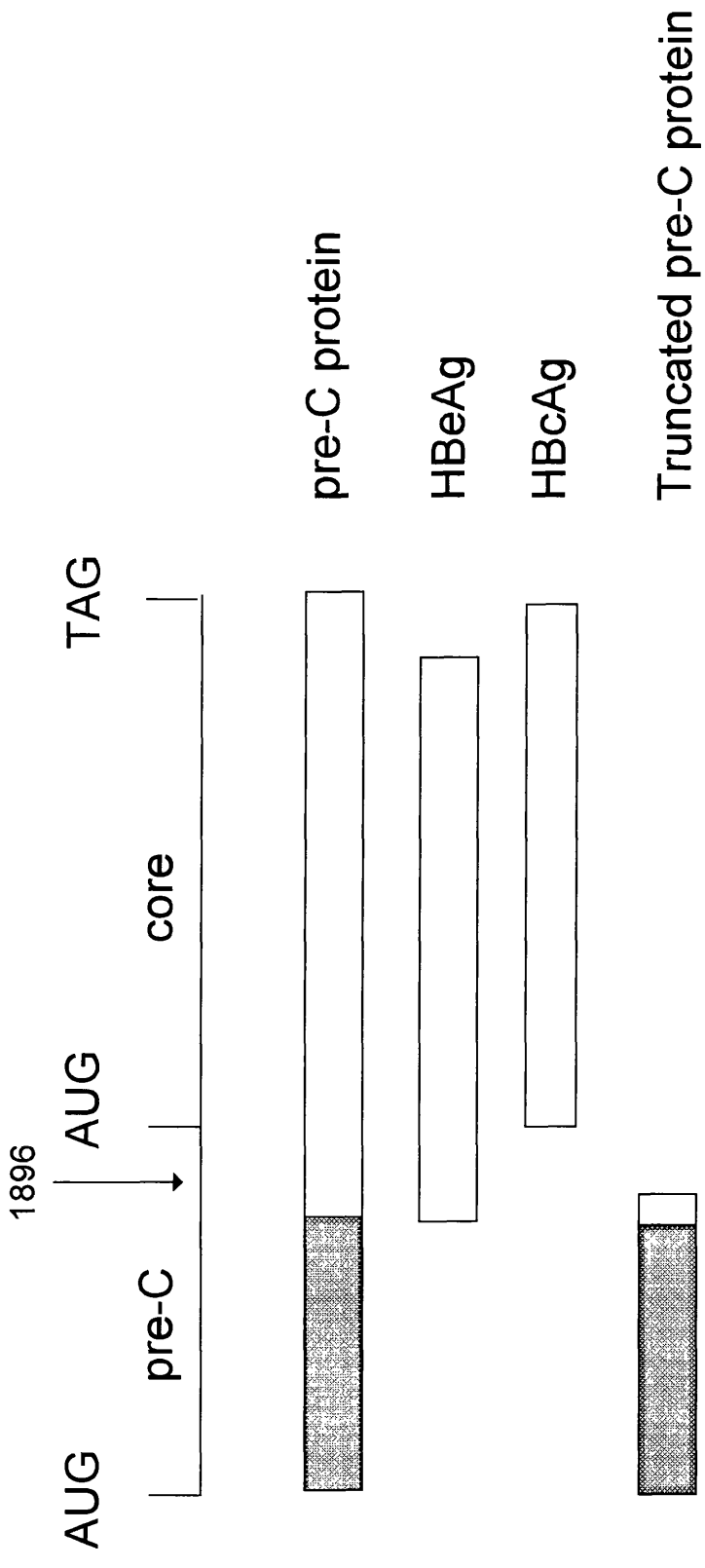


Figure 1.8 Schematic representation of the pre-C/C ORF. Translation from the first AUG produces HBeAg, translation from the second AUG produces pre-C protein, which subsequently cleaved at the signal peptide and at the N-terminus to produce HBeAg. Substitution of G to A at nucleotide position 1896 introduces a stop codon which subsequently terminates production of HBeAg. Shaded area represents the signal peptide

5.2 T-cell responses

5.2.1 Cytotoxic T lymphocytes (CTL)

The CTL response in acute self-limiting hepatitis is generally vigorous and multispecific (Penna *et al*, 1991). Ferrari *et al* (1991) showed that the major CTL response was to the products of the pre-C/C ORF, thus indicating that the immune response to HBcAg/HBeAg is an important contributory factor to viral clearance and liver cell injury in acute hepatitis (Penna *et al*, 1991; Bertoletti *et al*, 1991; Milich *et al*, 1991). HBcAg-specific responses are greater than HBsAg-specific responses and increase during active disease (Tsai *et al*, 1992; Vento *et al*, 1987). Rehermann *et al* (1995) showed that a CTL response against multiple epitopes within polymerase is also present in acute viral hepatitis. Usually, in chronic HBV infection, the CTL response is not detectable. When it is detectable, however, it is more highly focused and weaker compared to that observed in acute HBV infection (Penna *et al*, 1991, Nayersina *et al*, 1993). There is often a CTL response at low precursor frequency at the site of inflammation (Barnaba *et al*, 1989). The reason for this difference is not clearly understood but it is believed to represent a crucial determinant of the final outcome of infection with HBV, and indicates that CTL play a critical role in clearing HBV infection; thus, failure to recognise or respond to CTL epitopes may play a role in viral persistence.

Bertoletti *et al* (1991) identified an HLA-A2-restricted epitope in HBcAg, overlapping with HBeAg, spanning the residues amino acids 11 to 27 in patients with acute hepatitis. Again, the response from chronic hepatitis patients was significantly weaker or absent. The optimal sequence was later identified as being amino acids 18 to 27 (Bertoletti *et al*, 1991). Missale *et al* (1993) have described HLA-A2 and HLA-Aw68-restricted CTL epitopes within HBcAg, spanning amino acids 141 to 151, which also contain the nuclear localisation and encapsidation signals.

Rehermann *et al* (1995) described six HLA-A2-restricted CTL epitopes within the highly conserved reverse transcriptase and RNaseH domains of the polymerase protein; they showed that the CTL response to polymerase is polyclonal, multispecific and mediated by CD8⁺ T cells in patients with acute hepatitis, but that there is no detectable response in patients with chronic infection. DNA sequence analysis of the viruses, in patients who did not respond to peptide stimulation, indicated that CTL nonresponsiveness was not due to infection by viral variants that differed in sequence from the synthetic peptides. CTLs specific for one of the epitopes, were unable to recognise several naturally occurring viral variants, except at high peptide concentration, underlining the HBV subtype specificity of this response. Furthermore, CTL responses against polymerase, core and surface epitopes were detectable for more than a year after complete recovery and seroconversion, reflecting either the persistence of trace amounts of virus or the presence of long-lived memory CTL in the absence of viral antigen.

Since viral persistence is not due to escape from CTL recognition under these conditions, their data suggest that HBV may retreat into immunologically privileged sites from which it can seed the circulation and reach CTL-inaccessible tissues, thereby maintaining the CTL response in apparently cured individuals and, perhaps, prolonging the liver disease in patients with chronic hepatitis.

HBV-specific CTLs can persist in the blood for several decades after clinical and serological recovery from acute hepatitis B infection. This indicates that there is continuous priming of the CTL response by persistent virus or viral antigens long after apparent clinical recovery (Rehermann *et al*, 1996). Memory CTL responses (response in the absence of antigenic stimulation) apparently survive for much longer periods than was originally thought (Rehermann *et al*, 1996). Since an absence of CTL correlates with high viral load in patients with chronic

hepatitis B, the active maintenance of the CTL response may play a role in controlling HBV replication.

Spontaneous clearance of HBV-DNA and HBeAg seroconversion in chronic hepatitis is generally preceded by an exacerbation of liver disease activity and an increase in CD4⁺ proliferative T-cell responses is observed (Tsai *et al*, 1992; Jung *et al*, 1995), indicating that latent immune-mediated clearance mechanisms can become spontaneously activated in some individuals. Flares are also seen prior to HBsAg clearance by interferon alpha therapy (Alexander *et al*, 1987). These discoveries support the idea that HBV-specific CTL are present in chronically infected patients but are not effective enough to clear HBV, and that, in some individuals, this defect is reversible. Rehermann *et al* (1996) investigated this possibility; and their results indicate that chronically infected patients who have a spontaneous or interferon-induced remission, do develop a CTL response and that this response is similar in strength and specificity to that observed in acute hepatitis. Thus, specific immunotherapeutic enhancement of the CTL response in chronically infected individuals could lead to viral clearance and resolution of disease.

5.2.2 T helper lymphocytes

Depending on their lymphokine profiles, Th lymphocytes can be divided into at least three different subsets (Mossman *et al*, 1986): the Th1 cells secrete gamma interferon (IFN- γ), interleukin-2 (IL-2) and tumour necrosis factor (TNF) and promote cell-mediated effector responses; the Th2 cells produce IL-4, IL-5, IL-6 and IL-10, which influence B-cell development, enhancing humoral responses; and Th0 cells which release all of these cytokines. A subset of CD4⁺ T cells have cytotoxic functions *in vitro* (Jacobson *et al*, 1984; Erb *et al*, 1990).

Studies in mice have shown that the Th response to HBcAg and HBeAg supports the production of anti-HBs and anti-HBe which are virus

neutralising (Milich *et al*, 1987), as well as the generation of HBc/HBeAg CTL.

Barnaba *et al* (1994) investigated T-cells that infiltrate the liver in chronic hepatitis. They found a population of CD4⁺ cells that had cytotoxic properties when freshly isolated from tissue biopsy specimens. They had a Th1 cytokine profile and expressed CD56, also expressed on natural killer (NK) cells. These cells were not found in the peripheral blood of the same patients or of control subjects. These findings suggest that compartmentalisation of this Th subset in the liver may be an important pathogenic step, contributing to the lesions that occur during the active phase of hepatitis infection.

Using overlapping peptides, Ferrari *et al* (1991) studied the proliferative response of peripheral blood mononuclear cells from patients with acute hepatitis, and identified three peptides to which the majority of their patients reacted; amino acids 1 to 20, amino acids 50 to 69, amino acids 117 to 131, indicating non-HLA dependent binding. Diepolder *et al* (1996) - also using overlapping peptides - identified Th responses specific for amino acids 50 to 69, 81 to 105, 117 to 131, and 141 to 165, thus indicating that there are at least two dominant Th cell epitopes, with both areas being within HBcAg and HBeAg, and that the HBc-specific Th response is polyclonal within an individual and directed against multiple epitopes. In acute hepatitis, T-cell activation was closely associated with elimination of HBeAg and HBsAg from the serum (Jung *et al*, 1995). Recovery from chronic hepatitis is accompanied by increased T-cell responses to HBcAg and HBeAg (Tsai *et al*, 1992).

Pre-S2-specific T cells have been implicated in the suppression of anti-HBs, perhaps mediating the induction of chronic hepatitis. They lyse HBsAg specific B cells that present HBsAg peptides in the context of the

major histocompatibility complex (MHC) class I pathway (Barnaba *et al*, 1990).

The X protein has been shown to be immunogenic at the Th cell level (Jung *et al*, 1991). Several regions were identified: amino acids 11 to 45, 61 to 105, and 111 to 135. The significance of X-specific Th cells is not yet understood.

5.3 Interferon

The role of interferons in HBV clearance is unclear, although adding interferon to transfected cell cultures leads to reduced HBV-DNA production, implying a direct action. Interferon, in the acute infection, is produced in large amounts. Interferon increases expression of hepatocyte HLA antigens, assisting in the resolution of acute HBV infection. In those who develop chronic infection, interferon production is impaired and an appropriate immune response does not occur. If interferon was the sole factor in determining the development of chronic HBV infection, then all patients with chronic infection would respond equally well to therapeutic doses. This is not the case, however, and thus other factors must be involved. Interferon treatment causes a sustained loss of virus replication in only a proportion (approximately 40%) of HBeAg patients with non-cirrhotic chronic HBV infection.

5.4 Tolerance

Lymphocyte activation and tolerance are two possible outcomes of lymphocyte recognition of antigens. Antigens that generate immune responses are termed immunogens whereas those that induce tolerance are called tolerogens. Normally, all self-antigens are tolerogens but foreign antigens, depending on the dose, can act as tolerogens (Sarzotti *et al*, 1996). It was previously believed that tolerance is immunologically specific and is therefore due to the deletion or the functional inactivation

(or anergy) of antigen-specific T and/or B lymphocytes and hence inhibits lymphocyte activation by subsequent exposure to immunogenic forms of the same antigen. However, a study by Sarzotti *et al* (1996) on the induction of protective CTL responses in newborn mice by a murine leukaemia virus, indicated that immunological nonresponsiveness was not due to clonal deletion of CTL but induction of a nonprotective type 2 response. The understanding that immature or developing lymphocytes are more susceptible to tolerance induction than mature or functionally competent cells (Billingham *et al*, 1953) has recently been challenged. Several groups have apparently shown that the neonatal immune system (in mice) can be primed to recognise and attack foreign antigens if the antigen is introduced to the T cells under the right conditions (Sarzotti *et al*, 1996; Ridge *et al*, 1996; Forsthuber *et al*, 1996). The maintenance of tolerance requires the continuous availability of tolerogenic antigens to interact with immature lymphocytes as they develop from precursors. However, tolerance to foreign antigens can be induced even in mature lymphocytes when these cells are exposed to antigens under conditions that are inadequate for activation.

Murine experiments indicate that a Th cell-specific tolerance against HBcAg/HBeAg may be the basic immunological defect in neonates born to HBeAg-positive carrier mothers (Milich *et al*, 1990). The atypical immunological responses observed in neonates probably results from *in utero* exposure to HBeAg. HBeAg can cross the placenta (Lee *et al*, 1986) thereby gaining access to the thymus, inducing T-cell tolerance by deletion of the immature HLA class II-restricted HBeAg/HBcAg-specific Th cells. Tolerance of T lymphocytes is a particularly potent way for maintaining long-lived nonresponsiveness, as Th cells are critical control elements for all cellular and humoral immune responses.

To substantiate this in the human infection, Hsu *et al* (1992) investigated the cellular immunity to HBcAg in HBV carrier children and neonates born

to hepatitis B virus carrier mothers. Results showed a significant proliferative response of PBMCs to HBcAg in children with raised ALTs but not in those who were HBeAg-positive with normal ALTs.

T-cell anergy has also been proposed as a mechanism for the maintenance of self-tolerance and regulation of the immune response (Rocha *et al*, 1995). Diepolder *et al* (1996) investigated this possibility in HBV infection and showed that Th1 clones, but not Th0, isolated from a patient with acute hepatitis, could be anergised *in vitro* by stimulation with specific peptides.

For tolerance to be maintained, HBeAg must be continually present, as removal of the tolerogen would encourage the emergence of HBc/HBe-specific thymocytes. Consequently, although an infant may be exposed *in utero*, if they are not infected at birth then the longer the time between exposure to HBeAg and infection with HBV, the less chance there is of developing tolerance and therefore chronic hepatitis. The frequent loss of tolerance in young adult, chronic HBV carriers may be the result of a regressing thymus which no longer deletes HBeAg/HBcAg-specific T-cells.

It has been shown in the mouse model that the degree of T-cell tolerance to HBeAg depends on the MHC background of the mouse (Milich *et al*, 1991). Extrapolation of this finding to the human response may explain the different rates of spontaneous HBeAg seroconversion observed in chronic HBV carriers.

The infecting virus may also have a role in the development of tolerance given that perinatal transmission experiments in woodchucks show that the majority (70%) of animals infected with the HBeAg producing virus developed chronic infection, while woodchucks infected with the A₁₈₉₆ pre-

C mutant developed self-limited infection (Chen *et al*, 1992). A study by Raimondo *et al* (1993) confirmed these results in children.

Brunetto *et al* (1991) noted that loss of immunotolerance and seroconversion to anti-HBe was often associated with the appearance of an HBeAg-minus virus. Tarazawa *et al* (1991) highlighted two cases of fulminant hepatitis infection that was the outcome of babies born to anti-HBe-positive mothers who had mixed populations of the HBeAg producing virus and a HBeAg-negative mutant.

6. Genetic variability

Comparative sequence analysis of HBV DNA from a single chronic carrier has shown that the HBV core gene has a substitution rate of 2.2×10^{-5} /nucleotide/year. This figure is approximately 100-fold higher than that for herpesviruses, but 10-fold lower than for retroviruses (Okamoto *et al*, 1987) HBV, like other viruses with high mutation rates, can theoretically exploit variability as a strategy to evade the immune system within an infected host. However, due to its compact genetic organisation one would assume only a few of the mutations that arise during replication are admissible to the pool of viruses for selection. Despite this, patients with chronic and fulminant hepatitis have shown great diversity in the viruses cocirculating, with some regions of the genome being poorly conserved between different isolates (Kaneko *et al*, 1989).

6.1 Pre-C

The observation that some patients in the Mediterranean and the Far East had detectable HBV DNA in their serum yet were negative for HBeAg (Bonino *et al*, 1981, Lok *et al*, 1984), led to the discovery of a variant of HBV that was unable to produce HBeAg (Carman *et al*, 1989, Brunetto *et al*, 1989a). A single base substitution, of a guanine (G) to an adenine

(A), was found to occur at nucleotide 1896 (A_{1896} : codon 28 of the pre-C region), introducing a translational stop codon (Figure 1.9).

Changes in the pre-C region can theoretically lead to ten potential stop codon mutants. A_{1896} is by far the most common. The area, from nucleotide positions 1896 to 1899, is a likely hotspot for reverse transcription errors due to the four guanosine residues found together here. G to A mutations at nucleotides 1899 (Carman *et al*, 1989; Brunetto *et al*, 1989a; Fiordalisi *et al*, 1990) and 1898 (Carman *et al*, 1992) have been described but they do not affect translation of HBeAg. Other mutations have been found including loss of the pre-C initiation codon, (Tong *et al*, 1990) a G to A at nucleotide 1897 which also introduces a stop codon (Protzer *et al*, 1996) and nucleotide insertions and deletions resulting in frameshifts (Okamoto *et al*, 1990).

As this area is overlapped by ϵ , the 3' end of HBx and DR2, the absence of many potential stop codons in nature could result from reduced virus viability. Seven of these mutants have been constructed and tested for replication capacity (Tong *et al*, 1992). Five were able to replicate but at levels much lower than that of the prevalent stop codon mutant (A_{1896}) and the other two were completely defective in replication.

There has been some evidence that the genotype of HBV may influence the rate of occurrence of pre-C mutants. Li *et al* (1993) showed that genotype A was rarely A_{1896} . This could be explained by detrimental base-pairing in ϵ , whereas those with genotype D frequently selected A_{1896} (Rodriguez-Frias *et al*, 1995). The distribution of pre-C variants can probably be explained by their effects on ϵ .

It is clear that pre-C variants can be selected at or after seroconversion to anti-HBe, both in acute (Carman *et al*, 1991a) and chronic (Okamoto *et al*,

1990) infection, and during interferon therapy (Gunther *et al*, 1992; Fattovich *et al*, 1995).

Pre-C variants of HBV have not been shown unequivocally to be related causally with disease severity. For example, Liang *et al* (1991) and Kosaka *et al* (1991) have shown that patients with fulminant hepatitis and their infective anti-HBe-positive contacts are infected with an almost identical HBV isolate containing A₁₈₉₆, yet others have shown either that HBeAg positive cases are infected with HBeAg producing strains (Carman *et al*, 1991*b*) or even that the association in anti-HBe-positive patients is tenuous (Laskus *et al*, 1993). Similarly, pre-C mutants are found in chronic carriers who progress to severe hepatitis or have the infection resolve after seroconversion to anti-HBe (Okamoto *et al*, 1990; Tur-Kaspa *et al*, 1992). However, Lindh *et al* (1996), who examined liver damage histologically, found no association of A₁₈₉₆ with severe liver damage. On the contrary, HBcAg-negative patients with A₁₈₉₆ had less inflammation and fibrosis than those with G₁₈₉₆. This suggests a role for HBcAg in the necroinflammatory process, as withdrawal of HBcAg correlates with less liver damage. Transfection studies have failed to show differences in replication or encapsidation between strains with and without A₁₈₉₆ (Hasegawa *et al*, 1994).

Previously, in hepatomas, the cysteine residue at codon 23 in pre-C has been documented to be highly variable (Clementi *et al*, 1993, Manzin *et al*, 1992). A study by Hosono *et al* (1995) on HBV derived sequences from Taiwanese hepatomas failed to find any mutations at this position. Given that the earlier studies were from Italian hepatomas mutations at this amino acid may be related to the ethnic origin of the host.

Differences in the biology of HBV isolates have been demonstrated. For example, a study by Hasegawa *et al* (1994), showed that a virus taken from a patient with fulminant hepatitis exhibited an enhanced core particle

formation and more efficient encapsidation than the virus from a control case; mutagenesis studies confirmed that this was not due to A₁₈₉₆. Perhaps mutations in the X gene, enhancer regions, or the core promoter are important in this regard, but there is no firm evidence available to support this.

A recent case pair (Mphahlele *et al*, submitted) demonstrates that A₁₈₉₆ strains are independently transmissible and can lead in adults to acute hepatitis with viral clearance (Figure 4), with the appearance of anti-HBe (even though HBeAg was never produced).

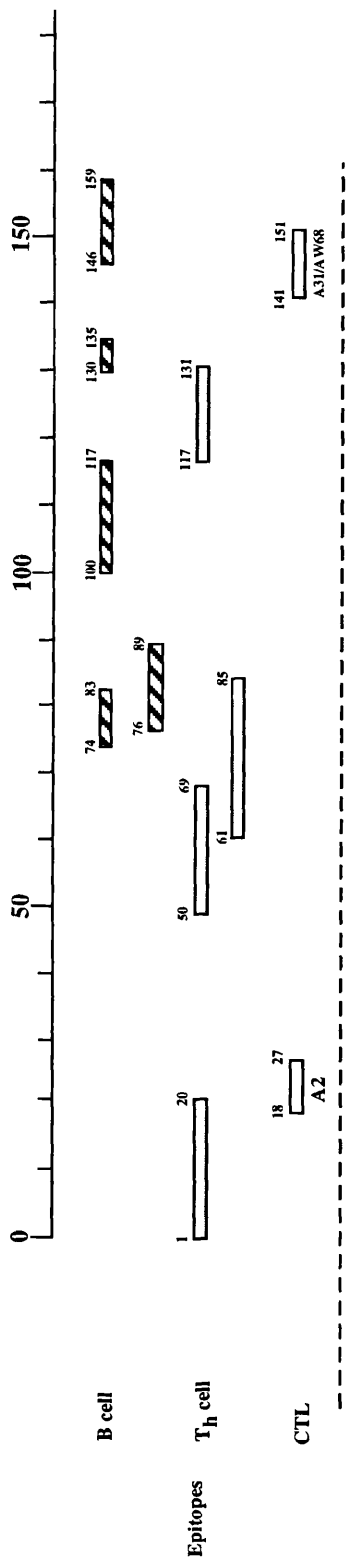
6.2 Core

HBcAg contains B-cell, Th and CTL epitopes (described in Chapter 1, section 5.2.2). Studies in recent years have attempted to show that the distribution and nonsynonymous nature of mutations observed in HBcAg is a nonrandom event and thus provides evidence of immune selection. These results will be discussed in greater detail.

Due to the different ways of analysing HBcAg mutations that have been employed by the various groups in this field, attempting to make direct comparisons is difficult and may be misleading. However, I have endeavoured to collate the current data (Figures 1.8 and 1.10).

Two cross-sectional studies by Rodriguez-Frias *et al* (1995) and Carman *et al* (1995) observed an association between amino acid substitution in HBcAg and the presence of a pre-C stop codon. This association was also identified by Akarca and Lok (1995). Rodriguez-Frias *et al*, (1995) identified two areas of high variability, amino acids 11 to 27 and 74 to 83. Amino acids 1 to 27 have been identified as a HLA-A2 epitope (Bertoletti *et al*, 1991; Penna *et al*, 1991) and it is known that alterations at amino acid positions 21 and 27 can inhibit CTL response in chronically infected

Figure 1.9 Variability in HBcAg observed in individuals with chronic HBV infection, excluding that in Figure 1.10. The 'ruler' represents amino acid positions of HBcAg. Epitopes are represented as: hatched boxes (B-cell epitopes at aa positions 74-83, 76-89, 100-117, 130-135 and 146-159); shaded boxes (Th epitopes at aa 1-20, 50-69 61-85 and 117-131) and open boxes (CTL epitopes at aa 18-27 and 141-151). Single amino acid codes are given in Appendix 2.



Areas of greatest mutation in patients

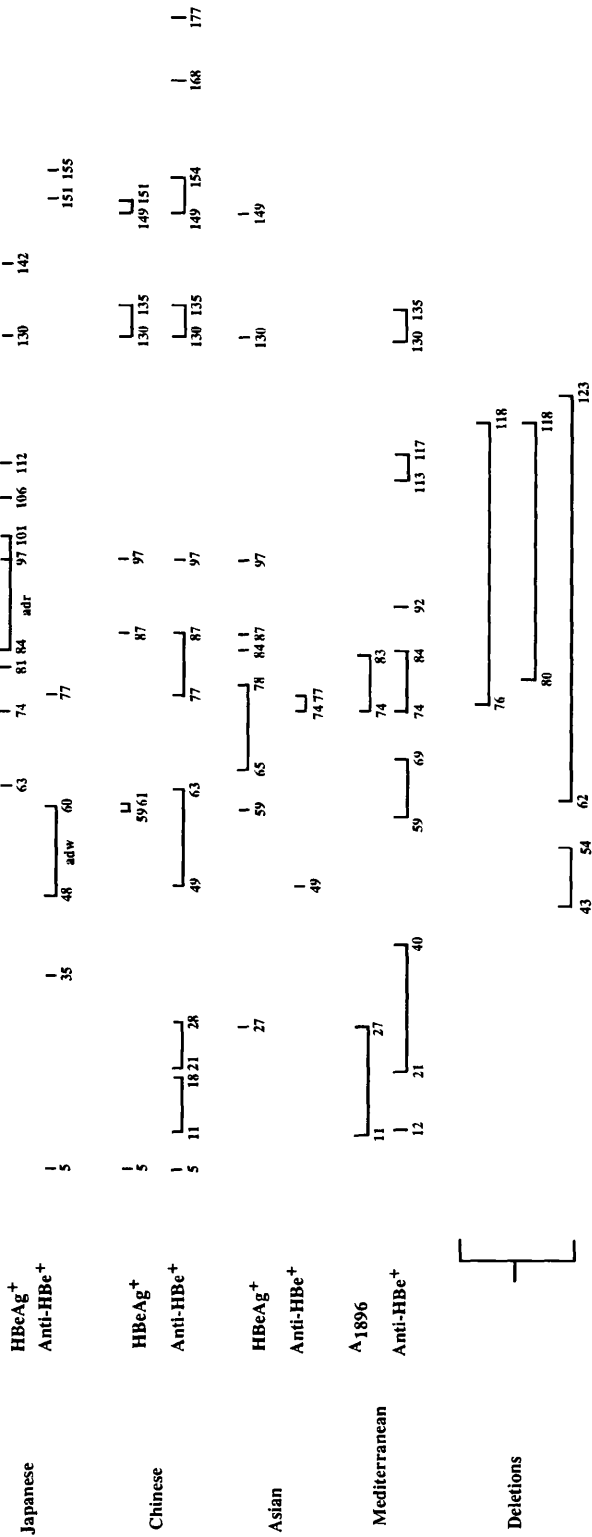
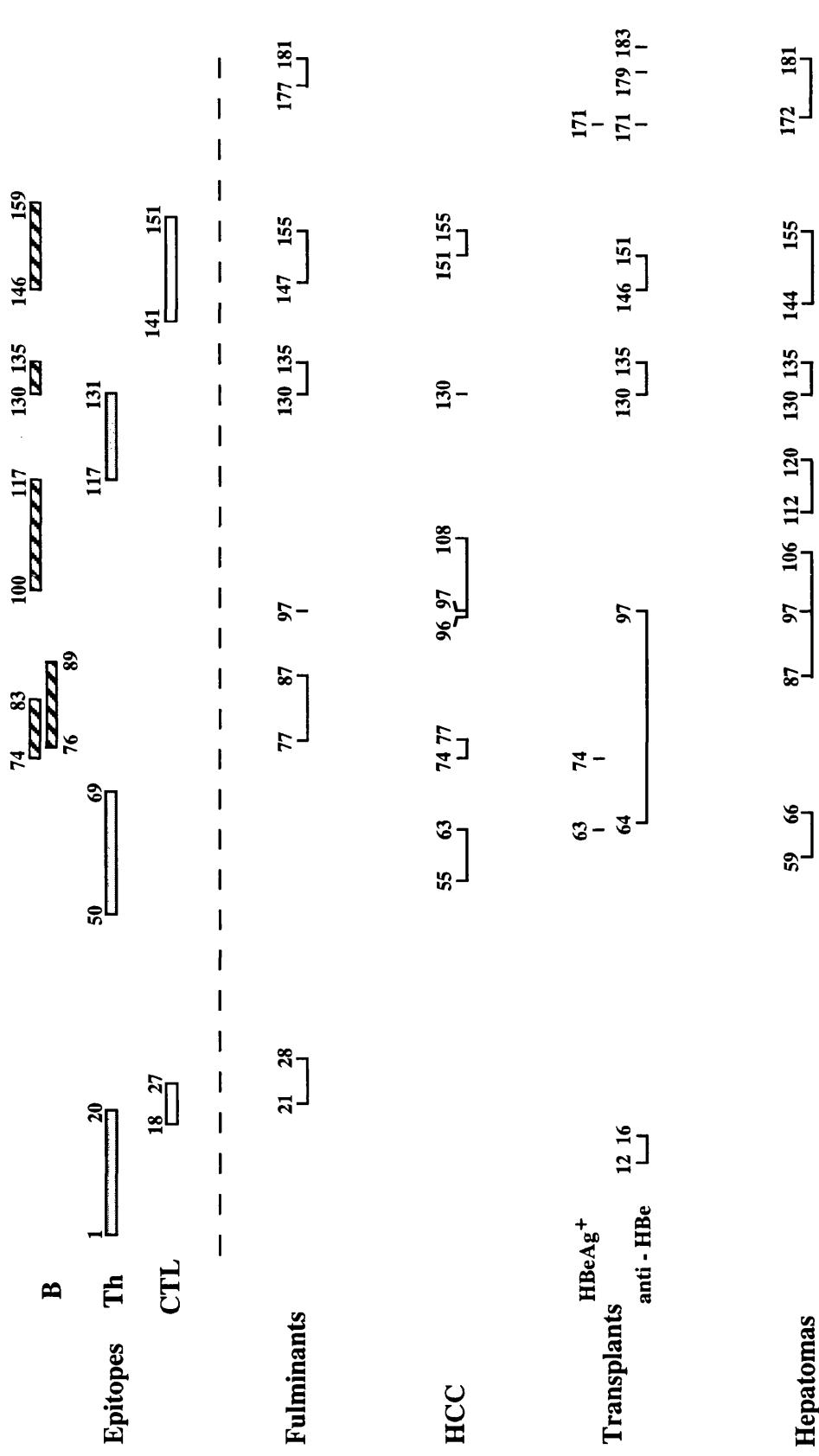
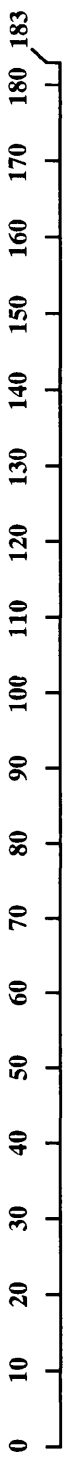


Figure 1.10 Variability in HBcAg in patients with severe hepatitis. The 'ruler' represents aa positions of HBcAg. Epitopes are represented as: hatched boxes (B-cell epitopes at aa positions 74-83, 76-89, 100-117, 130-135 and 146-159); shaded boxes (Th epitopes at aa 1-20, 50-69 61-85 and 117-131) and open boxes (CTL epitopes at aa 18-27 and 141-151). Single amino acid codes are given in Appendix 2.



people (Bertoletti *et al*, 1994). Mutations at these positions were observed in a few of their patients although no details on the HLA-type of the patients were given. The authors related the variation seen in the region amino acids 74 to 83 to genotype background (mutations in this area were only seen in those with genotype D) rather than to an escape mechanism. However, this area is the major B-cell epitope (Salfelds *et al*, 1989) and variation here has been described by two other groups (Carman *et al*, 1995; McMillan *et al*, 1996). Clusters of amino acid substitutions in other areas of HBcAg have been described, in particular at amino acids 84 to 101 and 147 to 155 (Chuang *et al*, 1993; Ehata *et al*, 1992). These patients were from Asia and the different clusters may be related either to the viral strain circulating in these areas or to the genetic (HLA) background of the patients.

Higher rates of variation in epitopic areas compared to non-epitopic areas have been described (Carman *et al*, 1995; Akarca and Lok, 1995). Primarily, they were observed in B and Th cell epitopes with little variation observed in the two described CTL epitopes. This result could be attributed to the lack of HLA-A2, -Aw68 types in the patients studied. However, neither of the groups examined the HLA type for all of their patients this has to be considered a possibility. Carman *et al* (1995) did give details of those patients who were HLA-A2, 10 out of 28 were HLA-A2 but only two of these selected any change in the HLA-A2 epitope. Other HLA types were not examined in this study. Of the patients in the Akarca and Lok (1995) study who did select mutations in this area, 77% of them were at amino acid 21 or 27. One selected a change (serine to asparagine) which has been shown to inhibit CTL recognition of the standard epitope. This change was not observed in any of the patients in the study by Carman *et al* (1995). Three patients (from the Akarca and Lok study) selected an isoleucine to valine substitution at amino acid 27; valine at this position has been shown to have a 10-fold increased binding when compared to isoleucine. As these latter three patients seroconverted

to anti-HBe, it may be that this substitution enhanced CTL recognition and thus HBe seroconversion. The substitutions observed by Carman *et al* (1995) at this position, valine to isoleucine, were only seen in patients who were anti-HBe with active disease. However, this analysis (by Carman *et al* [1995]) was a cross-sectional study and therefore the observed changes may not be true amino acid substitutions.

Hosono *et al* (1995) examined HBcAg sequences derived from the liver of hepatoma patients. Integrated DNAs contained fewer mutations than replicating HBV DNA present in the hepatomas, probably as a result of the tumour no longer being under selection pressure. There was a high rate of amino acid substitution within the Th epitope (amino acids 1 to 20) at positions 5 and 13. Mutations at these positions have also been described in HBeAg and anti-HBe-positive chronic patients (Asahina *et al*, 1996; Bozkaya *et al*, 1996; Lee *et al*, 1996). Hosono *et al* (1995) found these mutations to be mutually exclusive. This was also observed by Lee *et al* (1996). It is feasible that only one mutation is required to affect recognition or binding of the HLA class II site.

Until recently, most of the studies on variation in HBcAg have been cross-sectional, but now a few groups have analysed longitudinal samples from individuals, giving a more authentic picture of HBcAg selection. Asahina *et al* (1996) sequenced sequential whole genomes from patients with chronic hepatitis B before and after an acute exacerbation. The field of HBV variation has been eagerly anticipating a study of this nature, as this gives an all round impression of selection events in the genome and the effects on the different genes. However, the failing in this study was that only three patients were studied. To show statistical significance much greater number of genomes would have to be studied. The findings of this study showed mutations mainly located in the surface and pre-C/C genes of these patients. The common A₁₈₉₆ variant was selected in two of the

three patients and HBcAg mutations also present in two were not particularly associated with recognised epitopes.

Hur *et al* (1996) examined sequential samples from five patients, of Oriental origin, with chronic hepatitis. Gradual changes were observed in pre-C and HBcAg with a cluster of mutations around amino acids 84 to 97 detected in all cases. Although this area is not a described HBcAg epitope, it has been cited as an area of high mutation by other groups studying patients from the same geographical area. Thus it may be an epitope under immune pressure that is yet to be described.

Bozkaya *et al* (1996) analysed the sequential HBcAg sequences of 55 chronic hepatitis cases, the largest study published to date. Results from this study agreed with the findings of the cross-sectional analysis by Carman *et al* (1995), that the rate of HBcAg variation is lower in those who are HBeAg-positive with normal ALTs compared to those with raised ALTs or who are anti-HBe-positive.

The rates of nucleotide and amino acid substitutions observed were similar in interferon treated versus untreated patients and in responders versus non-responders. This is contrary to the findings of Naoumov *et al* (1995), but in accordance with Fattovich *et al* (1995).

The mutations described in the study by Bozkaya *et al* (1996), were associated with aminotransferase flares. However, the similar nature (rate and location) of the mutations between HBeAg-positives with raised ALTs and those who cleared HBeAg imply that these mutations were not responsible for seroconversion to anti-HBe. As half of the nucleotide changes resulted in amino acid changes, it is likely that immune pressure was influencing their selection.

Okumura *et al* (1996) sequenced HBcAg before, during and after an exacerbation of hepatitis, focusing on the central region of HBcAg. Their results indicate that there is a higher rate of mutation during the exacerbation compared with before and after the peak of ALT, concurring with the results of Bozkaya *et al* (1996). Changes were not observed in asymptomatic HBV carriers without flares of hepatitis during the same time frame. These results indicate further evidence for an unidentified epitope(s) in the central HBcAg region.

Some specific amino acid substitutions are notable but remain unexplained. For example, a threonine to serine at amino acid 12 is only found after selection A₁₈₉₆ in Mediterranean patients (Carman *et al*, 1995). Amino acid 12 is within a Th epitope, spanning amino acids 1 to 20, and the possibility that this mutation leads to escape from Th cell responses has been raised (Carman *et al*, 1995).

Several studies have shown selection of isoleucine to leucine at amino acid 97 (Horikita *et al*, 1994; Bozkaya *et al*, 1996; Hur *et al*, 1996; Okumura *et al*, 1996), the significance of this mutation remains unclear.

A longitudinal study by Naoumov *et al* (1995) indicated that specific mutations in HBcAg can interfere with T-cell function, particularly in patients with advanced chronic HBV infection and poor response to interferon (IFN). Patients who failed or relapsed after an initial response to IFN had more missense mutations before treatment, which can probably be accounted for by a longer duration of infection. It has previously been documented that HBeAg and HBsAg seroconversion occurs more frequently in patients who have had a shorter duration of infection (Perillo *et al*, 1990). Those that did respond not only had fewer missense mutations but they coexisted with wild-type sequences. One of the patients who failed to respond to treatment had mutations at amino acids 21 and 27. Mutations at these positions have been shown to act as

antagonists to the T-cell receptor (Bertoletti *et al*, 1994) and this perhaps indicates a possible mechanism for the failure to respond to IFN.

Deletions in HBcAg have been described in patients with chronic hepatitis (Wakita *et al*, 1991; Ackrill *et al*, 1993; Akarca and Lok, 1995) and in asymptomatic carriers (Akarca and Lok, 1995). Usually these deletions are in frame, thus truncated HBcAg is produced. However, such proteins are unable, on their own, to assemble into core particles (D. Williams, PhD thesis, University of Glasgow, 1997) and are therefore viral dead ends, but they may play a role in down-regulation of viral replication. Most of the deletions described are situated around the central region of HBcAg (between amino acids 70 and 120), the area which contains major B and Th cell epitopes (Figure 1.8).

The clinical significance of these defective HBcAg remains uncertain. Core deletions in patients with chronic hepatitis are of low prevalence but do appear to be maintained (Akarca and Lok, 1995). Frameshift deletions, resulting in early termination of HBcAg and in the loss of the first nine amino acid from polymerase, have been identified. Curiously, these viruses had a potential start codon for polymerase shortly upstream of the deletion (Akarca and Lok, 1995). A patient with an unusual serological profile, HBsAg and HBeAg positive but anti-HBc negative with severe liver damage, exhibited several frameshift deletions, varying between amino acids 39 and 130, with a HBcAg consisting of only the first 35 amino acids (Zoulim *et al*, 1996). Anti-HBc was detected intermittently at very low levels. This may be explained by full length HBcAg being present in the liver. Similar deletions were observed by Fiordalisi *et al* (1994) in patients with the same serological profile.

Interestingly, patients which exhibit a more severe form of HBV infection; such as fulminant hepatitis (Alexopoulou *et al*, 1996; Asahina *et al*, 1996 and Kaneko *et al*, 1995), those who have had liver transplants (McMillan

et al, 1996) or are suffering from HCC (Hosono *et al*, 1996), have multiple mutations in HBcAg with several documented in the C-terminus of HBcAg (Figure 1.10). Mutations in the C-terminus between amino acid 170 and 183 are seldom seen in chronic hepatitis (Figure 1.8). Unexpectedly, there are mutations described in the arginine-rich region that is involved in RNA binding (from amino acids 150 to 154). This region has many documented mutations (Akarca and Lok, 1995; Bozkaya *et al*, 1996; Asahina *et al*, 1996; Lee *et al*, 1996).

There does appear to be an association between the number of mutations in HBcAg and the outcome of disease, with the least being in HBeAg-positive individuals with minimal disease, and the most in anti-HBe-positive patients with severe hepatitis or those with fulminant hepatitis (Carman *et al*, 1995; Ehata *et al*, 1992; Chuang *et al*, 1993). However, this is not substantiated by all studies. Valliammai *et al* (1995) did not find any significant mutations in HBcAg in HBeAg-positive or anti-HBe-positive patients. However, the numbers of patients analysed in this study were very small therefore its significance is ambiguous. It is, however, unclear whether these substitutions predispose patients to progressive hepatitis or occur as a result of severe disease (perhaps mediated by immune pressure on HBcAg-expressing hepatocytes).

6.3 X gene and *cis*-acting regions

Figure 1.11 correlates variation within the X region with clinical and serological parameters

6.3.1 X gene

Deletions within the X gene have been detected in individuals from a variety of clinical backgrounds: chronic carriers with normal serological markers (Laskus *et al*, 1994; Kidd-Ljunggren *et al*, 1995), patients without normal serological markers (Liang *et al*, 1990; Uchida *et al*, 1995), and an

Figure 1.11 Variability in HBxAg and related cis-acting elements. The 'ruler' represents nucleotide positions on the hepatitis B virus genome (with numbering starting from the *Eco* R1 site). The relative positions of the X, P and C ORFs and cis-acting regions are depicted. HV=hypervariable. Single aa codes are given in Appendix 2.

anti-HBc-negative highly viraemic carrier (Repp *et al*, 1992). Deleted HBxAgS have also been found in sera from chronically infected woodchucks (Kew *et al*, 1993). Like the human virus counterpart, these deleted HBxAgS normally (if not always) co-exist with the full length form.

In their analyses of X gene sequences, Kidd-Ljunggren *et al* (1995), proposed a hypervariable area (from amino acid residues 26 to 52) and four conserved regions from residues: amino acid 13 to 25, amino acid 53 to 77, amino acid 89 to 115 and amino acid 132 to 143. One of these conserved regions (amino acid 132 to 143) has been implicated to be important for interaction of the X protein with cellular proteins (Takada and Koike, 1994) and encompasses one of the regions, from amino acids 118 to 142, found to be essential for its transactivating function. Their proposed hypervariable region overlaps the other region essential for transactivation, from amino acids 29 to 62 (Arii *et al*, 1992; Kim *et al*, 1993).

6.3.2 Core promoter and enhancers

The X gene contains important *cis*-acting elements, including DR2, the core promoter and enhancer II. The core promoter directs the transcription of the pre-C/C gene, leading to production of HBeAg, and it also has a role in the formation of pregenomic RNA. The proposed RNA polymerase binding site (nucleotide positions 1630 to 1636) also lies within the core promoter (Corden *et al*, 1980). Such an important control mechanism, one would imagine, would be fairly conserved. However, several groups have described variability in this region (Figure 1.11). A study on chronic HBV carriers with anti-HBe (Okamoto *et al*, 1994), revealed a large number of patients with mutations at or around the proposed RNA polymerase binding site but the transcription initiation site, which lies 25 nucleotides downstream, was highly conserved. 82% were found to have core promoter mutations, had an A to T change at nucleotide position 1762 and/or a G to A change at nucleotide position

1764, presenting a consensus sequence of AGG at these positions. In two of the chronic carriers studied, the core promoter mutated as they seroconverted to anti-HBe without detectable simultaneous mutations in the pre-C region. The authors proposed that such mutations would lead to a HBeAg negative phenotype through regulation of transcription. This was their explanation for the lack of pre-C mutants. In the study by Kidd-Ljunggren *et al* (1995), 90% of the HBeAg positive/anti-HBe negative patients had conserved sequences at nucleotide positions 1762 and 1764 and 4/5 HBeAg negative/anti-HBe positive patients had one or more mutations at these positions. This apparently supports the correlation between HBeAg/anti-HBe status and the presence or absence of mutations at these two positions in the core promoter. However, no information on their pre-C sequence was given in those patients who were HBeAg negative and had mutations at nucleotide positions 1762 and/or 1764. Mutations at nucleotide positions 1762 and 1764 have been described in people with fulminant hepatitis (Ogata *et al*, 1993; Hasegawa *et al*, 1994; Kaneko *et al*, 1995), chronic hepatitis, with or without anti-HBe, (Nishizono *et al*, 1995, Kidd-Ljunggren *et al*, 1996 and McMillan *et al*, 1995) in individuals who have serologically cleared HBV (Blum *et al*, 1991) but not in HBeAg-positive healthy carriers (Nishizono *et al*, 1995). Thus nucleotides 1762 and 1764 appear to be mutation hotspots.

Nishizono *et al* (1995) investigated the effect of mutations at nucleotide positions 1762 and 1764 on promoter and enhancer activities and found that they were not significantly different from wild type. The significance of mutations in the core promoter area has yet to be fully evaluated as similar point mutations and deletions in symptomatic (Kidd-Ljunggren *et al*, 1995) and non-symptomatic (Horikita *et al*, 1994), chronic HBV carriers in the core promoter have been described.

Another variant that occurs frequently is a T to C at nucleotide position 1727, which lies within the core upstream regulatory sequence. It has

been observed in fulminants (Repp *et al*, 1992; Alexopoulou *et al*, 1996) and in chronic carriers (Nishizono *et al*, 1995) but not in asymptomatic HBeAg-positive carriers (Nishizono *et al*, 1995), or those with acute HBV infection (Kaneko *et al*, 1995).

Mutations within enhancer I have been described in fulminants (Alexopoulou *et al*, 1996), and in a patient without normal serological markers (Uchida *et al*, 1995).

As the X region is involved in essential functions controlling viral replication mutations in this region could affect transcription and replication. As mentioned earlier the most common point mutations are T₁₇₆₂ and A₁₇₆₄, though it has not been shown to have a significant effect on core promoter and enhancer II functions (Nishizono *et al*, 1995). The techniques the study used could not distinguish between transcriptional levels of pre-C mRNA and pregenome RNA, thus unequivocal conclusions on protein expression could not be attained.

Baumert *et al* (1996) cloned and sequenced an HBV genome that was associated with an outbreak of fulminant hepatitis. The virus contained many mutations in all regions of the genome and was functionally characterised by more efficient encapsidation of the pregenomic RNA, leading to elevated levels of replication. Two adjacent point mutations in the core promoter, C to T at nucleotide position 1768 and T to A at 1770, were identified and investigated. These two mutations led to enhanced encapsidation and a high level of replication when compared with the wild type virus. Both these mutations lie outwith the encapsidation signal thus suggesting a *trans*-acting role for the core promoter in encapsidation and replication.

The possible effects of mutations in the X gene region were not appreciated at the time work began for this thesis. Therefore, no sequencing of this area was undertaken.

6.4 The role of CTL in genetic variability

CTL play an essential role in the control of virus infection. Host cell invasion is signalled to CTL by HLA class I molecules which bind short peptide fragments generated by the cytosolic processing of endogenously synthesised viral proteins and bring them to the surface of infected cells. Interaction of the T-cell receptor (TCR) with the peptide/HLA class I molecule complex leads to activation of the T-cell lytic function. Some of the amino acid residues of the peptide are crucial for binding to HLA molecules, normally amino acid 2 and amino acid 7, whereas the amino acid located in the central region are accessible to TCR recognition (Matsumura *et al*, 1992; Madden *et al*, 1993). One or two of these internal residues are usually crucial for TCR interaction since recognition of the peptide can be totally abolished by conservative substitutions at these positions (Evavold *et al*, 1992).

Substitution of TCR contact sites can also influence the CTL response by creating analogue peptides that can still interact with the TCR but may not be able to deliver a full stimulatory signal (Jameson and Bevan, 1995), thus inducing anergy (Sloan-Lancaster *et al*, 1993) or acting as partial agonists or antagonists of the TCR (Jameson *et al*, 1993; Racioppi *et al*, 1993).

Due to the molecular requirements for antigen recognition by T cells, it is conceivable that viral mutations that affect HLA binding or TCR contact sites might abolish viral recognition by destroying CTL epitopes or by inhibiting CTL function. It has also been reported that residues flanking

the immunogenic sequence could alter processing or transport of the viral peptide thus affecting its presentation (Couillin *et al*, 1994).

Experiments carried out in a transgenic mouse model of HBV replication indicate that CTL can cause the clearance of intracellular virus not only by lysing liver cells expressing HBV antigens but also by suppressing HBV gene expression without liver cell destruction (Guidotti *et al*, 1994). This effect is mediated by cytokines, principally IFN- γ and TNF- α , presenting the possibility of a more effective mechanism for HBV to escape immune surveillance, as a single mutation within the cytokine responsive regions of the HBV genome would abrogate the effect of the entire CTL population instead of abrogating the effect of the CTL response against individual epitopes.

Although no direct evidence has been documented, the above are possible strategies which HBV could adopt to evade immune surveillance and persist within the infected host. Escape from immunosurveillance by CTL targets is a biological possibility, as shown for lymphocytic choriomeningitis virus (Moskophidis and Zinkernagel, 1995) and HIV (Klenerman *et al*, 1996). Could this be happening in HBV?

Several studies have identified mutations scattered throughout the sequences of different HBV antigens, some of which appear to be within regions recognised by HBV-specific T cells (Akarca and Lok, 1995; Bertoletti *et al*, 1994; Rodriguez-Frais *et al*, 1995). However, in most of these studies the HLA haplotype of the infected patients was not determined, consequently, these findings do not prove that the mutations, shown to arise, emerged as a result of selective pressure exerted by T cells .

In patients with acute HBV infection who normally develop a strong polyclonal CTL response against multiple epitopes in the viral envelope,

nucleocapsid and polymerase (Nayersina *et al*, 1993), the likelihood of mutant selection by the CTL response is theoretically low. A number of CTL epitopes have been described within viral sequences that are critical for specific viral functions. For example, the nuclear localisation and genome encapsidation signals of HBcAg span amino acid residues 141 to 151 which also contain the CTL epitope recognised by HLA-A31 and HLA-Aw68-restricted CTL (Missale *et al*, 1993). Similarly, an important topogenic sequence in the transmembrane domain of HBsAg overlaps an HLA-A2-restricted CTL epitope located between amino acid residues 250 and 269 (Nayersina *et al*, 1993). Mutations in these epitopes might be lethal for the virus and therefore incompatible with escape from this arm of the immune system.

The multispecificity of the CTL response suggests that abrogation of the response against the epitope where the mutation arose should not render the infected cell invisible to CTL if other epitopes are simultaneously expressed on the same cell and other CTL specific for them can reach the infected cell.

In contrast to acute self-limited disease, the CTL response in chronic HBV is usually weak. If this response were also mono- or oligo-specific, the probability of mutant virus selection at this stage of infection would be greatly enhanced. This would depend on CTL responses being vigorous enough to exert selection pressure. In such cases mutations within critical epitopes would protect the infected cells from lysis by CTL. This is supported from results by a study by Bertoletti *et al* (1994) in which two patients with chronic HBV showed a strong HLA-A2-restricted CTL response narrowly focused on the amino acid residues 18 to 27 of HBcAg but failed to respond to any of the other HLA-A2-restricted CTL epitopes in HBV that are normally immunogenic in acutely infected individuals. On sequencing the infecting virus, the patients appeared to be infected by a homogenous population of variant virus carrying mutations within the

HBcAg epitope, from amino acids 18 to 27, that could reduce HLA binding and TCR recognition of the epitope. These variant epitopes were not recognised by the dominant CTL population present, which were only able to recognise the wildtype (or prototype) epitope. The variant epitopes appeared unable to induce a new population of CTL specific for themselves and hence were poorly antigenic. In addition, they were able to act as TCR antagonists by inhibiting the response to the prototype epitope when both variant and prototype peptides were simultaneously expressed on the surface of the same target cell (Bertoletti *et al*, 1994).

The world-wide prevalence of HLA-A2, A31 and -Aw68 together is approximately 65%.

These results reveal the scenario of antagonism, whereby the antagonism is protecting the infected cell from lysis by CTL specific for the prototype epitope thereby giving the variant virus time to replicate and infect other cells. However, this is dependent on whether cytokines which are being actively released by liver infiltrating cells, are unable to clear the variant virus before it has a chance to spread. At the same time the wild type virus will also spread but will be eliminated by the activated wild type-specific CTL. If the variant virus is strongly immunogenic it will prime a new CTL response which, if sufficiently effective, could clear the mutant virus. If, however, the new epitope is poorly immunogenic, as was found in the above two patients, the CTL response would be unable to clear the 'new' virus and the virus would persist. Such an outcome could be advantageous for both the virus and the host. It would allow survival of the virus and, if the mutations cause complete lack of recognition by CTL, then there would be no further liver cell damage and hence resolution of disease to a healthy carrier state.

The criticisms of the above study are that they failed to demonstrate the conversion of the wild type to the mutant sequence. Therefore, not

unequivocally demonstrating CTL escape and responses against other HLA-restricted epitopes were not measured and therefore cannot be ruled out. Yet it does illustrate how a mutant virus can persist in the chronically infected host despite a vigorous CTL response which is ineffective against it. Both these patients fulfil two crucial conditions for viral escape, a response against the prototype epitope is present and adequately strong to exert a selective pressure and the CTL repertoire is narrow and focused.

A study by Rehermann *et al* (1995) examined the 'frequency' of CTL escape mutants in patients with chronic HBV infection. They analysed eight separate HLA-A2-restricted epitopes, defined in patients with acute HBV infection, in twelve patients chronically infected with HBV. Four of the patients gave weak responses, while the other eight were totally undetectable. No sequence changes in the epitopes, differing from the prototype sequence, were found in any of the patients. Thus suggesting that CTL escape is not a common mechanism for HBV to evade immune recognition.

From the data currently available it appears unlikely that HBV variability plays an important role in viral persistence by evasion of CTL recognition. To address this possibility completely, further studies using improved CTL detection strategies that permit scanning of the entire HBV-specific CTL repertoire in infected patients should be performed in order to evaluate the frequency of CTL escape in chronically infected patients and their importance to viral persistence.

A much more effective strategy of escape than mutations within individual epitopes would be mutations within cytokine response elements. If cytokines prove to exert as strong an antiviral effect in natural HBV infection as is observed in the transgenic mouse model, this is an area

which may prove fruitful in explaining viral persistence and ongoing disease.

Whether these defective CTL responses, in chronic HBV patients, are due to an actual lesion of the hosts immune system or to viral mutations affecting immune surveillance and thereby virus escape, still remain open issues.

6.5 Interferon therapy and pre-C/C variability

A study by Naoumov *et al* (1995), addressed the question of whether variations in the pre-C/C gene affect the response to alpha interferon. They examined the sequence of the pre-C/C region of 12 patients before, during and after treatment with interferon. Of the 7 (2 of whom had cirrhosis) who responded to treatment (23 samples), 24 missense mutations were noted, with no significant changes observed after seroconversion. Of the 5 (all had cirrhosis) who failed to respond or reactivated after treatment was withdrawn (also representing 23 samples), 141 missense mutations were identified. This was shown to be statistically significant ($p=0.001$). Multiple variations in the pre-C/C region persisted during therapy and new mutations appeared with the relapse of hepatitis. Interestingly, the HLA-A2 epitope in HBcAg, from amino acids 21 to 27, was only mutated in the non-responders/reactivators. No mutations were observed in this area for those who responded. Substitutions in this region are known to diminish HLA-A2 restricted cytotoxic T-cell function, thus showing specific mutations in HBcAg that probably interfere with T-cell function occur frequently in patients with advanced chronic hepatitis and this may affect such individuals response to interferon.

A similar study by Fattovich *et al* (1995) showed that those who respond to interferon treatment were more likely to have mutations within the Th epitope, amino acids 50 to 69, and there was no correlation with outcome

to therapy and presence or absence of a pre-C stop codon. However, a study by Zhang *et al* (1996) investigated the influence of genotype and pre-C variability on response to interferon in anti-HBe chronic patients. The overall response rate of 49% was normal for this group of patients (Brunetto *et al*, 1989b and 1993). A higher response rate was observed in those with genotype A or who had wild type pre-C as compared to those infected with genotype D/E or who had A₁₈₉₆. This was independent of both the level of viral replication antecedent to therapy and interferon doses, thus raising the issue that prior characterisation of pre-C sequence or genotyping of virus present may help to predict the response to interferon in HBeAg negative patients.

7. Aims of thesis

The main aim of this thesis was to examine whether mutations in HBcAg, in those individuals who seroconvert to anti-HBe yet still have active disease, correlate with disease. Longitudinal studies would be carried out to examine variation in HBcAg in anti-HBe-positive patients. Mutations that were observed would be correlated with the known Th and B-cell epitopes. As it was envisaged that the HLA type of the patients would not be known, variation in CTL epitopes would not be addressed. The understanding that these mutations may represent immune escape by HBV would be tested.

Groups of patients from two geographical areas would be studied. The distribution of HBcAg mutations would be compared between these areas.

Chapter 2

Materials

2.1 Bacterial strains (*E.coli*)

Strain DH5 α

2.2 Bacterial growth medium

Luria-Bertani Liquid Medium (LB)

NaCl	10g/l
Bactopeptone	10g/l
Yeast extract	5g/l

Luria-Bertani Solid Medium

As for liquid plus 15g Bacto-agar. pH was adjusted to 7.0 prior to sterilisation. Before use LB media was supplemented with 100 μ g/ml ampicillin.

2.3 Chemicals

Acetic acid	Rathburn Chemicals Ltd, Walkerburn, Scotland
Acetonitrile	Rathburn Chemicals Ltd, Walkerburn, Scotland.

Agarose	Boehringer Mannheim, Lewes, E. Sussex.
Ammonia persulfate (APS)	Bio-Rad Laboratories, Richmond, California, USA.
Ampicillin (PenBritin)	SmithklineBeecham, Pharmaceuticals Ltd. Dublin, Ireland.
Ammonium solution	BDH Analar, Poole, UK.
t-amyl alcohol (TAA)	Rathburn Chemicals Ltd, Walkerburn, Scotland.
Anisole	Aldrich Chemical Company, Gillingham, Dorset, UK.
Boric acid	BDH Analar, Poole, UK.
Bromophenol blue	Sigma Chemical Company, Poole, UK.
Citric acid	BDH Analar, Poole, UK.
Deoxynucleoside triphosphates	Pharmacia LKB, Uppsala, Sweden.
Diethyl ether	Rathburn Chemicals Ltd, Walkerburn, Scotland
Dimethylformamide (DMF)	Rathburn Chemicals Ltd, Walkerburn, Scotland.
DIPEA	Aldrich Chemical Company, Gillingham, Dorset, UK.
EDTA	BDH Analar, Poole, UK.
Ethane di-thiol (EDT)	Aldrich Chemical Company, Gillingham, Dorset, UK.

L-Glutamine	Life Technologies Ltd., Paisley, UK.
Hydroxy benzotriazole (HOBT)	Novabiochem (UK) Ltd, Nottingham, UK.
Hydrochloric acid	BDH Analar, Poole, UK.
Isopropylthiogalactoside (IPTG)	Life technologies Ltd., Paisley, UK.
Lectin-Phytohaemmagglutinin	Sigma Chemical Company, Poole, UK.
Methanol, HPLC grade	Rathburn Chemicals Ltd, Walkerburn, Scotland.
Molecular biology grade water	BDH Analar, Poole, UK.
Penicillin/Streptomycin	Life Technologies Ltd., Paisley, UK.
Phenol	Sigma Chemical Company, Poole, UK.
Phenol/chloroform	Camlab Ltd., Cambridge, UK.
Piperidine	Rathburn Chemicals Ltd, Walkerburn, Scotland.
Proteinase K	Boehringer Mannheim, Lewes, E. Sussex, UK.
N,N'-methylene bisacrylamide	BDH Analar, Poole, UK.
NaOAc	BDH Analar, Poole, UK.
NaCl	BDH Analar, Poole, UK.
Na ₂ HPO ₄	BDH Analar, Poole, UK.

NaH ₂ PO ₄	BDH Analar, Poole, UK.
Repelcote	BDH Analar, Poole, UK.
SDS	BDH Analar, Poole, UK.
Sucrose	BDH Analar, Poole, UK.
N,N,N,N-tetramethylethylenediamine (TEMED)	Bio-Rad Laboratories, Richmond, California, USA.
Transfer RNA (tRNA)	Life Technologies Ltd., Paisley, UK.
Trifluoroacetic Acid (TFA)	Rathburn Chemicals Ltd, Walkerburn, Scotland.
Thioanisole	Aldrich Chemical Company, Gillingham, Dorset, UK.
Tri-isopropyl silane (TIPS)	Aldrich Chemical Company, Gillingham, Dorset, UK.
Tris base	Boehringer Mannheim, Lewes, E. Sussex, UK.
Fmoc ₈ K7 resin	Peptide and Protein Research, Exeter, Devon, UK.
(Fmoc Gly ₄)-Lys ₂ -Lys- Ala-Diamcryn KA	Peptide and Protein Research, Exeter, Devon, UK.
Ultrosyn C	Peptide and Protein Research, Exeter, Devon, UK.

2.4 Radiochemicals

All radiochemicals were purchased from Amersham International Ltd., Maidstone, UK.

The specific activities were 37Bq/mmol for [$\alpha^{35}\text{S}$]dATP and 60 Ci/mmol for [methyl- ^3H]Thymidine.

2.5 Enzymes

Restriction enzymes	Boehringer Mannheim, Lewes, E. Sussex, UK.
T4 DNA ligase	Boehringer Mannheim, Lewes, E. Sussex, UK..
Sequenase TM DNA polymerase (Version 2.0)	United States Biochemicals, Ohio, USA.
Taq TM DNA polymerase	Life Technologies Ltd., Paisley, UK.

2.6 Human sera

Negative (control) serum was donated by various members of lab 109 over the course of this thesis.

Test sera were taken in the normal course of treatment and had been stored for many years, therefore no patient consent was required. Sera were kindly supplied by Prof. A Lok (Chapter 5) and Prof. S. Hadziyannis (Chapter 6).

2.7 Buffers

Binding and wash buffer	Tris-HCl, pH 7.5	10mM
	EDTA	1mM
	NaCl	2.0M
Citrate/phosphate buffer	Citric acid	0.08M
	Na ₂ HPO ₄	0.1M

Denaturing buffer	Tris.HCl, pH6.7	100mM
	SDS	4.0%
	2-mercaptoethanol	1.4M
Gel loading buffer	Sucrose	4.0g
	Bromophenol blue	2.5g
	Dissolved above in 6ml	1xTE,
	brought to a final volume of 10ml with 1xTE.	
Hepes/bicarb buffer	Hepes	11mM
	NaHCO	10mM
	Dispensed into 80ml aliquots and autoclaved before use.	
PBS-Tween 20 solution x 10	NaCl	1.45M
	Na ₂ HPO ₄	0.075M
	NaH ₂ PO ₄	0.028M
	Glycerol	0.5%
	pH adjusted to 7.2 with NaOH or HCl.	
10xPBS	NaCl	255g
	Na ₂ HPO ₄	32.1g
	NaH ₂ PO ₄	11.1g
	Dissolved in 3l water.	
Reagent K	TFA	81.5%
	Thioanisole	5.0%
	Phenol	5.0%
	H ₂ O	5.0%
	EDT	2.5%
	TIPS	2.5%

10xSDE	EDTA	25mM
	NaOAc	250mM
SDS-PAGE running buffer	Tris base	52mM
	Glycine	53mM
	SDS	0.1%
10XTBE (for sequencing)	Tris base	242.0g
	Boric acid	123.66g
	EDTA	14.88g
	Dissolved in 2l pH=8.3.	
1XTE	Tris, 1M pH8.0	10mM
	EDTA, 0.5M	1mM
50xTAE	Tris base	242.0g
	Acetic acid, glacial	57.1ml
	EDTA, 0.5M pH 5.0	100ml
	Made up to a final volume of 1l with water.	

2.8 Kits

QIAquick gel extraction kit	QIAGEN, Dusseldorf, Germany.
QIAquick spin PCR purification kit	QIAGEN, Dusseldorf, Germany.
Blood extraction kit	QIAGEN, Dusseldorf, Germany.
QIAGEN DNA kit	QIAGEN, Dusseldorf, Germany.

Geneclean	Bio101, La Jolla, USA.
Sequenase (Version 2.0) DNA sequencing kit	United States Biochemicals, Ohio, USA.

2.9 Plasmids

pUC containing inserts of either wild type core (A) or pre-C mutant core (C) were available in the lab.

For cloning of HBcAg (Chapter 8)

pET3a	A. Abbots, Institute of Virology, Glasgow.
pKK223.3	Clontech Laboratories, California, UK.
pRK5	Prof. H. Will, Hamburg, Germany.
pT7Blue	AMS Biotechnology, Oxon, UK.

2.10 Miscellaneous

Betaplate printed filtermat	LKB Wallac, Pharmacia, St. Albans Herts, UK.
Betaplate sample bag	LKB Wallac, Pharmacia, St. Albans Herts, UK.
Betascint	National Diagnostics, Aston Clinton, Bucks, UK.
Dynabeads	Dynal, Oslo, Norway.
Foetal calf serum (FCS)	Advanced Protein Products, West Midlands, UK.

Immulon 2 plates	Dynatech Laboratories, Billingshurst, West Sussex, UK.
Iscove's modified Dulbecco's medium	Life Technologies Ltd., Paisley, UK.
Kodak X-Omat film	Kodak Ltd., UK.
100bp Ladder	Life Technologies Ltd., Paisley, UK.
1Kb Ladder	Life Technologies Ltd., Paisley, UK.
Lymphoprep™	Nycomed, Oslo, Norway.
Mineral oil	Sigma Chemical Company, Poole, UK.
96 well plates and lids for sequencing	Costar, High Wycombe, Bucks, UK.
96 well plate for lymphoproliferation	Gibco (Nunclon), Paisley, assay UK.
RPMI 1640, 10x	Life Technologies, Ltd., Paisley, UK.
Sequagel	BS and S, Edinburgh, UK.
Sequagel-6	BS and S, Edinburgh, UK.
Whatman paper	Whatman International Ltd., Maidstone, UK.

Chapter 3

Methods

3.1 DNA extraction from serum

3.1.1 Proteinase K method

25 μ l of human serum was extracted at 37 $^{\circ}$ C overnight in the presence of 10 μ l 10xSDE, 10 μ l proteinase K (10mg/ml), 7.5 μ l 6%SDS, 1 μ l tRNA (or glycogen) and 46.5 μ l H₂O. The following morning the DNA was extracted once with phenol:chloroform (1:1), and twice with chloroform alone. The DNA was then precipitated from the aqueous layer in the presence of 2 volumes 100% ethanol, 10% v/v NaOAc and washed; once with 100% ethanol and once with 70% ethanol. Finally, the ethanol was allowed to evaporate completely and the DNA resuspended in 30 μ l molecular biology grade water (hereafter referred to as H₂O). DNA was stored at -20 $^{\circ}$ C for future use.

3.1.2 NaOH method

20 μ l of 0.2M NaOH was added to 20 μ l of serum and incubated overnight at room temperature. The following morning 19.6 μ l of 0.2M HCl was added. The extracted DNA was diluted 1:10, in 1xTE, prior to use in the pulse chain reaction (PCR).

3.2 Removal and purification of oligonucleotides from column

Oligonucleotides were synthesised on a Biosearch 8600 DNA synthesiser. They were removed from the resin by washing 1.5ml ammonia solution (high grade) through the cartridge, subsequently deprotected at 55 $^{\circ}$ C for 5

hours (h) and dried overnight in the speedivac. The oligonucleotide was then resuspended in 100 μ l H₂O. Prior to use for PCR, the OD₂₆₀ was read and pmol/ μ l was calculated. Oligonucleotides that were used for the PCR or sequencing were not purified further.

Oligonucleotides used for cloning purposes were purified on a 6% acrylamide, 1xTBE, 9M urea gel. 80ml of the gel mix was polymerised with 160 μ l of 25% (w/v) APS and 60 μ l TEMED. This was poured between two 20x22cm glass plates separated by 1.5mm spacers, and a 10 tooth comb was then inserted at the top. 30 to 50 μ l of the oligonucleotide was mixed with an equal volume of gel loading buffer and then heated at 99^oC for 10 minutes (min) before being loaded immediately onto the gel. In a separate well 10ml of gel loading buffer was loaded to act as a molecular weight marker. The gel was run at 10mA for 2-3 h in 1xTBE. To visualise the oligonucleotide, the gel was removed, wrapped in cling-film and viewed against a white chromatographic plate by UV shadowing.

In a successful synthesis a predominant band, with several lower molecular weight bands, was observed. The top band was excised with a sterile scalpel, 'chopped-up' and incubated at 37^oC for 16 h in 1ml elution buffer (0.5M ammonium acetate, 1mM EDTA, 0.5% (w/v) SDS). The tube was centrifuged at 2,000rpm for 2 min and the aqueous layer removed. The oligonucleotide was phenol:chloroform extracted (500 μ l was added), ethanol precipitated, washed in 70% ethanol, dried and finally redissolved in H₂O. Concentration of the oligo was determined either by running it on a 1% agarose gel or reading the OD₂₆₀ as before.

3.3 Polymerase chain reaction

3.3.1 First round PCR

In a 50 μ l reaction, 5 μ l of extracted DNA was added to 1x buffer (Tris/KCl - supplied as 10x by the manufacturer), 1.5mM MgCl₂, 250 μ M of each

deoxynucleotide triphosphate (dNTP), 50pmol of each of the relevant primers (see Appendix 1), 2 units (U) *Thermus aquaticus* (*Taq*) DNA polymerase, made up to the final volume with H₂O. The reaction mix was overlaid with 2-3 drops of mineral oil to prevent evaporation and was then cycled as follows:

initial denaturation of 3 min at 95°C, followed by 5 cycles of:

denaturation 1 minute at 95°C

annealing 1min 15s at 55°C

elongation 1min 30s of 72°C

This was followed by 35 cycles of:

denaturation 1min at 90°C

annealing 1min 15s at 55°C

elongation 1min 30s of 72°C

This was followed by a final elongation step of 5 min.

Lowering the denaturation temperature to 90°C after the first 5 cycles was thought to prolong the *Taq* activity. Subsequently, the protocol was modified to a final reaction volume of 20µl.

3.3.2 Second round PCR

1µl of first round product was added to a 50µl reaction mix containing the following: 1x buffer, 1.5mM MgCl₂, 250µM of each dNTP, 50pmol of relevant primers (Appendix 1), 1U *Taq* polymerase. The reaction mix was then made up to the final volume with H₂O. The reaction was overlaid with 2-3 drops of mineral oil and cycled as above, with the exception of only 25 cycles of the 90°C, 1min. denaturation step performed.

Biotinylated primers were employed occasionally in the PCR for specific sequencing applications (3.6.2); these are identified where appropriate.

3.3.3 PCR mutagenesis

The ser15 oligonucleotide, 66 nucleotides in length (Appendix 1), was used to amplify a 280bp pre-C fragment, introducing a codon for serine at codon 15. The PCR product was purified using the QIAGEN PCR purification kit (Boner *et al*, 1995).

3.3.4 Screening for inserts using the PCR

With 15 μ l of 2M NaOH, 50 μ l of an overnight LB plus ampicillin culture, was heated to 65 $^{\circ}$ C for 5 min. 100 μ l of Tris (pH7.5) was subsequently added and 1 μ l of this mixture was then added to a 20 μ l PCR reaction (3.3.2)

3.4 Purification of PCR products and DNA fragments

3.4.1 GeneClean method

Prior to sequencing, PCR products were purified using the GeneClean kit to remove excess primers and nucleotides. Briefly, 20-30 μ l of PCR product was made up to 50 μ l using 1xTBE, 150 μ l (i.e. 3 volumes) of sodium iodide and 5 μ l glass milk were added and incubated at room temperature for 5 min, centrifuged at 12,000rpm for 1 minute and the supernatant discarded. The pellet was resuspended in 500 μ l NEW wash, centrifuged at 12,000rpm for 1 minute and supernatant removed. This was repeated three times. After the final wash the pellet was resuspended in 20-30 μ l of H₂O, and DNA was eluted by incubation at 55 $^{\circ}$ C for 3 min. After spinning for 1 minute; the supernatant (DNA) was then removed to a fresh eppendorf tube and stored at -20 $^{\circ}$ C.

Restriction enzyme digests, prior to electrophoresis on a minigel submarine agarose gel, were also purified in this way.

DNA fragments excised from agarose gels were purified by one of the following methods:

3.4.2 Using the Geneclean kit

Three volumes of sodium iodide was added to the weighed 1xTAE gel slice and incubated at 55°C until the gel had melted (approximately 5 min). 5µl of glass milk was added and the procedure continued as detailed above. If a 1xTBE gel was used 0.5 volumes of TBE modifier (supplied with the kit) and 4.5 volumes of sodium iodide were added and the procedure followed as above.

3.4.3 QIAquick gel extraction kit

After excision, the gel slice was weighed and three volumes of buffer QX1 was added to one volume of gel. This was then incubated at 50°C for 10 min. To aid dissolution of the gel, the tube was inverted 2-3 times during this period. The correct pH (<7.5) is important for efficient binding, and thus if the pH>7.5, 10µl 3M sodium acetate (pH 5.0) was added. The sample was loaded onto the QIAquick column and centrifuged at 12,000rpm for 60s. The flow-through fraction was discarded and 0.75ml of buffer PE was loaded onto the QIAquick column and allowed to stand for 5 min. The column was then centrifuged for 30-60s, the flow-through discarded and the column centrifuged again for 60s. To elute DNA, 30µl of 10mM Tris-HCl (pH 8.5) or water was added, ensuring the filter was covered, and centrifuged for 1 minute. DNA was collected and stored at -20°C.

3.5 Mini-gel submarine agarose electrophoresis

This method was used for several purposes:

1. To visualise the result of the PCR.
2. PCR products were visualised on a 1% 1xTBE gel giving an estimate of the volume of PCR product to be purified for DNA sequencing.
3. Restriction enzyme digests were run on a 1% 1xTAE or 1% 1xTBE gel, excised and purified.
4. DNA fragments and vector were quantified prior to ligation.

Gels were prepared by boiling 1% agarose in 1xTBE or 1xTAE. When cool, 0.05mg/ml ethidium bromide was added and the agarose was poured directly into the mini-gel kit (either Bio-Rad or Uniscience). Once set, the combs were removed and the appropriate volume of 1xTBE or 1xTAE was added to the tank. Samples were run at 65v for 15-45 min. For DNA fragments which were manipulated further, long-wave ultra violet (UV) was used to visualise the DNA; otherwise short-wave UV was used. Either 1kb and 100bp (1µg/ml) ladders were run alongside the DNA to enable confirmation of the PCR products and vector/fragment sizes.

3.6 DNA sequencing

3.6.1 Using the Sequenase™ version 2.0 DNA sequencing kit

2.5µl of appropriate primer (Appendix 1) was added to 5µl of purified DNA heated to 95°C for 2 min, the temperature was reduced to 70°C (allowing time for centrifugation to bring the DNA/primer mix back to tip of tube), again temperature was increased to 95°C for 30s and finally reduced to 37°C for 15 min. DNA/primer mix was then placed on ice. 2µl label mix (diluted 1:5 with water), 2µl reaction buffer, 1µl DTT, 5mCi ³⁵Sα dATP and 2µl Sequenase™ DNA polymerase (diluted 1:8) were added to the annealed DNA and incubated at 19°C for 2 min. 3.5µl of the label reaction was added to 2.5µl of each ddNTP and incubated at 37°C for 5 min. Finally, 4µl of stop solution was added. Reactions were stored at -20°C for up to 2 weeks.

3.6.2 Biotinylated sequencing

Preparation of streptavidin coated beads (Dynabeads) and single stranded DNA for sequencing. 20µl (equivalent to 200µg) of Dynabeads was added to a well of the microtitre plate, placed on magnet and the supernatant was removed. Dynabeads were resuspended in 20µl of binding and washing (BandW) buffer, mixed gently and the supernatant removed. The beads were resuspended in 40µl B and W buffer; 40µl of amplified,

biotinylated PCR product was then added to the washed beads and incubated at room temperature for 15 min. The immobilised product was placed on the magnet, the supernatant removed and washed with 40 μ l of B and W buffer. The wash was then repeated. Finally, the beads were resuspended in 8 μ l of a freshly prepared 0.1M NaOH solution and incubated at room temperature for 10 min. The DNA strands were separated using the magnet and the biotinylated DNA/dynabeads were washed once with each of the following: 50 μ l 0.1M NaOH, 40 μ l B and W buffer and 50 μ l 1x TE.

The annealing and labelling reaction steps were followed as detailed in 3.7.1. Once extension was completed, the product was placed on the magnet and the supernatant removed. The newly synthesised DNA strands were eluted by the addition of the sequenase stop solution and heating the DNA to 72^oC for 2 min. The DNA strands were finally separated using the magnet.

3.6.3 Running the sequencing reactions

An inner and outer pair of glass sequencing gel plates were cleaned and the shorter inner plate was coated with Repelcote, a silicon based compound. This ensured the gel only attached to the larger (outer) plate. 0.4mm spacers were positioned between the plates, at each side, and the plates were taped together.

Sequencing gels were prepared using either of the following methods:

Sequagel. A 5% gel was normally prepared. This entailed mixing together 18ml of concentrate, 7.5ml of buffer and 49.5ml of buffer. Immediately prior to pouring the gel, 600 μ l of APS and 60 μ l of TEMED were added (to polymerise the gel mix).

Sequagel-6. To prepare a 6% gel, 60 ml of Sequagel-6 was added to 15ml of buffer and 60 μ l of APS added immediately prior to pouring the gel.

Shark-toothed combs were inverted and inserted into the top of the gel, to form the wells, and the gel was left at room temperature (RT) to polymerise (approximately 30 min). Once set, the tape was removed and the gel placed in a sequencing apparatus, 500ml of 1xTBE was added to top and bottom reservoirs and the gel was pre-run at 70W for 30min thus allowing it to 'warm-up'.

2.5 μ l of sequencing reactions, denatured at 90 $^{\circ}$ C for 5 min, were added to the appropriate wells and run for either 1hour 45 min or 4h 30 min. On completion of the run, the gel was fixed in 10% acetic acid, 10% methanol for 15 min, blotted onto 3mm Whatmann paper and then dried in a Biorad gel drier for 1hour. The gel was then exposed to X-omat film and developed following 1-10 days exposure. Each gel was read twice using the Sequence Boy machine (Integra Biosciences, Woburn, MA)

3.7 Restriction enzyme digestion of DNA

The manufacturer's recommended buffers and conditions were used for each individual restriction enzyme. Generally, the DNA was incubated with 1x appropriate buffer and 2-3U of restriction enzyme for 2 h at 37 $^{\circ}$ C. To stop the reaction 0.2 volumes of gel loading buffer was added to the digest prior to running on an agarose gel and where appropriate. DNA was purified as described in section 3.4.

3.8 Ligation

Both the vector and the insert were cut with the appropriate restriction enzymes. The vector and insert were purified as described in section 3.4. In all cases, cohesive ends were ligated. The vector and the insert (at a molar ratio of 1:5) were ligated at 16 $^{\circ}$ C for 3h in the presence of 1x ligation buffer and 1U of ligase, in a total volume of 10 μ l.

3.9 Preparation of competent cells

10 μ l of a glycerol stock of *E. coli* DH5 α was added to 10mls of LB broth and grown overnight at 37 $^{\circ}$ C in a shaking incubator. 8mls of the overnight culture was added to 500mls of pre-warmed LB broth and grown at 37 $^{\circ}$ C in a shaking incubator until the O.D. measured between 0.2-0.4. The culture was transferred to pre-chilled polypropylene tubes and kept on ice for 10 min. The cultures were spun at 2,800rpm for 10 min at 4 $^{\circ}$ C and the supernatant decanted. The pellet was resuspended in 80ml 100mM CaCl₂ and kept on ice for 2h. This spin was repeated and the pellet carefully resuspended in 10ml 100mM CaCl₂. After a minimum of 30min glycerol was added, to give a final concentration of 15% and the cells were snap frozen in liquid nitrogen and stored at -70 $^{\circ}$ C in 1ml aliquots.

3.10 Transformation of *E.coli*

100 μ l of DH5 α was added to 10 μ l of ice-cold ligation reaction and incubated on ice for 40 min. The cells were heat shocked, 42 $^{\circ}$ C for 2 min, and immediately returned to ice for a further 3 min. 100 μ l of LB broth was added to the cells and incubated at 37 $^{\circ}$ C for 1 hour. 100 μ l of the transformation reaction was then plated onto a LB agar plus ampicillin plate and incubated overnight at 37 $^{\circ}$ C. Linearised (cut) vector was also transformed as a control (to measure background re-ligation).

3.11 DNA purification from plasmid (Mini-prep)

1.5 μ l of an overnight LB plus ampicillin culture was centrifuged at 2,000rpm for 1 minute and the supernatant discarded. The pellet was resuspended in 100 μ l of solution I (GET buffer) and left at RT for 5 min. 200 μ l of solution II was then added, mixed gently by inversion, and left on ice for approximately 3 min, but for no longer than 5 min. 150 μ l of solution III was added, vortexed and left on ice for 5 min. 50 μ l of phenol/chloroform

was added, the mix centrifuged at 2,000rpm for 1 min. and the supernatant removed and discarded. Two volumes of 100% ethanol was added, incubated at RT for 2 min. and subsequently centrifuged at 2,000rpm for 15 min. Two volumes of 70% ethanol was added, centrifuged briefly, the ethanol was removed and the pellet dried in a speedivac. The pellet was resuspended in 50 μ l 1xTE and stored at -20°C.

3.12 Peptide synthesis

All peptides used were made by continuous flow Fmoc chemistry on either (Fmoc Gly-Gly-Gly-Gly)-Lys₂-Lys-Ala-Diamcryn KA or Ultrosyn C resin, using a Novabiochem peptide synthesiser.

This involves the sequential addition of Fmoc protected amino acids. The Fmoc group was removed at the end of every cycle using 20% piperidine in dimethyl formamide (DMF) for 15 min. PyBOP esters in conjunction with HOBt were used for the coupling of amino acids.

Side chain protecting groups were as follows: trityl (trt), side chains of asparagine, cysteine, glutamine and histidine; butyloxycarbonyl (Boc), side chains of tryptophan and lysine; tertiary-butyl (t-Bu), side chains of aspartic acid, glutamic acid, serine and tyrosine, pentamethylchromanesulphonyl (pmc), side chain of arginine.

3.12.1 Synthesis of tetrameric peptides

These peptides were synthesised on a branched polylysine core allowing the formation of 4 chains of homologous peptides.

3.12.2 Synthesis of monomeric peptides

These peptides were synthesised on Ultrosyn C. This resin is designed to make peptide carboxy amides.

In all cases, the scale of synthesis was approximately 0.1 mmol of free Fmoc groups at a concentration of 0.1 mmol per gram of functionalised resin for peptides. During the synthesis of the overlapping series of peptides, three peptides were synthesised at the same time. In this case the amount of resin was adjusted to keep the total scale at 0.1mmol. Prepacked vials containing 0.5 mmol of Fmoc amino acid esters were used giving a 5-fold excess of amino acid during coupling reactions.

3.13 Cleavage and deprotection of peptides

After synthesis, the peptide resin was removed from the column and transferred to a sintered glass funnel and washed with the following solvents (in order): DMF, t-amyl alcohol, acetic acid, t-amyl alcohol and finally diethyl ether. The resin was then transferred to a 100ml round bottomed flask and dried in an evacuated dessicator. The resin was weighed approximately 10% was cleaved as a test cleavage, and the remaining resin was stored at -20°C.

The resin to be cleaved, along with 20 to 25ml of Reagent K was added to a 100ml round bottomed flask and left at room temperature for 1.5 to 2h. The resin/Reagent K mixture, with the peptide now in solution, was then transferred to a sintered glass funnel and allowed to drain into a 250ml round-bottomed flask. Once all the solution had completely drained a small amount of TFA was added to wash the funnel. TFA was then evaporated under vacuum from the peptide solution using a rotary evaporator until approximately 5ml remained. This was transferred to a 50ml falcon tube, diethyl ether added to a final volume of 40ml, to precipitate peptide, and centrifuged at 2,000rpm for 15 min to pellet the peptide. Diethyl ether was carefully poured off. The peptide was washed a further two times with diethyl ether. Finally, the peptide was redissolved, as appropriate, shell frozen and dried overnight.

3.14 Analysis of peptides

3.14.1 Mass spectrometry

The molecular weight of the monomeric peptides was determined by M-Scan Ltd., Ascot, England, UK, using the fast atom bombardment (FAB-mass) ionisation technique (Barber *et al*, 1981).

3.14.2 Amino acid analysis

Amino acid analysis was carried out on the branched peptides by Cambridge Research Biochemicals Ltd, Cheshire, England, UK. This provides the molar ratio of each amino acid contained in the peptide.

3.15 Reverse phase high pressure liquid chromatography (HPLC)

The purity of each peptide was determined by reverse phase HPLC monitored at 225nm on a Beckman System Gold HPLC using a dynamax 300 A C8 analytical column (4.6mm internal diameter x 250mm) A gradient of 0-95% acetonitrile (plus 0.05% TFA) in water (plus 0.1% TFA) was run over 5 min at a flow rate of 0.5ml/min

3.16 Purification of peptides

Peptides were purified by reverse phase HPLC on a Beckman System Gold system using a Vydak-FB1520 preparative column (20mm internal diameter x 50cm) and a gradient of 0-95% acetonitrile (plus 0.05% TFA) in water (plus 0.1% TFA), run over 20 min at a flow rate of 10ml/min. Fractions were analysed by reverse phase HPLC.

3.17 Storage of peptides

Peptides were stored at -20°C in a dry, sealed universal. Peptides were warmed to room temperature prior to opening to prevent moisture damaging the peptide.

3.18 Dissolution of peptides

Vials containing peptides were brought to room temperature before opening. The required amount of peptide was removed and dissolved in one of the following ways:

1. In H_2O . The desired volume of sterile water was added to the peptide.
2. By bubbling of ammonia (NH_3) gas. The desired volume of sterile water was added to the peptide and NH_3 gas bubbled through until dissolution was observed.
3. By addition of acid. Varying concentrations of acid (either 10%, 33% or 100%) was added dropwise to the peptide until dissolution was observed. Sterile water was then added until the required volume was achieved.

Details of the solubility of the constructed peptides are given in Table 7.3

3.19 Enzyme-linked immunosorbant assay (ELISA)

Peptides were dissolved as described in Table 7.3 at the desired concentration and adsorbed to 96 well microtitre ELISA plates (Immulon II) overnight at 37°C . The plates were then washed five times in PBS-Tween 20 solution and blocked with 2% BSA in PBS for 1 hour at 37°C . Antibodies or human sera were diluted in PBS containing 0.5% BSA and $50\mu\text{l}$ of the appropriate dilution was added to the wells. Plates were incubated at room temperature for 1 hour and then washed five times in PBS-Tween 20 solution. Horseradish peroxidase conjugated-goat anti-mouse or horseradish peroxidase conjugated-protein A was diluted

1:1000 in PBS containing 0.5% BSA and 50µl added to each well. Plates were incubated at room temperature for 1 hour and washed a further ten times in PBS-Tween 20 solution and 100µl of a 50mg/ml solution of enzyme substrate; 2,2', Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) in citrate phosphate buffer, containing 0.01% hydrogen peroxide, was added. After 15 min of colour development, the absorbance at 405nm was read on a Multiskan plate reader. The background absorbance for each serum dilution was determined by incubating the sera on a well with no peptide. This value was subtracted from the appropriate reading.

3.20 Separation of peripheral blood mononuclear cells from whole blood

20ml of blood was collected into 0.2ml preservative free heparin and gently mixed. 10ml of blood was carefully layered onto 15ml Lymphoprep in a sterile universal (repeated for the remaining 10ml). The universals were centrifuged at 1400rpm for 40 min, the plasma removed and using a sterile pastette the mononuclear cells were carefully harvested from the gradient interface and placed in a fresh universal.

To an aliquot of hepes/bicarb buffer: 20ml RPMI 1640 (10x concentrated), 50IU/ml of penicillin, 50mg/ml of streptomycin and 1mM L-glutamine was added and the pH adjusted to 7.2 using 5N NaOH. The mononuclear cells were washed with this medium and centrifuged at 1400rpm for 5 min. The supernatant was decanted into another universal, more medium was added to the first universal and both were centrifuged at 1400rpm for 5 min.

The supernatants were decanted from both universals and the cells resuspended using vigorous finger flicks, but never vortexed, to loosen the cell pellet. The cells were pooled and made up to a volume of 1ml with medium.

All work was carried out in a class 2 safety cabinet to maintain sterility and protect the operator. The cells were counted, in white cell counting fluid, and the cell count adjusted to 1×10^6 cells/ml.

3.21 Lymphoproliferation assay

3.21.1 Using whole blood

1ml of blood, test and control, was added to 9ml Iscove's medium in appropriately marked universals and gently mixed. To each well, of a 96 well plate, 100 μ l of diluted blood was added. Row A contained no mitogen/stimulant. Rows B to D contained phytohaemagglutinin (which acted as a positive control). The contents of the other rows were specific for each experiment, the details are given in the appropriate chapter. Triplicate wells were set up for each variable parameter. The plates were sealed, using adhesive plate sealers, and incubated at 37 $^{\circ}$ C in 5% CO $_2$. Three days later the cells were 'pulsed' with 1 μ Ci of tritiated thymidine. The plates were resealed and incubated for a minimum of 4h.

The cells were then harvested onto a filter mat using a Betaplate 96-well cell harvester and dried overnight on the bench. The following day, the filter was placed in a sample bag, 10ml of Betascint was added and the bag sealed. Betascint was rolled out to cover the entire filter, removing any bubbles and resealing if necessary. The filter was read in the Betaplate counter using the appropriate programme.

All work was carried out in a class 2 safety cabinet to maintain sterility as bacterial contamination would result in distortion of the results, either by interfering with the response of the lymphoid cells or by excess label being incorporated into the bacterial DNA.

3.21.2 Using separated peripheral blood mononuclear cells

The method used for testing the lymphoproliferation of separated peripheral blood mononuclear cells was similar to that of the whole blood assay, except cells and mitogen are mixed together before adding to the 96 well plate.

15 μ l RPMI medium and 10 μ l heat inactivated foetal calf serum (FCS) were added to all wells. To row A, 75 μ l of RPMI and cell mixture were added and to rows B to D, 75 μ l of PHA concentrations and cell mixture. To the other rows, 75 μ l of appropriate mitogen concentration and cell mixture were added, the exact contents were dependent on the experiment and details given in the appropriate chapter. The plates were sealed and incubated at 37 $^{\circ}$ C in 5% CO $_2$. Three days later the cells were pulsed with 1 μ Ci tritiated thymidine, the plates were resealed and incubated at 37 $^{\circ}$ C in 5% CO $_2$ for a minimum of 4h. The cells were harvested onto a filter mat using a Betaplate cell harvester. The filters were dried on the bench overnight and read the following day as detailed in 3.21.1.

The stimulation index (SI) was calculated as the ratio between the counts per minute obtained in the presence of antigen and those obtained without antigen. An SI above three was considered significant.

3.22 Cloning of U₁₈₅₆ in vaccinia virus

A vaccinia virus with the wild-type HBeAg (containing proline at amino acid 15 of pre-C) as a backbone, previously described (Schlicht and Wasenauer, 1991), was used to generate the U₁₈₅₆ (serine at amino acid 15 of pre-C) recombinant virus by conversion of CCT to TCC by PCR mutagenesis (Boner *et al*, 1995). The pre-C fragment was then end-filled, using Klenow polymerase, cut with *Sal* I and *Cla* I and recloned into a vaccinia virus expression vector containing the complete HBV pre-C/C gene downstream from the vaccinia virus 7.5K early/late promoter. The

pre-C/C gene used for expression was taken from a construct containing a synthetic HBc gene, which included many restriction enzyme sites not present in the wild-type gene (Schlicht *et al*, 1989). Before use in any experiments, the whole recloned fragment was sequenced to verify the mutations and to exclude the possibility of any further mutations introduced by the PCR.

3.23 Sera and antibodies used in vaccinia analysis.

The three mouse MAbs used were provided by M. Noah (Behring, Germany). One of these antibodies (03) was specific for the HBe1 epitope, and the other two (152 and 158) were specific for the HBe2 epitope. These are the only two well-characterised HBe epitopes, therefore these antibodies were considered sufficient for analysing antigenicity. The polyclonal anti-HBc-positive human serum (obtained from a chronically infected patient) was strongly HBeAg positive and had an anti-HBc titre of $\sim 10^4$, as determined by end point dilution using a radioimmunoassay (RIA). The HBc/e specific polyclonal rabbit serum was generated by multiple intramuscular injections of recombinant HBc protein (100 μ g/dose) and denatured by boiling for 5 min. in PBS containing 1% SDS and 1% 2-mercaptoethanol. No adjuvant was used. This serum reacts efficiently with all known HBc gene products, including HBeAg. The suitability of these antibodies for the analysis of HBc gene products by immunoprecipitation has been described (Nassal, 1988).

3.24 Immunoprecipitation and Western blotting of vaccinia-generated proteins

For the expression and serological characterisation of both HBe proteins, HepG2 cells were infected with recombinant vaccinia virus at a multiplicity of infection (MOI) of 10 in 1 ml of serum free medium. After 90 min at 37^oC, the inoculum was removed, and 2ml of fresh medium containing

10% FCS was added. Supernatants were collected 48h after infection and used for immunoprecipitation and Western blotting. This allowed secretion of the expressed HBe protein to be detected.

2ml of medium was used to prepare a single Western blot sample. Immunoprecipitation was performed using the well characterised Mabs 03, 152 and 158 and an exclusively HBc-specific polyclonal rabbit antiserum specific for both HBcAg and HBeAg. 5 μ l of polyclonal serum (human or rabbit) was adsorbed to 25 μ l of preswollen protein A-Sepharose. When mouse monoclonals were used, MAb solution (5 μ l; immunoglobulin concentration, 1mg/ml) was adsorbed to 25 μ l of preswollen protein A-Sepharose with 5 μ l of goat anti-mouse immunoglobulin. Immunoprecipitation was carried out for 16h at 4^oC. The samples were washed twice with TNE, boiled in sample buffer with 2% 2-mercaptoethanol for 5min, and separated on a 12.5% polyacrylamide gel. After Western transfer, the filter was blocked with 1% bovine serum albumin and incubated with polyclonal rabbit serum (end dilution, 1:2000), and bound antibody was detected by incubation with alkaline-phosphatase-labelled protein A and subsequent alkaline-phosphatase staining.

Chapter 4

Results: Section 1

4.1 Reconstruction experiments: Testing sensitivity of detection of mixed populations by sequencing

The success of the work carried out in this thesis would depend on the sequencing results. It was considered likely that mixed populations existed in the patients we wanted to examine. The relevance and importance of such mixed populations was not known but we thought it unwise to ignore them.

Dilution of PCR products to a single population and subsequent sequencing would be a highly accurate method of determining the presence of sub-populations but, due to the number of samples we intended to sequence, this was thought to prove too time consuming. A quicker method would be to sequence the PCR products directly. Thus it was considered fundamental to determine the sensitivity of detecting mixed populations by this method.

To test the sensitivity of sequencing directly from PCR products two plasmids (A and C; where A contains A₁₈₉₆ and C has G₁₈₉₆), available in the lab, were mixed together at varying dilutions, amplified by PCR (3.3), the DNA was purified using the GeneClean method (3.4.2) and then sequenced (3.6.1).

4.1.1 Methods

A dilution of 1 $\rho\text{g}/\mu\text{l}$ was prepared for both plasmids. This was the elucidated value equivalent to the amount of DNA in the number of virions commonly seen in a low level viraemic carrier in 25 μl of serum. Mixtures of plasmids A and C were prepared as detailed in Table 4.1

1 μl of the indicated mixtures were amplified using primers BC1 and C3 (see Appendix 1 for primer sequences). These primers allowed amplification of the pre-C region, an area of approximately 100 base pairs (bp).

Successful amplification was observed by running 5 μl of the PCR products on a 1% TBE agarose gel. 25 μl of the PCR products were then purified by the GeneClean method, as outlined in 3.5.1, and resuspended in 20 μl H₂O. 5 μl of each mixture was then sequenced, as outlined in 3.6.1, using primer BC1. The sequencing results are given in Figure 4.1.

4.1.2 Results

The lower limit for observing mixtures by sequencing directly from PCR products is 5%, as shown in Figure 4.1. This was considered to be adequate for the purpose of this study as populations below this limit were not considered to be present at sufficient levels to play an important role in the biology of, or the host response to, HBV.

4.2 Extraction of DNA from serum

A second important factor in the success of the study would be the method used to extract DNA from the serum. Concerns here were the sensitivity of the procedure and prevention of contamination between samples. Some samples would have a high titre of viral DNA, thus the possibility of contamination was always present.

Table 4.1 Relative mixtures of plasmids A and C

PLASMIDS	
A (%)	C (%)
97.5	2.5
95	5
92.5	7.5
90	10
75	25
50	50

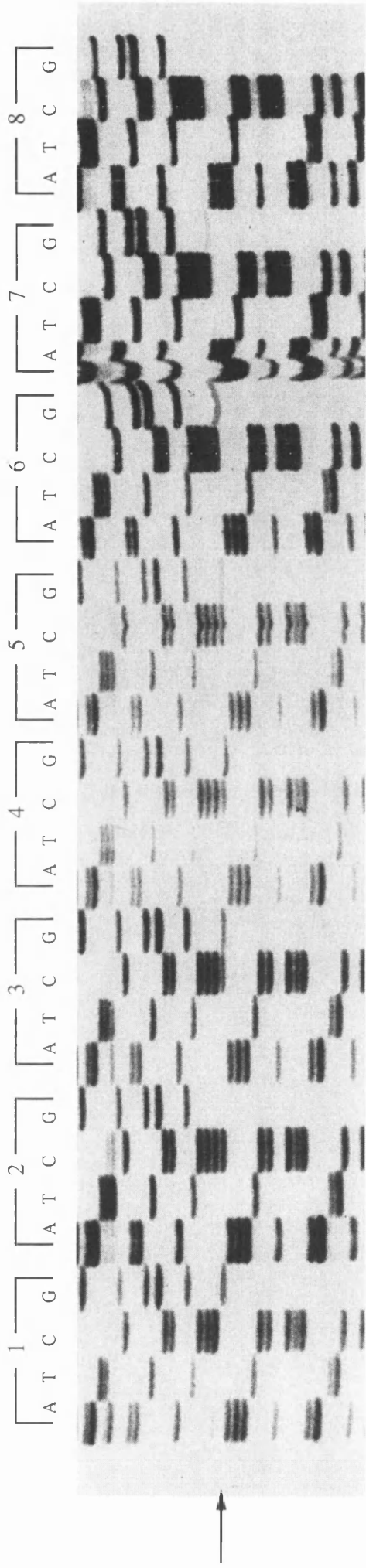


Figure 4.1 Autoradiograph of part of the pre-C region sequenced directly from BC1/C3 PCR products of plasmids A and C, indicating the level of detection of A_{1896} . Lane order: ATCG, 1-plasmid A (A_{1896}), 2-plasmid C (G_{1896}), 3 to 8- mixtures of plasmids A and C, 3- 50/50 (C%/ A%), 4- 75/25, 5- 90/10, 6- 92.5/7.5, 7- 95/5, 8- 97.5/2.5. Sequencing of the mixtures indicates that the level of detection of mixtures is 5%. Arrow indicates nucleotide 1896

The standard method that was currently employed in the lab is detailed in 3.1.1. This is a multi-step procedure thus increasing the chance of contamination between samples.

A second method, detailed in 3.1.2, involved fewer steps, thus decreasing the risk of contamination between samples.

The sensitivity of this method was compared to the standard extraction method.

4.2.1 Methods

A previously extracted serum sample, thus known to be PCR positive, was employed as the test sample. This sample was diluted in negative serum, in 10-fold dilutions from 10^{-1} to 10^{-10} and 25 μ l of each dilution was extracted using both methods.

5 μ l of each extracted DNA was then amplified for the core region by first (3.3.1) and second (3.3.2) round PCR, using C1/C2 and C3/C4 primers respectively (Appendix 1). After the PCR, 5 μ l was run on a 1% TBE agarose gel.

4.2.3 Result

Figure 4.2 shows 5 μ l of each first round PCR product run on a 1% TBE agarose gel. Figure 4.3 shows the results from the second round PCR. The standard method employed in the lab had a hundred-fold increased sensitivity compared to the shorter, less 'hands on' method.

4.4 Conclusions

The lower limit of detecting mixed populations directly from amplified PCR products was 5%, therefore it was decided that all our sequencing data could be gathered using this method.

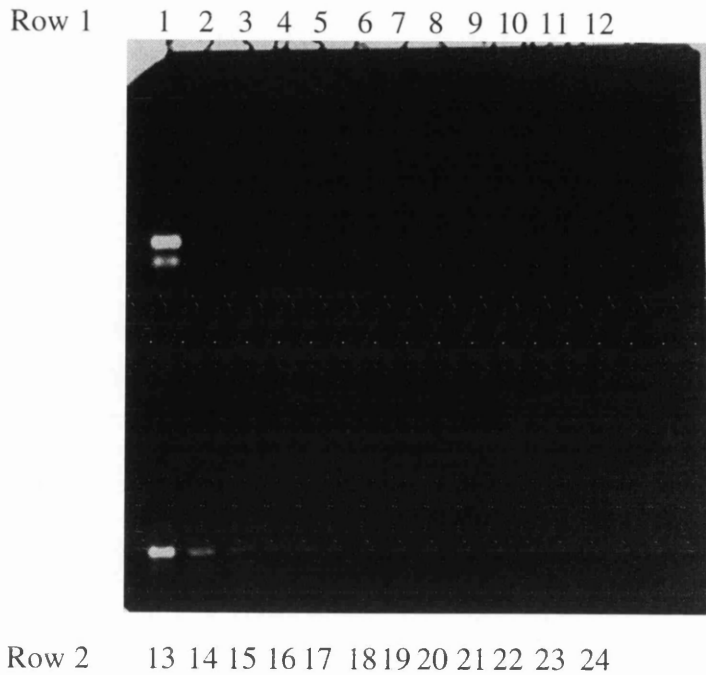


Figure 4.2 Ethidium bromide stained 1% (1xTBE) agarose gel showing the C1/C2 PCR products of DNA extracted by the NaOH method (top wells - row 1) and the proteinase K method (bottom wells - row 2). Lanes 1 and 13 serum dilution 10^{-2} , Lanes 2 and 14 serum dilution 10^{-3} , Lanes 3 and 15 serum dilution 10^{-4} , Lanes 4 and 16 negative serum control, Lanes 5 and 17 serum dilution 10^{-5} , Lanes 6 and 18 serum dilution 10^{-6} , Lanes 7 and 19 serum dilution 10^{-7} , Lanes 8 and 20 negative serum control, Lanes 9 and 21 serum dilution 10^{-8} , Lanes 10 and 22 serum dilution 10^{-9} , Lanes 11 and 23 serum dilution 10^{-10} , Lanes 12 and 24 negative serum control.

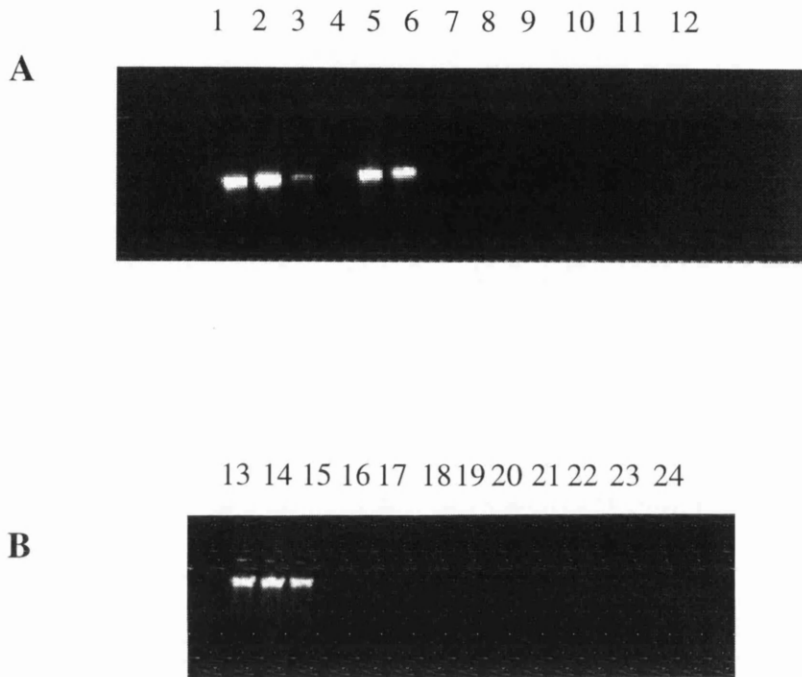


Figure 4.3 Eithidium bromide stained 1% (1xTBE) agarose gel showing the C3/C4 PCR products of DNA extracted by the NaOH method (A) and the proteinase K method (B). Lanes 1 and 13 serum dilution 10^{-2} , Lanes 2 and 14 serum dilution 10^{-3} , Lanes 3 and 15 serum dilution 10^{-4} , Lanes 4 and 16 negative serum control, Lanes 5 and 17 serum dilution 10^{-5} , Lanes 6 and 18 serum dilution 10^{-6} , Lanes 7 and 19 serum dilution 10^{-7} , Lanes 8 and 20 negative serum control, Lanes 9 and 21 serum dilution 10^{-8} , Lanes 10 and 22 serum dilution 10^{-9} , Lanes 11 and 23 serum dilution 10^{-10} , Lanes 12 and 24 negative serum control.

A recent paper by Alexopoulou *et al* (1997) assessed changes in the pre-C/C region by direct sequencing and sequencing pre-C/C clones from different time points of an anti-HBe positive patient. Their cloning results indicate the presence of quasi-species in all the samples investigated and highlight the superiority of sequencing from clones as a further four amino acid changes was observed using this method.

It was decided to continue with the standard serum extraction method taking the usual precautions (i.e. using plugged tips, changing gloves between each manoeuvre, interspersing every third sample with a negative control and adding the DNA last) to prevent inter-sample contamination. As this method is time consuming it was decided, as a further precaution, not to extract more than 6 serum samples at a time. In this way there would be no temptation to 'cut corners', perhaps by not changing gloves or not including as many negative samples as one should.

The quicker NaOH method was employed in repeat extraction where the sample was known to be strongly PCR positive.

Chapter 5

Results: Section 2

Chinese HBcAg study

5.1 Background

This study was based on results by Carman *et al* (1992) in which they addressed the prevalence of pre-C mutants in HBeAg and anti-HBe positive Hong Kong Chinese patients with chronic HBV infection. To correlate the clinical course with variations in the pre-C sequence and to determine whether the presence or absence of mutant strains parallels fluctuations in HBe status.

The results indicated the presence of two mutually exclusive pre-C mutants in this group of patients: A₁₈₉₆ and T₁₈₅₆. The latter causes an amino acid change, of proline to serine, at amino acid 15. A₁₈₉₆ was found, as the predominant strain only in the anti-HBe phase of infection, while T₁₈₅₆ was also found during the HBeAg phase. As selection of the T₁₈₅₆ mutation was not observed it is likely that this mutation is present in the infecting virus. Two other pre-C mutations were found, A₁₈₉₉ and A₁₈₉₈, only in association with A₁₈₉₆ and T₁₈₅₆ respectively, and neither correlated with disease severity.

HBeAg is believed to induce immunological tolerance against HBeAg and HBcAg; nonetheless, a strong immune response is eventually mounted against HBeAg. Selection of variants that are unable to produce HBeAg

may be a means of survival employed by the virus. It was of particular interest that, as shown in previous work, A₁₈₉₆ and T₁₈₅₆ are mutually exclusive (Carman *et al*, 1992; Lok *et al*, 1994). Since loss of HBeAg appears to be beneficial to the virus, T₁₈₅₆ must inhibit selection of a pre-C inactivating mutant, or selection of a pre-C stop codon is not necessary because similar functions can be ascribed to the two mutations. For instance, T₁₈₅₆ results in the introduction of serine at amino acid 15, normally a highly conserved proline, into the signal peptide of HBeAg. In the original paper it was hypothesised that the exclusive nature of A₁₈₉₆ and T₁₈₅₆ may be due to the mutant HBeAg being secreted into the serum and not remaining on the surface of hepatocytes; thus, it would not be a target for B cells and, therefore, would not come under immune pressure. Alternatively, it was hypothesised that it may be different antigenically (perhaps because of an altered secondary structure) and therefore would not be recognised; thus there would be no pressure to select an inactivating mutation.

There has been much interest in attempting to link core variation and disease. It appears that amino acid substitutions or deletions are more common in HBeAg patients with severe disease (Ehata *et al*, 1992; Chuang *et al*, 1993), and Carman *et al* (1995) have shown that anti-HBe positive individuals with progressive hepatitis are more likely to select a large number of amino acid substitutions in HBcAg, particularly after the emergence of A₁₈₉₆. Such substitutions occur significantly more often in B and Th cell epitopes than in inter-epitopic regions of HBcAg.

5.2 Aims

The overall aim of this section of work was to compare the degree and distribution of evolution of HBcAg strains with either of the two pre-C variants in those who seroconverted from HBeAg to anti-HBe to those who were continuously anti-HBe, and to delineate their distribution.

The original paper also raised several questions which were addressed:

1. Why are A₁₈₉₆ and T₁₈₅₆ never observed on the same genome?

The mutual exclusivity of A₁₈₉₆ and T₁₈₅₆ was examined by analysing the effects of U₁₈₅₆ on HBeAg synthesis, secretion and antigenicity.

2. What is the phylogenetic relationship between A₁₈₉₆ and T₁₈₅₆?

As both variants are equally present in this community they could represent separate lineages.

5.3 Patients

Serial serum samples from 10 Hong Kong Chinese (Ch) patients with chronic hepatitis were studied. Patient details are given in Table 5.1. Group I consisted of three patients who were continuously anti-HBe positive, had persistent or episodic increase in serum ALT levels and were known, from the previous study, to have A₁₈₉₆. Group II consisted of three patients who were continuously anti-HBe positive, had persistent or episodic increase in serum ALT levels and were known to have T₁₈₅₆. Group III comprised four patients who had spontaneous HBeAg to anti-HBe seroconversion but had continued elevation of ALT after clearance of HBeAg. Each case had at least 2 or 3 sequential serum samples available for study.

5.4 Methods

5.4.1 DNA extraction from serum

DNA extraction was carried out as detailed in 3.1.1 with a slight modification for a few samples where only 5µl of serum was available. In such cases the same extraction method was used with extra H₂O added to compensate for the reduced volume of serum and the DNA was resuspended in a final volume of 10µl.

Table 5.1 Patient characteristics. Anti-HBe, antibody to HBeAg; WT, wild type (G at nucleotide 1896 and proline at codon 15 of pre-C); A₁₈₉₆, G to A mutation; C₁₈₁₅ mutation of the start codon leading to failure to initiate translation; U₁₈₅₆ translates as proline at codon 15 of pre-C.

	AGE / SEX	SAMPLE DATES	HBe STATUS	PRECORE SEQUENCE (serial samples)
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Group I : anti HBe and HBV DNA positive, elevated ALT

case 1,	40 / F	6 / 84	anti - HBe	WT / A 1896
		11 / 87		A 1896
		10 / 89		A 1896
case 2,	61 / M	2 / 86	anti - HBe	A 1896
		7 / 87		A 1896
		9 / 89		A 1896
case 3,	39 / M	7 / 86	anti - HBe	WT
		9 / 89		A 1815

Group II : anti HBe and HBV DNA positive and fluctuating ALT

case 4,	28 / M	9 / 87	anti - HBe	U 1856
		6 / 89		U 1856
case 5,	29 / M	7 / 88	anti - HBe	U 1856
		5 / 89		U 1856
case 6,	28 / F	3 / 87	anti - HBe	U 1856
		6 / 89		U 1856

Group III : HBeAg to anti - HBe positive, HBV DNA positive and fluctuating ALT

case 7,	23 / F	5 / 85	HBeAg	U 1856
		3 / 90	anti - HBe	U 1856
case 8,	31 / M	7 / 85	HBeAg	U 1856
		12 / 87	anti - HBe	U 1856
case 9,	36 / M	5 / 85	HBeAg	U 1856
		5 / 86	anti - HBe	U 1856
		6 / 87	anti - HBe	U 1856
case 10,	32 / M	11 / 84	HBeAg	WT
		2 / 90	anti - HBe	WT

5.4.2 PCR and sequencing

First and second round PCR was carried out as detailed in 3.3.1 and 3.3.2 using primers C1/C2 and C3/C4 respectively (Appendix 1). Products were purified (3.4.1) and sequenced as outlined (3.6) using primers C1, C α , 19, C4 and BC1 (Appendix 1).

5.4.3 Recombinant vaccinia viruses.

Using HBeAg containing proline at amino acid 15 of pre-C in a vaccinia backbone as previously described (Schlicht, 1991), the T₁₈₅₆ (serine at amino acid 15 of pre-C) was generated. Although serine can be encoded by the triplets UCC or UCU, all natural cases examined had the codon UCC. Thus, this was the codon introduced. This was achieved by conversion of CCT to TCC by PCR mutagenesis using standard PCR procedures (3.3.3) where the ser15 primer acted as the mutagenic primer.

5.4.4 Cloning, expression and analysis of T₁₈₅₆ in vaccinia virus

As detailed in 3.22, 3.23 and 3.24.

5.4.5 Phylogenetic analysis

Whole pre-C/C sequences were aligned using the Clustal V program (Higgins *et al*, 1992) as implemented in the Genetic Data Environment software package (provided by S. Smith). Alignments were checked visually. Phylogenetic trees were reconstructed using a number of methods taken from the PHYLIP software package (Felsenstein), particularly one invoking maximum likelihood inference (program DNAML). To assess the level of support for each node, trees were also reconstructed using the neighbor-joining clustering algorithm (program NEIGHBOR (Felsenstein)) with bootstrap resampling (1000 replications; programs SEQBOOT and CONSENSE (Felsenstein)). The distances between each sequence were corrected for multiple substitutions using a method that allows different base frequencies and different rates of transition and transversion (program DNADIST).

5.5 Results

5.5.1 Sequencing

Figure 5.1 shows the distribution of amino acid substitutions which are related to the known Th and B cell epitopes. The Th epitope (amino acids 50 to 69) is recognised by essentially all patients studied by other groups (Ehata *et al*, 1993; Akarca and Lok, 1995; Valliammai *et al*, 1995; Carman *et al*, 1995, Bozkaya *et al*, 1996) and was recognised by all the patients in group I, patient 4 (group II) and patient 8 (group III). In group I, multiple substitutions occurred in the core gene when A₁₈₉₆ emerged; however, few appeared thereafter. The initial samples were anti-HBe positive, so the presence of anti-HBe alone was not sufficient for selection of core substitutions. Although case 3 did not select A₁₈₉₆, an equivalent mutation (T to C₁₈₁₅) arose. This mutant would also prevent production of HBeAg by inactivating the pre-C start codon. Again, the substitutions arose at or after the emergence of C₁₈₁₅. A mean of 9.6 substitutions occurred in this group: 79% occurred within known Th or B cell epitopes.

In contrast, cases with T₁₈₅₆ (serine), whether they were continuously anti-HBe positive (group II) or seroconverted from HBeAg to anti-HBe (group III), selected few HBcAg substitutions (mean: 3.1/case) with only 36% of these within epitopes. Thus, in contrast to subjects with A₁₈₉₆, subjects in both groups II and III had ongoing selection without obvious influence of the seroconversion event. Subject 10 (group III) had proline at amino acid 15 but did not select A₁₈₉₆ during the study period. His HBcAg substitution pattern was indistinguishable from those with serine 15. The mean value of substitutions in group II was skewed by case 6. This patient selected a total of 7 substitutions in contrast to the 2 or 3 substitutions seen for all the other cases in these groups. The possibility that two sera from different patients had been extracted was addressed by re-extracting and sequencing both these samples again. The resultant sequences were identical to the original. To exclude wrong labelling on collection, two

Epitopes	Th	*	Th	B ₁	B ₂	B ₃
Core AA	1 20	21 40	50 89	74 97	100 117	130 135
Case	13	21 27 28 33	41 45 48 49 50 53 58 59 63	73 78 84 87 95 96 97 99	100 104 112 113 114	126 129 131 132 135 149 160 177
Consensus	V	S I R A	A P C S P L A I G S	S D L S L K I Q	L H R E T I P	A Y P V T Q
1	6/84 11/87 10/89		P T T	V L I/L G I I G I I	T R/H L L L H L V L H V V	R/T P E E E E E E
Group I	2/86 7/87 9/89	T S S	G T L V A S I G A S I G	A S L G L G		
3	7/86 9/89	N S I D N L	P A S P A X T G	L S A R		A G L
4	9/87 6/89		V G	S R		
Group II	7/88 5/89	V I		M P I L	G R	
6	3/87 6/89	V A	E S D T	I L	P A	
7	5/85 3/90	V I				P S
8	7/85 12/87		A G			
Group III	5/85 5/86 6/87				E E D	R R P
10	11/84 2/90		S T	I M		

Figure 5.1 Distribution of amino acid substitutions in HBcAg. Only positions at which changes were observed are noted. * region previously shown to have higher than expected variability but not known to be a B-cell epitope (Carman *et al*, 1995). Th, described T-helper cell epitopes in HBcAg ; B1, B2 and B3, described B-cell epitopes in HBcAg or HBeAg. Single letter amino acid codes are given (Appendix 2).

further samples from this series were also extracted and sequenced; these again confirmed the original sequences. Interestingly, all of the substitutions occurred in inter-epitopic regions. It has subsequently been shown that superinfection can sometimes occur accounting for genotype conversion events (Bollyky *et al*, submitted, 1997)

Using the Mann-Whitney U test, we have shown that the total number of substitutions within epitopes in group I was significantly greater than in groups II and III combined ($p = 0.02$). Selection of a glycine at amino acid 63 in the Th epitope was seen in 4 of the 5 cases who selected any change in this antigenic region (amino acids 50 to 69). However, glycine is normally found at amino acid 63 of *adr*, *ayr*, and *adw* subtypes, and thus the significance of this substitution is unclear.

5.5.2 Serine15 prevalence

We examined the prevalence of serine15 at this position in the pre-C by constructing a Pileup (using the GCG package) of all the pre-C sequences currently available in the Genbank database. As can be seen from Figure 5.2, serine is not usually found whereas proline is normally highly conserved at this position.

It was noted that this is the only proline found in the pre-C region; however it cannot be essential to the virus otherwise our patients would not have been infected.

5.5.3 Functional studies on HBeAg

To analyse whether the proline to serine exchange at amino acid 15 had any influence on the biosynthesis or antigenicity of the respective HBeAg molecules, recombinant vaccinia viruses containing either of these variants were generated. This recombinant virus was then used to express the protein in HepG2 cells, a human hepatoblastoma cell line. After immunoprecipitation with monoclonal or polyclonal sera, the samples

Xxhepav ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCTTGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
Xxhepa ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCTTGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
Hbvaywmcg ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCTTGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTAGGAC
Hpbhbvaa ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCTTGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTAGGAC
Hpbmut ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCTTGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTAGGAC
Hbvdna ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCTTGTTCATGT**CCC**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
Hbvadw2 ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCTTGTACATGT**CCC**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
Hbvadw ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCTTGTACATGT**CCC**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
Hvhepb ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCTTGTACATGT**CCC**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
Hpbadwzcg ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCTTGTACATGT**CCC**ACTTTCAGCCTCCAAGCTGTGCCTTGGATGGCTTTGGGGC
Hpbcgadr ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCATGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
Hbvadrm ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCATGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
Hpbadricg ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCATGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
Hbvadr4 ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCATGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
Hpbadra ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCATGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
Hpbcg ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCATGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
Hehbvayr ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCATGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
Hbvadr ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCATGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
... AAC. TTTTTCACCTCTGCCTAATCATCTCATGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
Hpbadrc ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCCTGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTAGGAC
Hpbetnc ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCTTGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTAGGAC
Hpbadw3 ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCATGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
Hpbadwz ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCATGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
Hpbadw1 ATGCAAC. TTTTTCACCTCTGCCTAGTCACTCTTGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
Hpbadw2 ATGCAAC. TTTTTCACCTCTGCCTAATCATCTCATGTTCATGT**CCT**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC
Hbvadw4a ATGCAAC. TTTTTCACCTCTGCCTAATCATCTTTGTTCATGT**CCC**ACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC

Figure 5.2 The pre-C region of 23 HBV sequences from Genbank database. Codon 15 is highlighted in bold. CCT = proline, CCC = proline.

were analysed by SDS-PAGE and Western blot using a rabbit polyclonal anti-HBc/e. As shown in Figure 5.3, there were no obvious differences between the proline-containing HBeAg lanes (1-5) and serine-containing HBeAg lanes (6-10). Both proteins reacted with the HBeAg-specific Mabs (lanes 1-3 and 6-8) and with the HBc/e-specific polyclonal rabbit antiserum (lanes 5 and 10) but not with the exclusively HBcAg-specific human serum (lanes 4 and 9), indicating that it is HBeAg and not HBcAg being produced. By the nature of the experiments it is clear that both proteins were being produced and with equal efficiency. Molecular size also appeared to be unaltered.

5.5.4 Phylogenetic analysis

The unrooted maximum likelihood tree for the 23 pre-C sequences is presented in Figure 5.4. The patients with the proline 15 and A₁₈₉₆ (cases 1 and 2) are clearly separated from T₁₈₅₆ (serine 15) strains (cases 4 to 9). Case 10 also separated with this group. This finding was also apparent in trees reconstructed using the neighbor-joining method, by which the separation was strongly supported under the bootstrap criterion. The single sequence with the mutated start codon (case 3) is also clearly distinct.

5.6 Discussion

At the time this work was undertaken it was assumed that one of the main targets for the immune system is the HBcAg. As yet there is no direct evidence to support this theory but several sequencing studies over the past few years have provided indirect evidence of this (Carman, 1995, Akarca, 1995): changes in sequence observed in the core are often associated with known epitopes in HBcAg.

In this study we have shown that Hong Kong Chinese who have pre-C mutations that inactivate HBeAg translation also accumulate core amino

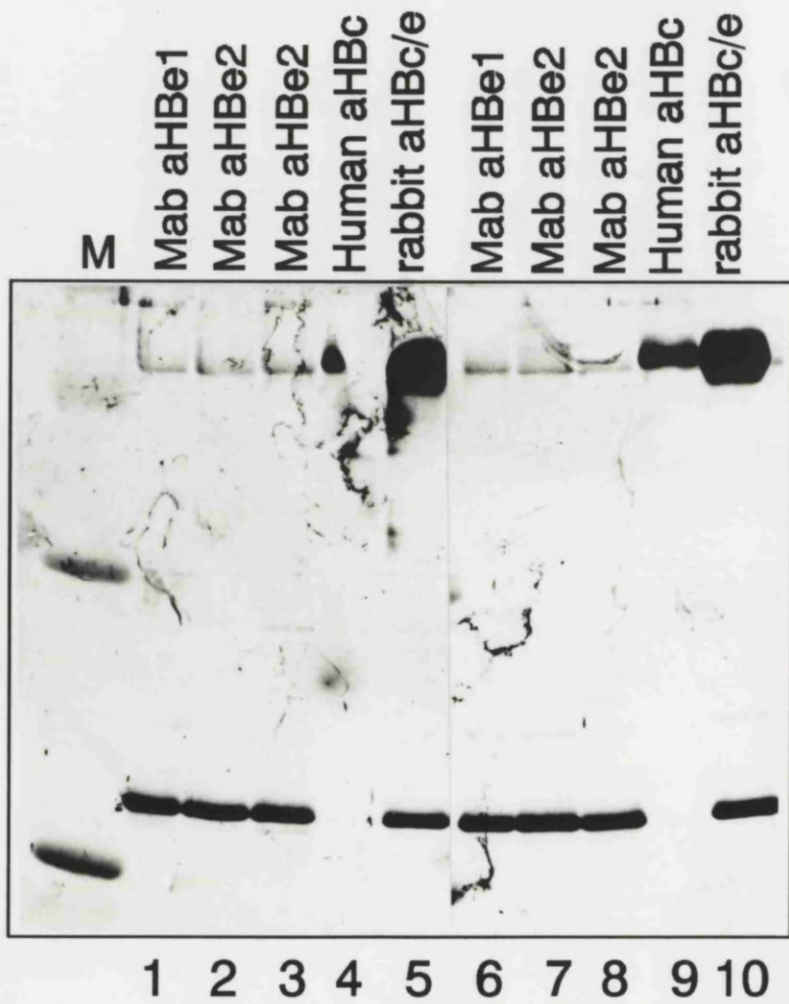


Figure 5.3 Comparisons of molecular size and antigenicity of wild type HBeAg and proline to serine mutant. Lanes 1-5, proline samples; 6-10, serine 15 samples. M = molecular size marker. Both proteins respectively reacted with HBeAg-specific Mabs (Mabs; lanes 1-3 and 6-8) and with hepatitis core/e (HBeC/e)-specific polyclonal rabbit anti-serum (lanes 5 and 10) but not with exclusively HBeAg-specific human serum (lanes 4 and 9).

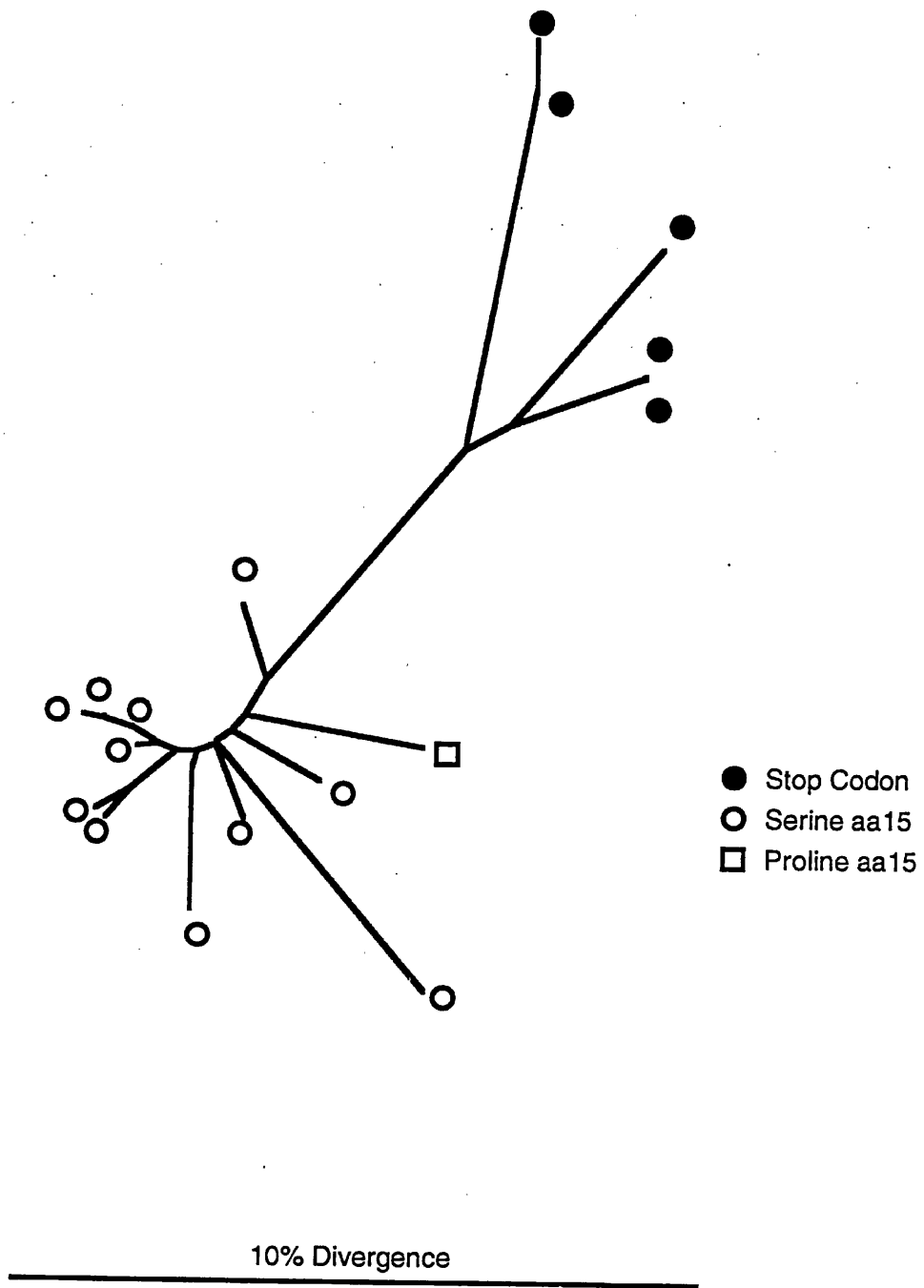


Figure 5.4 Unrooted maximum likelihood tree of 23 HBV sequences. All branch lengths are to scale. Symbols next to branches indicate status of sequences with respect to amino acids at positions 15 (proline or serine) and position 28 (stop codon) of pre-C.

acid substitutions. The underlying mechanism behind this may relate to loss of the tolerance mediated by HBeAg. HBeAg and HBcAg contain identical stretches of protein sequence (Figure 1.9), so it is possible they may share some common epitopes (Chapter 1, section 5); thus, loss of tolerance would lead to an active immune response against core. HBeAg and HBcAg contain B cell, Th cell and CTL epitopes. There is no formal evidence that the substitutions observed arose as a result of escape from immune pressure from either T or B cells. However, previous results from our laboratory (Carman *et al*, 1995) and those presented here show that substitutions are more likely to occur in Th and B cell epitopes. As there are many possible CTL epitopes, it seems unlikely that substantial escape could occur in enough epitopes to allow continued survival (Chapter 1, section 6.4). In this regard, it is also relevant that CTL have proved very difficult to find in patients with chronic HBV infection (Bertoletti *et al*, 1991, Missale *et al*, 1993).

The fact that the serine15 virus is not a predisposing factor to such selection pressures is not entirely surprising. It is found in both the HBeAg- and anti-HBe-positive phases, so it is likely that there is always some HBeAg production. Good evidence for circulating complexes of HBeAg/anti-HBe in patients with recurrent reactivation has been shown by Maruyama *et al* (1993). Thus, in these patients the tolerogenic effect of HBeAg would never be completely lost. The finding that case 10 (who had proline at amino acid 15 but no pre-C stop codon and is thus still capable of producing HBeAg) had an amount of core substitution similar to that in the serine15 infected cases would support this conclusion.

The fact that HBeAg with serine15 is synthesised and secreted and is antigenically similar, if not identical (although this was not formally assessed) to that with proline at amino acid 15, indicates that the previous hypotheses explaining the exclusive nature of T₁₈₅₆ and A₁₈₉₆ were probably incorrect. We now believe that the mutual exclusivity on

genomes of A₁₈₉₆ and T₁₈₅₆ can be explained by the resultant perturbation in the encapsidation signal (ϵ). This is outlined in Figure 5.5. From the predicted structure of ϵ , codons 15 and 28 would appear to lie opposite one another in the first stem. The wild-type sequence for proline (C₁₈₅₆CU) already introduces a degree of mismatch with tryptophan (UG₁₈₉₆G). Introduction of the stop codon (A₁₈₉₆) would have the effect of stabilising the stem, as there would now be perfect base pairing. Serine (U₁₈₅₆CC) would also give a perfect match with G₁₈₉₆ but would introduce a mismatch between T₁₈₅₆ and G₁₈₉₈. Thus, we propose that a subsequent mutation at nucleotide 1896 to A (the stop codon) would result in too many imperfect base pairs and, therefore, instability in the stem resulting in collapse of the structure or, at best, poor encapsidation. Thus, viruses that had these two mutations would fail to encapsidate efficiently and would be taken over by viruses with a replicative advantage. Studies by Tong *et al* (1992 and 1993) have shown that variants in ϵ can influence encapsidation efficiency (Chapter 1, section 4.8) supporting our hypothesis as to why we never observed serine15 and A₁₈₉₆ on the same genome. This does not explain why other mutations, that do not involve ϵ but do inhibit translation of HBeAg, occur only infrequently (e.g. case 3) in these cases.

It appears that strains containing either A₁₈₉₆ or T₁₈₅₆ have an equal replicative and infectious potential, as they have been found in approximately equal numbers in anti-HBe-positive carriers in the Chinese community in Hong Kong. This finding is consistent with the phylogenetic analysis presented here, which shows that such strains are separate lineages (Figure 5.4).

If the development of A₁₈₉₆ is an important part in the biology of HBV during chronic infection, then the reason why viruses with serine15 fail to introduce a different stop codon - there are theoretically nine other stop codons - still remains unclear. Carlier *et al* (1995) have shown that

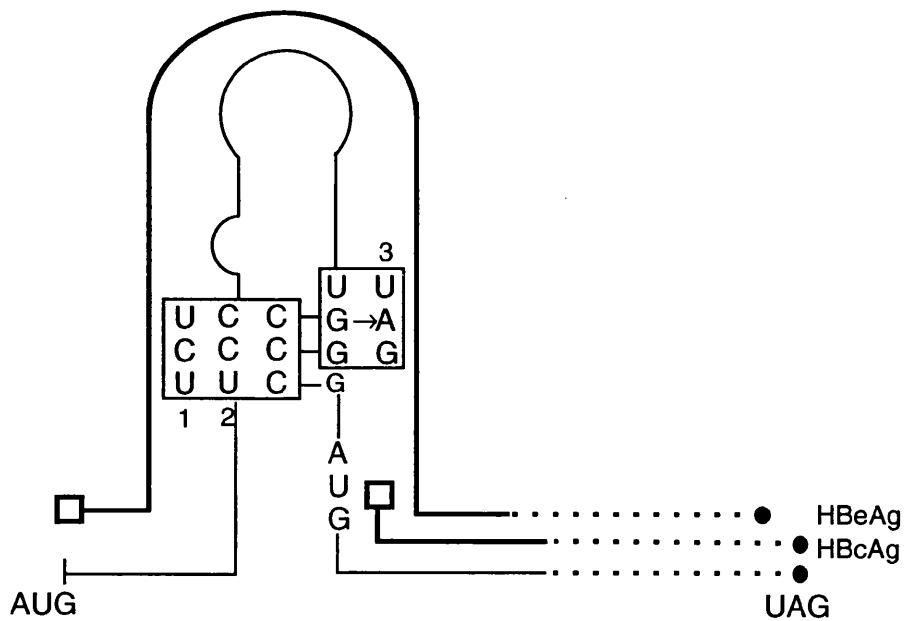


Figure 5.5 The predicted RNA structure of the encapsidation signal of HBV. Translation (bold lines) from the first AUG (□) results in HBeAg. Translation from the second AUG results in HBcAg. Codons 15 and 28 are highlighted in boxes. CCC at codon 15 is proline. UCU or UCC codes serine. Codon 28 is UGG in HBeAg producing strains, substitution of A at position 1896 leads to a stop codon at codon 28.

secretion efficiency of HBeAg could be greatly reduced by truncation of the C-terminal end. Therefore, it could be envisaged that a truncation or mutation(s) in this arginine rich area could have a similar biological effect as A₁₈₉₆. However, none of the samples studied had truncations or common mutations in this area.

That severe disease occurs in patients with serine15 (indistinguishable from those with A₁₈₉₆) who do not have large numbers of core amino acid substitutions, indicates that core variation is not the sole cause of severe disease but a result of immune pressure, which appears less active or less focused in those with the serine15 variant.

5.7 Further plan of investigation

5.7.1 Mutating the encapsidation signal.

Testing of the encapsidation theory was always intended to be included in the study. An attempt at introducing the serine15 into plasmid A (Chapter 4) using PCR mutagenesis was made at the beginning of the study but this failed to prove as straight forward as anticipated. However, due to the lack of category III facilities, I was unable to conduct the desired experiments, and this line of work was abandoned. If the facility was to become available it would be desirable to pursue this avenue of investigation. A study by Tong *et al* (1993) indicates that our hypothesis of the effect these mutations have on ϵ are probable, so this would be purely for academic purposes.

5.7.2 Variability in other HBV proteins.

Variation in the other HBV proteins was not addressed as part of this study. There are no documented studies of the above nature that have looked for substitutions in other HBV proteins and formally associated these with epitopes and progression of disease. Polymerase is unlikely to be a strong immune target as this is a protein which has evolved to serve

several purposes (Chapter 1, section 4.3.4). Variants in HBsAg, particularly in the a determinant, are now well documented (Wallace and Carman, 1997); whether or not such variants could contribute to disease severity is speculation. Perhaps this is an area that could be investigated.

5.7.3 Mutations in the core promoter

Two mutations within the core promoter, at nucleotide positions 1762 and 1764, are well documented (Chapter 1, section 6.3.2) and have been implicated in an HBeAg negative phenotype without a pre-C stop codon (Okamoto *et al*, 1994, Kidd-Ljunggren *et al*, 1996). The effect of these mutations has not been fully investigated, though Nishizono *et al* (1995) investigated their effect on promoter and enhancer activities and found that they were not significantly different from wild type.

Such a mutation in the serine15 viruses could explain the lack of any pre-C stop codons. Thus, sequencing of the serine15 viruses in this area would be a useful exercise.

Chapter 6

Results: Section 3

Mediterranean study

6.1 Aims

As shown in Chapter 5, there is an association with the appearance of HBeAg non-producer strains and core mutations in anti-HBe positive Chinese patients. Patients with severe disease have multiple flares of hepatitis with corresponding peaks of transaminases. As the immune response is believed to be increased during these flares, it is possible that a 'new' mutant is selected with each peak. The work described in this chapter examines in more detail the temporal emergence of core substitutions, answering the following questions:

- at which time point do mutations occur in core?
- do they arise with each ALT peak?
- are they associated with particular regions?
- are the flares selecting 'new' viruses or inversely are the flares themselves caused by the presentation of 'new' viruses to the immune system?

6.2 Selection of patients

The study consisted of patients with chronic hepatitis B infection from Greece and Italy. No patient had undergone interferon therapy. Prof. Hadziyannis (Athens, Greece) kindly provided us with the Greek sera,

which are coded as H followed by the identity number of sample. The sera were from a well followed-up series, taken over several years and encompassed several flares of hepatitis. All the Italian samples, coded A followed by the identity number of the sample, were taken from an earlier study and had been sequenced by either Graham McIntyre or Kathryn Colman in our laboratory.

Two groups were selected from the samples available: 6 HBeAg to anti-HBe seroconverters and 21 continuously anti-HBe positive patients with ongoing hepatitis. For the latter group, initial samples were either the first samples available or were taken as soon after anti-HBe seroconversion was noted, with later samples taken when the patient still had active disease. In the former group, three went on to active disease and three went into clinical remission, losing DNA as measured by hybridisation after seroconversion. These two groups were chosen because although both situations are associated with active hepatitis, the immune effector mechanisms may be different. Times between samples are given in Figures 6.1 and 6.2.

To time the appearance of mutations that emerged between the first and final samples in the continuously anti-HBe group, intermediate samples were sequenced in order of collection only in the regions where mutations had been detected (i.e. the complete HBcAg was not determined). Samples were sequenced in order of collection; no further samples in the series were analysed once the appearance of all the mutations detected in the final sample (compared to the first) had been timed. An additional analysis was performed on a subset of continuously anti-HBe-positive patients to address the possibility of transient mutations. Such mutations would subsequently be cleared by the immune response, so they would not be found in the final sample; this could account for the multiple flares of hepatitis observed in these patients. For this purpose, three of the continuously anti-HBe positive patients had the complete core sequence

Patient	Sex	Age	HBe Status	ALT	Sample Interval	Pre-C Sequence	Epitope																									
							T	50	51	54	57	58	59	62	63	64	65	66	68	74	75	89	90	92	93	107	118	124	138			
							V	S	P	H	A	Q	A	I	W	G	E	N	L	N	N	N	M	F	V	F	V	P	G	R	Q	
remission after seroconversion	A27	F	18	HBeAg Anti-HBe	115 45	0 48	WT A ₁₈₉₆										D E			K N	M/V V	E D										
	A28	M	18	HBeAg Anti-HBe	30 40	0 43	WT A ₁₈₉₆	M R	N H		I V	G D								H/N N		E/D D			L/P P							
	A31	F	22	HBeAg Anti-HBe	110 33	0 51	mix A ₁₈₉₆			Q H	A V	L W	E D																			
active hepatitis after seroconversion	A34	F	41	HBeAg Anti-HBe	145 125	0 47	WT mix	A AS																	V VL							
	A35	M	39	HBeAg Anti-HBe	209 95	0 52	mix A ₁₈₉₆																									
	H20	M	58	HBeAg Anti-HBe Anti-HBe	37 184 337	0 4 22	WT mix A ₁₈₉₆			C R R			T L L							N N NT	N/V V V					P/Q Q Q						

Figure 6.1 Clinical data, number and distribution of amino acid substitutions in patients who seroconverted to anti-HBe during the period of observation. ALT= alanine aminotransferase (Normal<40iu/l), F= female, M= male, WT= G₁₈₉₆, MIX= G₁₈₉₆ and A₁₈₉₆. Only positions at which changes were observed are noted. T, described T-helper cell epitopes in HBcAg ; B, described B-cell epitopes in HBcAg or HBeAg. Mixtures of amino acids are shown as the two observed amino acids separated by a slash. Sample interval is in months. Single letter amino acid codes are given (Appendix 2).

Figure 6.2 Clinical data, number and distribution of amino acid substitutions in continuously anti-HBe positive patients. ALT= alanine aminotransferase (Normal<40iu/l), F= female, M= male, WT= G₁₈₉₆, MIX= G₁₈₉₆ and A₁₈₉₆, X= indicates unresolved sequence at that position. Only positions at which changes were observed are noted. T= described T-helper cell epitopes in HBcAg ; B= described B-cell epitopes in HBcAg or HBeAg. Mixtures of amino acids are shown as the two observed amino acids separated by a slash. Sample interval is in months. Single letter amino acid codes are given (Appendix 2).
*= Patients for which the entire core open reading frame from intermediate samples were sequenced to detect transient mutations.

74 88 107 118 128 138

74 75 77 79 80 81 82 87 92 93 95 96 99 105 110 113 116 117 118 121 130 131 135 137 140 143 147 150 151 153 171 177 180 182

S N E P A S R S N M L K Q I F E L E Y S P A P P L L T R R G P Q E C

G O T
V E A
V E A
V E A

A
V
V
V

N
H
H

P S
Q A
Q A
Q A

A W G
T R S
T R S
T R S

G O AT
V P A

V
A
V
T

Y/C
C
C

M
V
V

NH L
H LI
H I
H I

T A
N M
N M
N M

K
T/K

M
V

F
F
I

E
E/Q
C

HL
L
L
L

E V
Q I

L
I

A
A
A
P

S
Y
Y

P/Q
Q
Q

P
P
A
A

T
A
A

Q P T
P T A
P T A
P T A

A Q
P P

P
P
A
A

S
P
P

F
L
L
L

L S
I C
I C
I C

S
T
T
T

R I
G G

H
H
O

Q
N
N

T
X

E
A

D
D/N

T
T/S
S

P/Q
C
Q

T
A

I
V
V

S/C
C
C

T
AT

Q
O
E

Patient	Sex/Age	ALT	Sample interval	Pre-C Sequence	Epitope T										T																									
					1					20					50					69																				
					12	13	14	18	20	21	23	26	27	28	29	30	31	35	38	40	45	49	50	54	57	58	59	62	64	67	69									
					Consensus S V E L P S F S I R D L L S Y E P S P L Q A I W E N A																																			
H24	F/46	33	0	MIX											V/A	D/E						T						D/E												
		249	13	A1896											A	E						S						E												
		25	17	A1896											A	E						S						E												
H25	M/43	362	0	WT											Y	G						L						N												
		338	2	A1896											T	V						X						WT												
		25	8	A1896											T	V						X						T												
		14	14	A1896											T	V						Q						T												
H27	M/49	31	0	WT																R																				
		119	6	A1896																L																				
		35	34	A1896																L																				
H30	M/30	20	0	MIX	T																				I					E/D										
		312	6	A1896	S																				I					D										
		34	24	A1896	S																				V					D										
H31	M/38	66	0	MIX	V	E				A/S																Q	A													
		305	2	A1896	V	E/Q				A/S																H	V													
		39	22	A1896	L	Q				A																H	V													
		34	29	A1896	L	Q				A																H	V													
H32	M/39	19	0	MIX											Y/F	X						P						G												
		383	15	A1896											Y/F	T						A						A												
		774	35	A1896											F	T						A						A												
H35*	M/52	34	0	MIX	T										V						S	Y	T	S	N/P															
		1368	5	A1896	S										V						A	F	D	T	P															
		490	17	A1896	S										V						A	F	D	T	P															
		19	43	A1896	S										A						A	F	D	T	P															
H37	M/38	38	0	WT	T															G																				
		188	34	A1896	T															G/V																				
		56	37	A1896	S															V																				
		36	65	A1896	S															V																				
A13	M/38	84	0	WT	T/S																				F	D					D									
		41	37	A1896	S																				Y	E					N/S									
																														F	T	A								
H36	M/65	42	0	WT																																				
		142	5	WT																																				
		44	50	WT																																				
A11	F/35	76	0	WT																					F	Q	T					E/D								
		135	26	WT																					Y	E	S					T/P								
																																			D					
A19	M/40	347	0	WT																																				
		96	41	WT																																				
H33*	M/47	15	0	A1896																					F						Q									
		905	23	A1896																					Y						Q									
		468	62	A1896																					Y						N									
H28	F/53	31	0	A1896																																				
		158	25	A1896																																				
		26	64	A1896																																				
H21*	M/62	49	0	A1896																																				
		198	7	A1896																																				
		435	36	A1896																																				
H38	M/63	69	0	A1896																					T/S															
		154	5	A1896																					S															
		556	7	A1896																					S															
A10	M/48	495	0	A1896																																				
		63	46	A1896																																				
A12	M/38	88	0	A1896																																				
		46	52	A1896																																				
A20	M/26	160	0	A1896																																				
		54	43	A1896																																				
A21	M/33	246	0	A1896																																				
		60	57	A1896																																				
A23	M/32	306	0	A1896	T																																			
		54	47	A1896	S																																			

analysed from four or five samples each. Clinical details are given in Figures 6.1 and 6.2. Representative patient profiles are supplied in Figure 6.3.

The greater number of patients in this study, in comparison to the Chinese study (Chapter 5), augmented the reliability of statistical analyses.

6.3 Methods

6.3.1 Extraction and PCR

Extraction was carried out as detailed in 3.1.1. First and second round PCR was carried out as detailed in 3.3.1 and 3.3.2, using primers C1/C2N and C3/C4N respectively (Appendix 1).

6.3.2 Sequencing

Products were purified (3.4) and the entire pre-C/C region sequenced as outlined (3.6.1), using primers C3, C α , 19, C4, KC1 and BC1 (Appendix 1). If sequencing ladders were of poor quality, the first round PCR products were re-nested using a biotinylated primer (C4N) and sequenced as detailed in 3.6.2.

6.4 Statistical analysis

Substitutions observed in sequential samples were correlated with the known CD₄ restricted T-helper epitopes (aa 1-20 and 50-69) and with the known anti-HBc and anti-HBe B-cell epitopes (aa 74-83, 76-89, 107-118, 130-138 and 128-135). The region from amino acid 74 to 123 of core has been described as a B cell epitope (Tordjeman *et al*, 1993): however, this was not included in the analysis as it was considered too large.

The rate of mutation within the defined epitope was compared to the rate of mutation in the remaining core sequence. The relative risk of an amino

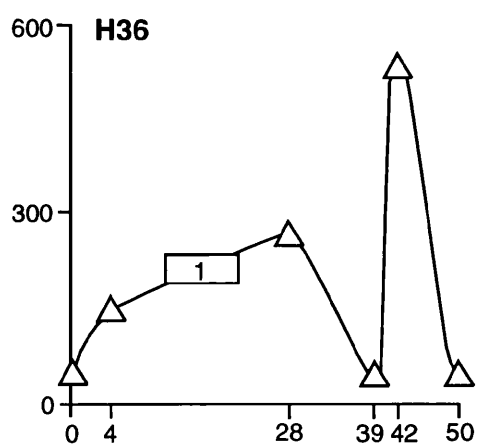
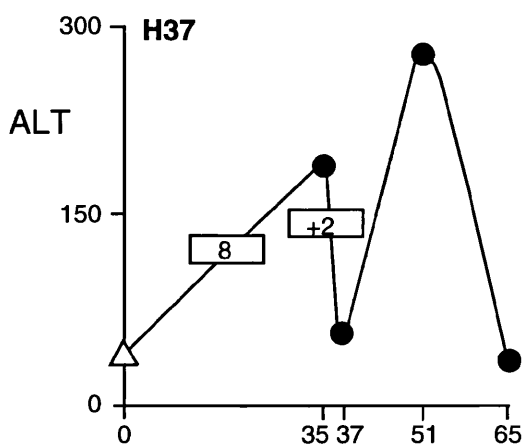
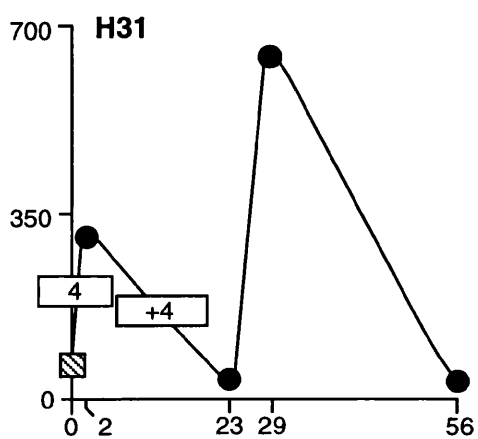
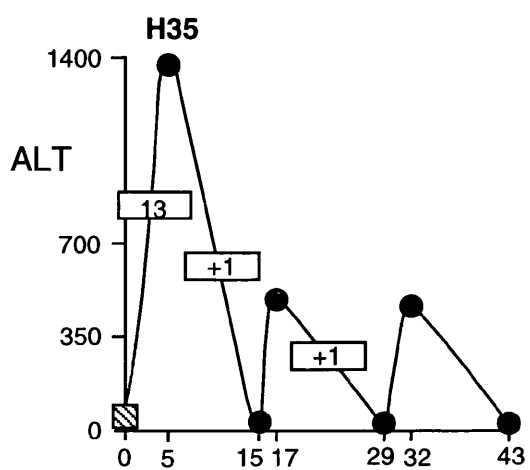
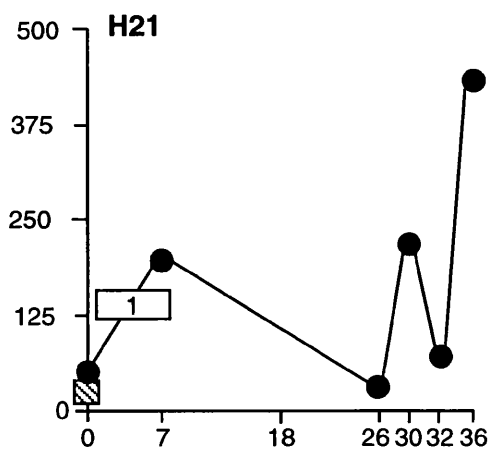
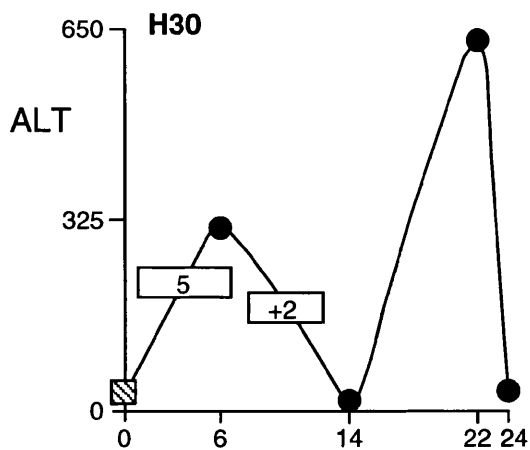
acid substitution occurring within an epitope was calculated using the crude odds ratio. 95% confidence limits were calculated using the Exact method. p values were calculated with the Chi squared test or, when an element with value less than 5 was present, the Fisher's exact method. Comparison of the initial number of amino acid substitutions with the number of substitutions which occurred later was made using the paired t-test.

6.5 Results

6.5.1 Relationship between pre-C and core mutations

There was a strong temporal association between the selection of pre-C mutants that do not allow translation of HBeAg and core protein amino acid substitution (Figures 6.1 and 6.2), as was also observed in Chapter 5. Figure 6.3 demonstrates this in pictorial fashion, highlighting a representative sample of patients. For example, of the 15 substitutions observed for patient H35, 13 occurred as A₁₈₉₆ was evolving, with only 2 subsequent substitutions. Conversely, patient H36 - who failed to develop any pre-C stop codon - developed only one substitution during the period of observation. In the continuously anti-HBe positive cases (H21, H28, H33, H38, A10, A12, A20, A21 and A23) in which A₁₈₉₆ was present in the initial sample, few HBcAg amino acid substitutions appeared between the initial and final samples (range of 1-4, mean 2.1). This contrasts with the other patients in this group, H24, H25, H27, H30, H31, H32, H35, H37, and A13, who selected A₁₈₉₆ between the initial and final samples (range of 5-16, mean 9.0). In the single HBeAg to anti-HBe seroconverter, where multiple samples were available (H20), all observed substitutions appeared by the time a mixture of G₁₈₉₆ and A₁₈₉₆ had emerged. Hence, complete take-over by A₁₈₉₆ is not necessary for the core protein selection process.

Figure 6.3 Serological profiles and HBc amino acid changes in selected anti HBe positive patients with active hepatitis. Alanine aminotransferase levels (ALT) are shown for each time point studied in patients H21, H30, H31, H35, H36, and H37. The pre-C sequence at nucleotide 1896 is indicated by an open triangle (G_{1896}), closed circle (A_{1896}) or hatched square (a mixture of G_{1896} and A_{1896}). The number of HBc amino acid substitutions observed between any two points is shown in the open boxes, but the actual changes can be found in Figure 6.2. A plus sign before numbers indicates that the HBc changes seen in the previous sample are still present in subsequent samples and that the indicated number of further changes have appeared. X-axis is in months.



Months

- △ G1896
- A1896 (pre-core stop)
- ▨ Mix of G1896 and A1896

Interestingly, 95% of the nucleotide changes, both in this Chapter and Chapter 5, led to amino acid substitutions. The functional aspects of the nucleotide changes were not formally addressed. However, in a separate study by Mr. E. Dornan, where the distribution of HBcAg in the cell was examined, in the patients studied in this Chapter, it was shown that there was shift in the distribution of HBcAg from nucleus to the cytoplasm. This occurred in patients who had changes within the nuclear localisation site. Although all the changes observed in this region led to amino acid substitutions it is likely that it is the nucleotide rather than the amino acid change that is having an effect on the distribution of HBcAg.

6.5.2 Clinical correlates and distribution of mutations in HBe to anti-HBe seroconverters

Figure 6.1 shows the results for those who seroconverted, during the period of observation, to anti-HBe. With regard to clinical outcome, patients who went into remission not only selected the greater number of total substitutions - 19 compared to 9 in those who progressed - but also had a higher proportion of substitutions in the common Th epitope from aa 50 to 69 compared to the remaining core sequence (odds ratio 5.64 [95% CI 2.12-14.85]; $p = 0.00045$). Statistical results for all epitopes are given in Table 6.1. In the group with ongoing disease (Table 6.2), there is no apparent correlation between mutations and epitopes. However, if earlier and later samples for A34 and A35 had been available for these patients, we believe we may have observed a greater number of mutations as was the case for the continuously anti-HBe patients (Chapter 6, section 6.5.3).

6.5.3 Timing and distribution of mutations in continuously anti-HBe positive patients

Figure 6.2 shows the distribution of the substitutions in the core protein; overall, a total of 110 amino acid substitutions were observed for all 21 patients. It is important to note that only those positions where changes were detected are noted, so the horizontal distances in Figure 6.2 do not

Table 6.1 Statistical analysis of amino acid substitutions in patients who seroconverted from HBeAg to anti-HBe during the period of observation and went into clinical remission.

Epitope	T/B	Odds ratio	95% confidence interval	p value
1-20	T	0.81	0.13-3.74	0.99
50-69	T	5.64	2.12-14.85	0.00045
74-83	B	0	-	-
76-89	B	0	-	-
107-118	B	1.42	0.02-6.69	0.65
128-135	B	1.04	0.02-7.06	0.99
130-138	B	0.92	0.02-6.18	0.99

The odds ratio and 95% confidence intervals represent the likelihood of amino acid substitutions occurring in the epitope in comparison to the remaining HBc sequence. p values are calculated using the χ^2 test or Fishers exact method. T/B=Th or B cell epitope.

Table 6.2 Statistical analysis of amino acid substitutions in patients who seroconverted from HBeAg to anti-HBe during the period of observation and sustained active disease.

Epitope	T/B	Odds ratio	95% confidence interval	p value
1-20	T	0	-	-
50-69	T	2.37	0.23-12.84	0.25
74-83	B	2.20	0.05-17.36	0.4
76-89	B	0	-	-
107-118	B	1.80	0.04-14.09	0.46
128-135	B	2.81	0.06-22.45	0.33
130-138	B	3.85	0.08-31.46	0.26

The odds ratio and 95% confidence intervals represent the likelihood of amino acid substitutions occurring in the epitope in comparison to the remaining core sequence. p values are calculated using the χ^2 test or Fishers exact method. T/B=Th or B cell epitope.

add up to the full length of the gene. The timing of substitutions was examined in 13 of the 21 cases (H21, H24, H25, H27, H28, H30, H31, H33, H35, H36, H37 and H38) where more detailed series of sera were available (Table 6.3). Most (53/77) substitutions in these 13 cases occurred between the initial and second samples (range 1-15, mean 5.9) rather than between the second and final samples (range 1-4, mean 1.85) $p = 0.0069$ (paired t test). Of the 110 substitutions in all 21 patients, 61 occurred in B- or Th cell epitopes (odds ratio 1.69 [95% CI 1.13 - 2.52]; $p = 0.007$). Table 6.4 shows the statistical analysis of the correlation with particular epitopes. Significant numbers of substitutions were found in the B-cell epitopes from amino acids 74 to 83 (odds ratio 2.63 [95% CI 1.36-4.74]; $p=0.00067$), from amino acids 128 to 135 (odds ratio 2.15 [95% CI 0.94-4.37]; $p=0.03$) and from amino acids 130 to 138 (odds ratio 2.56 [95% CI 1.35-4.80]; $p=0.0014$). The number of mutations found in the common Th epitope from amino acids 50 to 69 verged on the level of significance (odds ratio 0.55 [95% CI 0.28-1.08]; $p=0.06$). Figure 6.3 shows that even though multiple flares of hepatitis occurred in these patients, extra substitutions seldom emerged before or after each peak.

Finally, the entire pre-C/C region of 5 sequential serum samples from each of patients H21 and H33, along with 4 sequential samples from patient H35, were sequenced to search for transient substitutions: none were found.

6.6 Discussion

In this section of the thesis, the emergence of core substitutions was examined in continuously anti-HBe patients; their appearance was timed and correlated with known humoral and Th cell epitopes. These data were compared to those generated in HBeAg to anti-HBe seroconverters because although both situations are associated with active hepatitis, it is possible that different immune effector systems are in operation.

At the beginning of the study, seven of the continuously anti-HBe patients were infected with HBeAg producing strains and five had a mixture of producer and non-producer strains, confirming results from previous studies (Chapter 5) that there is an initial burst of core substitutions with the simultaneous emergence of a pre-C mutant. This is most likely due to the loss of immunotolerance, as HBeAg is believed to induce immunotolerance both against itself and HBcAg (which shares much of the protein sequence), with the result that loss of HBeAg leads to an enhanced immune response, and thus selection pressure, against HBcAg. Ongoing selection does occur after this event, but at a greatly reduced rate. Most of the substitutions detected occurred between the first and second samples (Table 6.3) and, although there were subsequent substitutions, these could not be associated temporally with the ALT peaks observed in these patients. It seems unlikely, therefore, that a 'new' virus gives rise to a fresh episode of disease, as one would then expect a novel virus at each peak. Despite several episodes of disease, however, it was noted that once a substitution arose, it remained until the final sample. Also, few substitutions were observed subsequent to the initial burst. The correlation in this case appears to be with the emergence of a stop codon rather than a flare of hepatitis.

The flaw in only sequencing the area in which substitutions had arisen (enabling us to time their appearance) is that this would only highlight viruses that were maintained, and not those that were cleared by the immune system. To address this possibility, three patients (H21, H33 and H35), who had experienced several transaminase flares, were selected and the entire pre-C/C sequence from all the samples available, was sequenced. This did not reveal any transient substitutions. This is not what we would predict were the immune system being stimulated (causing the hepatitis flares) by a 'new' virus.

Table 6.3 HBc amino acid changes in sequential samples from continuously anti-HBe positive patients with active hepatitis.

Pre-C sequence	G ₁₈₉₆ /mix→A ₁₈₉₆			G ₁₈₉₆ (no change)			A ₁₈₉₆ (no change)		
	T	B	O	T	B	O	T	B	O
Distribution	(39)*	(36)*	(107)*	(39)*	(36)*	(107)*	(39)*	(36)*	(107)*
Total no of patients	9 patients			3 patients			9 patients		
1st to last sample	19	27	35	2	3	4	5	5	9
No of patients with >2 samples in each group	8 patients			1 patient			4 patients		
1st to last sample	16	21	27	0	0	1	3	2	6
1st to 2nd sample	11	17	25	0	0	0	0	2	5
2nd to last sample	5	4	2	0	0	1	3	0	1

- T = Number of changes within T helper cell epitopes;* Figure in brackets denote the number of core amino acids encompassed by T helper epitopes
- B = Number of changes within B cell epitopes;* Figure in brackets denote the number of core amino acids encompassed by B cell epitopes
- O = Number of changes outside epitopes;* Figure in brackets denote the number of core amino acids outside epitopes
- A₁₈₉₆= Pre-C stop codon
- G₁₈₉₆= No stop codon in pre-C sequence
- G₁₈₉₆/mix→A₁₈₉₆ Indicates that there was no A₁₈₉₆ or a mixture of G₁₈₉₆ and A₁₈₉₆ in the first sample but A₁₈₉₆ was found in a subsequent sample

Table 6.4 Statistical analysis of amino acid substitutions in HBcAg in patients with active disease who remained anti-HBe positive throughout the period of observation.

Epitope	T/B	Odds ratio	95% confidence interval	p value
1-20	T	0.86	0.88-1.52	0.59
50-69	T	0.55	0.28-1.08	0.06
74-83	B	2.63	1.36-4.74	0.00067
76-89	B	1.21	0.59-2.43	0.56
107-118	B	1.31	0.61-2.71	0.45
128-135	B	2.15	0.94-4.37	0.03
130-138	B	2.56	1.35-4.80	0.0014

The odds ratio and 95% confidence intervals represent the likelihood of amino acid substitutions occurring in the epitope in comparison to the remaining HBc sequence. p values are calculated using the χ^2 test or Fishers exact method. T/B=Th or B cell epitope.

These results hint at the immune effectors that force viral escape during chronic hepatitis. In earlier cross-sectional analyses, where comparisons were made to a consensus sequence, anti-HBe-positive cases with active disease had greater numbers of mutations in the commonly recognised Th epitope (between amino acids 50 to 69) as well as in two of the three anti-HBc/e B-cell epitopes. In contrast, in the sequential samples analysed in this chapter, where sequences were compared to their predecessors, patients who went into remission appeared to select more mutations in the Th epitope and those with ongoing disease selected mutations in B-cell epitopes. There may be some pressure on the common Th epitope as the number of mutations in this area was verging on significance. In the cross-sectional analysis the number of Th mutations represented the accumulated mutations over time which are therefore consistent with these current data.

The influence of substitutions in the other three proteins was not addressed in this study, but the results indicate that the mutant strains are escaping from the immune response against core protein, rather than causing peaks of hepatitis.

6.7 Implications

In acute HBV infection, there is a strong CTL response which clears infected hepatocytes (Bertoletti *et al*, 1991, Penna *et al*, 1991, Missale *et al*, 1993); however, this is absent in chronic hepatitis. These results may help to explain why these patients have ongoing disease in the absence of CTL. In those who remit, we hypothesise that the virus has escaped the Th response, allowing disease-free persistence. The reason why there is not a high level of viraemia in these patients is not clear, but it is possible there are two independent Th responses, one of which may stimulate production of effective antibodies. In Figure 6.1 it can be seen that in the clinically remitted cases, no amino acid changes were seen in the Th

epitope from amino acids 1 to 20 or in the B-cell epitopes. However, in those with ongoing disease after seroconversion, we believe there is an ineffective antibody response against infected hepatocytes which does not clear infection from all cells, but kills a proportion of infected hepatocytes. This puts the virus under selection pressure, manifested by focused substitution in B-cell epitopes. The few mutations in Th epitopes in these cases is in keeping with this hypothesis.

If such a finding were upheld there is a clear implication for therapeutic vaccination: vaccines that mainly stimulate antibody are unlikely to clear virus from infected carriers. A vaccine which includes either novel Th epitopes, which may be needed to enhance antibody effectors, or one that stimulates CTL may be more effective in clearing virus from chronic HBV infected individuals.

Chapter 7

Results: Section 4

Overlapping peptide series

7.1 Aims

There has been much debate as to whether the proposed immune selection pressure on HBcAg is humoral or T-cell mediated (or both). In Chapters 5 and 6, we showed that the number of substitutions in B-cell epitopes in continuously anti-HBe positive patients was statistically significantly greater compared to other regions of the core protein, yet in those who seroconverted to anti-HBe and went into remission, there was selection in Th cell epitopes. To provide direct evidence for such immune escape we decided to construct a series of peptides, covering the entire core region, to use in an ELISA system to test for humoral escape, and in lymphoproliferation assays to test for Th cell escape.

7.2 Preliminary study

7.2.1 Background

A number of groups have used peptides to delineate epitopes (humoral, CTL and Th) in both HBcAg (Sallberg *et al*, 1991, Salfeld *et al*, 1991, Penna *et al*, 1991, Bertoletti *et al*, 1993) and HBsAg (Manivel *et al*, 1992). Several of these groups had difficulty in showing reactivity of patients' sera to peptides. As the use of patients' sera would be the basis of our experiments, this would clearly be a major problem for our studies.

In previous studies, peptides were fairly short, around 10 to 15 amino acids in length. The major B-cell epitope (from amino acids 74 to 83) is believed to be part of a larger conformational epitope, therefore we propose that such short peptides may have failed to fold into the correct conformation. Second, all the groups had used monomeric peptides. We had the facility to construct peptides with more than one branch (multiply antigenic peptides [MAPs]). This technique was originally developed by Tam (1988) who observed that antibodies could be detected with a lower amount of branched peptides than monomeric peptides (Tam and Zavala, 1989). Further characterisation of branched peptides was carried out by C.A. Robertson (PhD thesis, University of Glasgow, 1994). One of the probable reasons for increased sensitivity may be bivalent binding between the two arms of the antibody and epitopes on two arms of the branched peptide, providing increased stability of the bond. However, an epitope in close proximity to the polylysine core may not be able to bind to the two paratopes on the two arms of the reactive antibody. Therefore, Robertson (1994) tested the effect of varying the distance between the epitope and the polylysine core by the introduction of glycine residues (acting as spacers) and came to the conclusion that a spacer of four or five glycine residues markedly increased the reactivity in the system tested.

As a preliminary experiment, reactivity of anti-HBc positive serum to peptides was tested. Large peptides can have problems such as folding back on themselves during synthesis which can result in;

1. the peptide not achieving its full complement of amino acids
2. steric hindrance caused by close proximity of the peptide chains
3. poor solubilisation of the final product.

7.2.2 Oligopeptides

As the previous studies employed short monomeric peptides without much success and the facilities were available to construct branched peptides,

we decided to test long, branched peptides against known anti-HBc positive sera.

7.2.3 Methods

A peptide of 30 amino acids in length, spanning the described major epitope of HBcAg between amino acids 74 and 83, was synthesised in monomeric (peptide 414-a) (3.12.2) and branched: two (peptide 414-b), four (peptide 414-c) and eight (peptide 414-d) branch configurations (Table 7.1) (3.12.1). Four glycine spacers were inserted between the lysine core and the peptide.

7.2.3.1 Cleavage and dissolution of peptides

Peptides were cleaved (3.13) and dissolved as follows: peptide 414-a was soluble in water, while peptides 414-b, -c and -d required the addition of 33% acetic acid to achieve dissolution (3.18). Peptides were analysed by reverse-phase HPLC (3.15) but not purified further.

7.2.3.2 ELISA

The method followed is detailed in 3.19. The comparative reactivity of peptides 414-a, -b and -c, were tested in ELISA with anti-HBc positive and negative human sera (supplied by the diagnostic virology department, Western Infirmary, Glasgow). A range of peptide concentrations (0.156-80 µg/well) was tested against sera at 1:50, 1:100, 1:200, 1:400 and 1:800 dilutions.

7.2.4 Results

Figure 7.1 shows the results at a serum dilution of 1:50. The monomeric peptide gave the poorest reactivities at all concentrations. Although the branched peptides gave higher reactivities, it was not as high as we would have anticipated (from work in other viral systems).

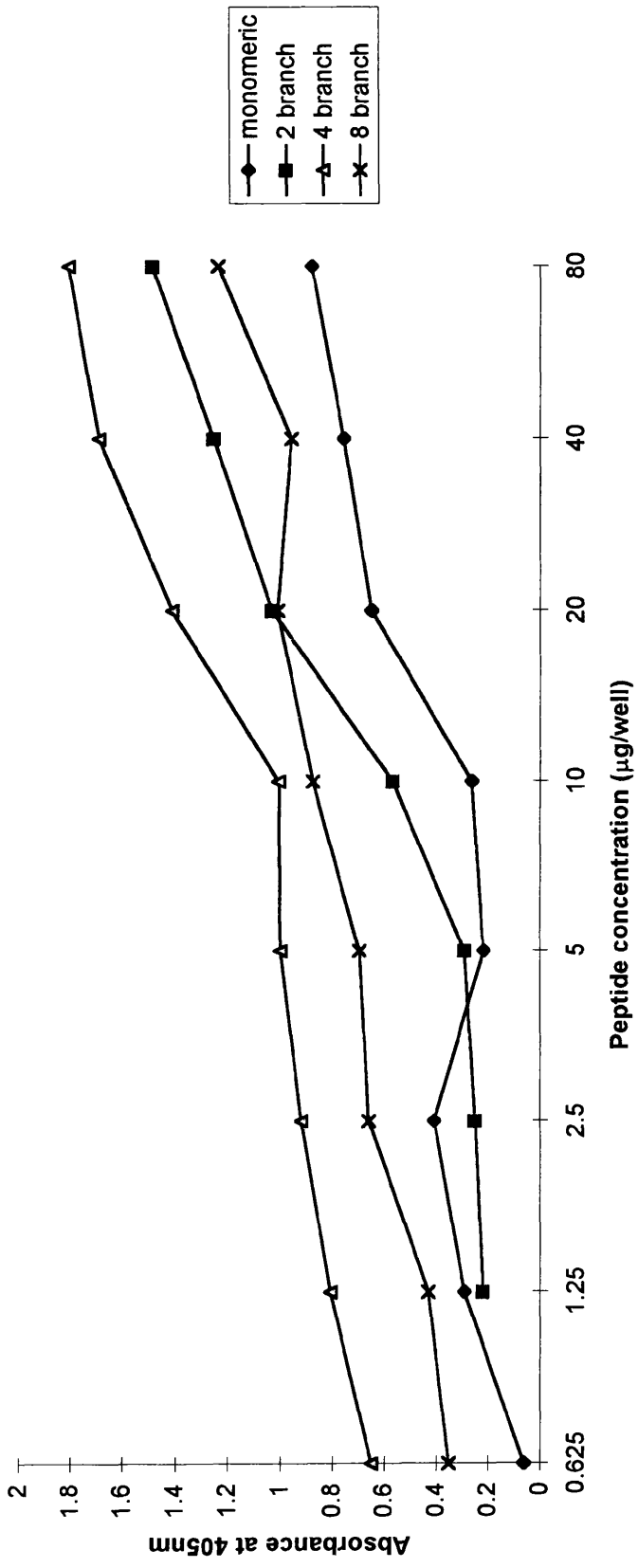


Figure 7.1 Reactivity of anti-HBc positive serum with varying amounts of peptides (414-a, -b, -c and -d). Serum diluted at 1:50. 414-a = monomeric peptide, 414-b = 2 branched peptide, 414-c = 4 branched peptide, 414-d = 8 branched peptide.

Table 7.1 Configuration, sequence and charge of peptides used in preliminary study

Peptide	Branched or monomer	aa	Sequence	Charge
414-a	M	68-97	LATWGGNLEDPISRDLVSYVNTNMGLK	-2
414-b	B(2)	68-97	LATWGGNLEDPISRDLVSYVNTNMGLK	-2
414-c	B(4)	68-97	LATWGGNLEDPISRDLVSYVNTNMGLK	-2
414-d	B(8)	68-97	LATWGGNLEDPISRDLVSYVNTNMGLK	-2

aa=amino acid

Single letter aa codes are given in Appendix 2

M= monomeric peptide

B= branch chain peptide

Figure in brackets = number of branches

Figure 7.2 shows the results at a serum dilution of 1:200. The monomeric peptide gave almost negligible reactivities at this dilution. Again, the branched peptides (414-b, 414-c and 414-d) exhibited better reactivities when compared to 414-a; 414-c and 414-d demonstrated greater reactivities than 414-b.

7.2.5 Conclusions of the preliminary study

The results indicated that it was possible to achieve reactivity using serum and that the branched peptides were more reactive against anti-HBc than the monomeric peptide. Therefore, an overlapping series of peptides of 30 amino acids in length was synthesised, with four glycine spacers in a four branch configuration. As there was little apparent difference in antibody binding between the four and eight branches, it was hoped that the four branch configuration may behave better in terms of folding and solubility.

7.3 Overlapping peptide series: Synthesis

7.3.1 Peptide synthesis

All peptides were synthesised in a four branch configuration (3.12.1) and were cleaved as detailed in 3.12. Peptide sequences are detailed in Table 7.2

Four other peptides were also synthesised. Results from Chapters 5 and 6 and a previous study by Carman *et al* (1995) showed three frequent amino acid changes; from threonine (peptide Th₁) to serine (peptide Th₂) at amino acid 12, from glutamic acid to aspartic acid at amino acid 64 (peptide ThP₆₄) and from threonine to asparagine at amino acid 67 (peptide ThP₆₇). Peptide ThP_n had glutamic acid at amino acid 64 and threonine at amino acid 67. As all of these amino acids reside in Th epitopes (amino acids 1 to 20 and 50 to 69), we decided to construct these 'mutant' peptides to test them in lymphoproliferation studies.

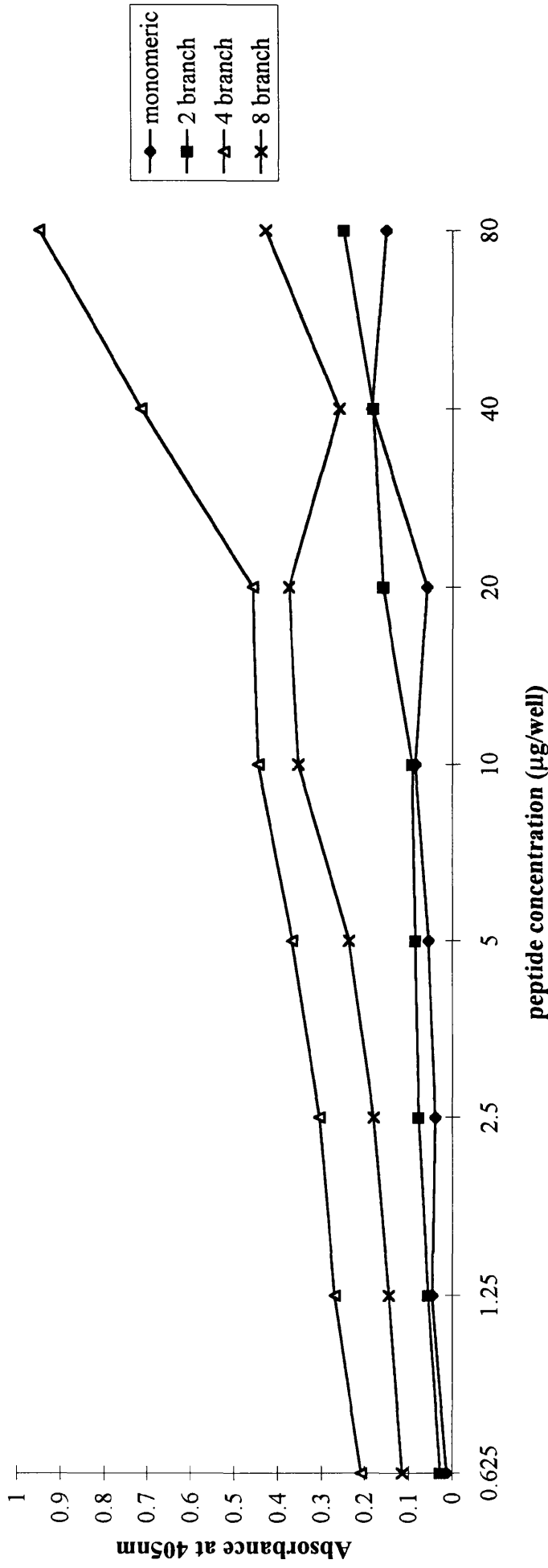


Figure 7.2 Reactivity of anti-HBc positive serum with varying amounts of peptides (414-a, -b, -c and -d). Serum diluted at 1:200. 414-a = monomeric peptide, 414-b = 2 branched peptide, 414-c = 4 branched peptide, 414-d = 8 branched peptide.

Table 7.2 Peptide name, sequence and charge from the initial overlapping peptide series

Peptide	aa	Sequence	Charge
P22-4/Th ₁	1-23	MDIDPYKEFGATV ELLS FLPSDF	-4
P22-5	4-33	DPYKEFGATV ELLS FLPSDFFPV RD LLDT	-4
P22-6	14-43	ELLSFLPSDFFPV RD LLDTASALYREALE	-4
P22-7	24-53	FPSV RD LLDTASALYREALESP EH CSPHHT	0
P22-8	34-63	ASALYREALESP EH CSPHHTALRQAILCWG	+2
P22-9	44-73	SPEHCSPHHTALRQAILCWGELMTLATWVG	+2
P22-10	54-83	ALRQAILCWGELMTLATWVG GN LEDPISRD	-2
P22-11	64-93	ELMTLATWVG GN LEDPISRDLVSYVNTNM	-3
P22-12	74-103	GNLEDPISRDLVSYVNTNMGLKFRQLLWF	0
P22-13	84-113	LWSYVNTNMGLKFRQLLWFHISCLTFGRE	+3
P22-14	94-123	GLKFRQLLWFHISCLTFGRETVIEYLV S FG	+2
P22-15	104-133	HISCLTFGRETVIEYLV S FGWIRTPPAYR	+2
P22-16	114-143	TVIEYLV S FGWIRTPPAYRPPNAPILSTL	+1
P22-17	124-153	WIRTPPAYRPPNAPILSTLPETT V RRRG	+4
P22-18	134-163	PPNAPILSTLPETT V RRRGRSPRRRTPSP	+6
P22-19	144-173	PETT V RRRGRSPRRRTPSPRRRRSQSPRR	+12
P22-20	154-183	RSPRRRTPSPRRRRSQSPRRRRSQSRESQC	+12
Th ₂	1-23	MDIDPYKEFGA S V ELLS FLPSDF	-4
ThP _n	44-73	SPEHCSPHHTALRQAILCWGELMTLATWVG	+2
ThP ₆₇	44-73	SPEHCSPHHTALRQAILCWGELM N LATWVG	+2
ThP ₆₄	44-73	SPEHCSPHHTALRQAILCWG D LMTLATWVG	+3

All peptides were synthesised in a four branched configuration, aa= amino acid position in HBcAg
 Single letter aa codes are given in Appendix 2

7.3.2 Dissolution of branched peptides

It was envisaged that, due to the length and branched nature of the peptides there may be problems with solubility. Figure 7.3 shows a hydrophobicity/philicity plot of HBcAg; peaks above the horizontal line indicate areas with a positive charge and peaks below indicate areas which are negatively charged. As solubility depends on the charge of the peptide, possible problem areas were the positively charged carboxy terminal, a highly arginine-rich region (which would also have the problem of side-chain protecting groups), and the negatively charged central region. To aid dissolution of positively charged peptides, bubbling of NH_3 gas is often employed; addition of acid can aid dissolution of those that are negatively charged.

It is usual to begin a synthesis at the carboxy terminal when making an overlapping series. On synthesis of peptides P22-18, -19, -20, all were soluble in water without the addition of NH_3 . However, peptides P22-10, -12, -13, -14, -15, and P22-16 proved to be insoluble even with the addition of acid (Table 7.3 details solubilities of peptides from the initial synthesis).

In consultation with Dr. H. Marsden (Institute of Virology, Glasgow), three lysine or three aspartic acid residues were added to peptides P22-10, -12, -13, -14, -15, -16 with the aim of increasing solubility.

Solubility results of these latter peptides are given in Table 7.4. Addition of three lysine residues to P22-12 did improve its solubility but the other peptides remained insoluble. Peptides P22-10, -13, -14, -15, -16 were then resynthesised as 20 mers (in a four branch configuration) and renamed as P22-10N, -13N, -14N, -15N, -16N but all proved to be insoluble in water, acetic acid and NH_3 .

Table 7.3 Solubilities of peptides from the initial synthesis

Peptide	charge	Solubility		
		H ₂ O	acetic acid	NH ₃
P22-4	- 4	-	-	+
P22-5	- 4	-	-	+
P22-6	- 4	-	-	+
P22-7	0	+	-	-
P22-8	+ 2	+	-	-
P22-9	+ 2	-	+	-
P22-10	- 3	-	-	-
P22-11	- 3	-	-	+
P22-12	0	-	-	-
P22-13	+ 2	-	-	-
P22-14	+ 3	-	-	-
P22-15	0	-	-	-
P22-16	+ 1	-	-	-
P22-17	+ 4	+	-	-
P22-18	+ 6	+	-	-
P22-19	+ 12	+	-	-
P22-20	+ 12	+	-	-

Table 7.4 Solubility results for peptides on addition of three lysine or aspartic acid residues.

Peptide	charge	SOLUBILITY +K ₃			SOLUBILITY +D ₃		
		H ₂ O	acetic acid	NH ₃	H ₂ O	acetic acid	NH ₃
P22-10	- 3	-	-	-	-	-	-
P22-12	0	+	-	-	-	-	-
P22-13	+ 2	-	-	-	-	-	-
P22-14	+ 3	-	-	-	-	-	-
P22-15	0	-	-	-	-	-	-
P22-16	+ 1	-	-	-	-	-	-

Lysine = K

Aspartic acid = D

7.3.3 Monomeric synthesis

As the branched configuration, which have an increased charge, was probably contributing to the insolubility of the peptides and we wished to maintain the overlapping series, peptides P22-10, -13, -14, -15, -16 were synthesised as monomers (3.12.2), firstly as 30 mers, but as these also proved to be insoluble, subsequently as 20 mers (P22-10M, -13M, -14M, -15M, -16M) (Sequences given in Table 7.5).

7.3.4 Dissolution of monomeric peptides

When synthesised as monomeric 20 mers the peptides were soluble in acetic acid though with much reduced yields (Table 7.6).

7.3.5 Purification of peptides

Purification can sometimes improve the solubility, thus it was decided to purify all the peptides. All peptides were subsequently purified by Dr. Ania Oswianka (Institute of Virology, University of Glasgow) by reverse phase high pressure liquid chromatography (HPLC) using a Vydak-FB1520 column (3.16).

7.3.6 Amino acid analysis of peptides

The amino acid content of the monomeric peptides (3.14.1) and branched peptides (3.14.2) were analysed. Except peptide P22-8, all peptides had the correct amino acid content. P22-8 was resynthesised and reanalysed and subsequently found to have the correct amino acid content.

7.4 Functional analysis of HBcAg peptides

The peptides were used to examine the T- and B-cell response in patients with HBV infection. Primarily, we wanted to examine the nature of the ALT flares observed in patients with a similar clinical background as those studied previously (Chapters 5 and 6) but other outcomes of infection (i.e. acute and fulminant) were also studied. In this section of the work we

Table 7.5 Peptide name, sequence and charge from the monomeric peptide synthesis

Peptide	aa	Sequence	Charge
P22-10M	54-83	ALRQAILCWGELMTLATWVGGNLEDPISRD	-2
P22-13M	84-103	LVSVYVNTNMGLKFRQLLWF	+2
P22-14M	94-113	GLKFRQLLWFHISCLTFGRE	+3
P22-15M	104-123	HISCLTFGRETVIEYLVSFG	0
P22-16M	114-133	TVIEYLVSFGVWVRTPPAYR	+1

All peptides were synthesised as monomers, aa= amino acid position in HBcAg
 Single letter aa codes are given in Appendix 2

Table 7.6 Solubility results for the 30- and 20-mer monomeric synthesis

Peptide	charge	30-mer monomer			20-mer monomer		
		H ₂ O	acetic acid	NH ₃	H ₂ O	acetic acid	NH ₃
P22-10	- 3	-	-	-	-	-	+
P22-13	+ 2	-	-	-	-	+	-
P22-14	+ 3	-	-	-	-	+	-
P22-15	0	-	-	-	-	+	-
P22-16	+ 1	-	-	-	-	+	-

collaborated with Professor Hadziyannis (Athens, Greece) as we did not have access to many hepatitis cases (over a period of six months there were only two acute cases in Glasgow). Professor Hadziyannis has a large repertoire of patients and expertise in this area of study.

7.4.1 Lymphoproliferation studies: results

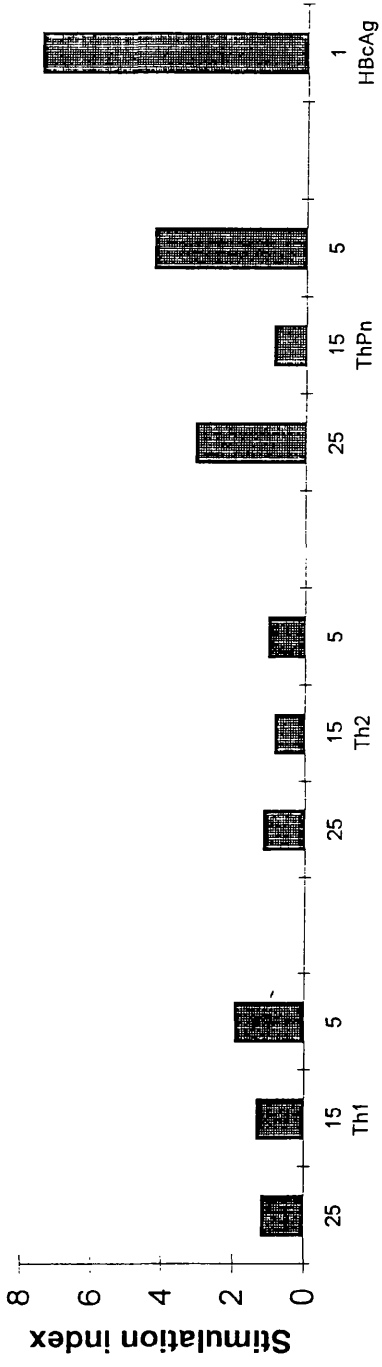
Study 1(Glasgow) The lymphoproliferative response of five non-symptomatic chronic carriers against peptides Th₁, Th₂ and ThP_n were tested using the whole blood assay (3.21.1), the results are given in Figure 7.4; the results of stimulation with phytohaemagglutinin (PHA) are given in Figure 7.5. The PHA results are plotted separately as the values obtained far exceeded those obtained for the peptides. When the PHA and peptide results are plotted together the graph produced is meaningless.

A stimulation index (SI) > 3 indicates a response to the antigen tested. Patients 4 and 5 failed to respond to either peptides or HBcAg, but as their SIs to PHA are low this is not surprising. Patients 1, 2 and 3 responded well to PHA and HBcAg. However, they failed to respond to Th₁ or Th₂ but there was a response to ThP_n.

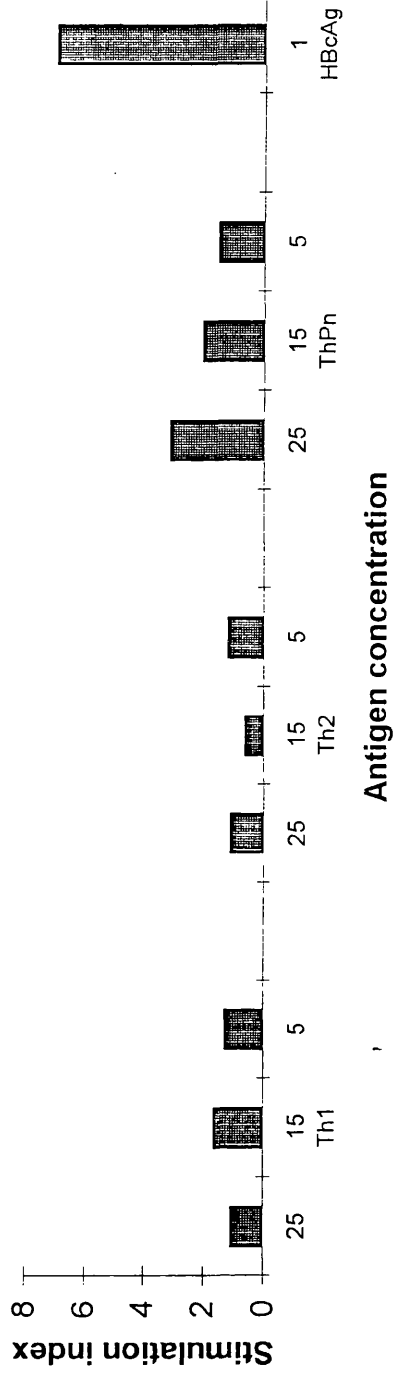
The PBMCs of two acute hepatitis patients were tested for lymphoproliferation (3.21.2) to Th₁, Th₂, ThP_n and P22-16M (this peptide contains amino acids 117 to 131, described as a Th epitope by Ferrari *et al* (1991) [Figure 7.6]). Unfortunately, due to a breakdown in the cell harvester, the cells from one of the patients could not be read.

The other patient responded well to PHA (43820cpm.). In contrast to the results observed with the chronic patients, the acute hepatitis case failed to respond to ThP_n. However, there was a response seen with Th₁, Th₂ and P22-16M.

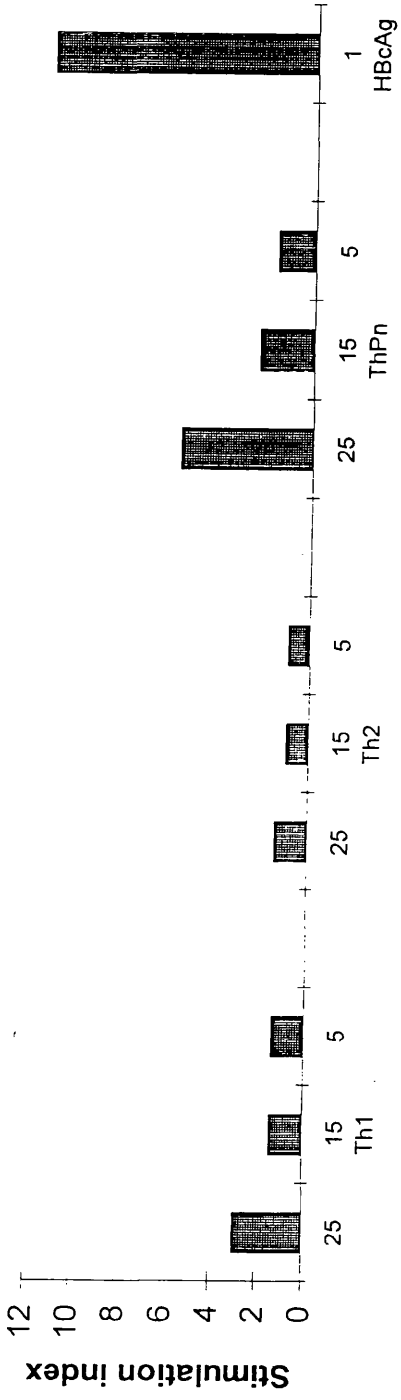
Patient 1



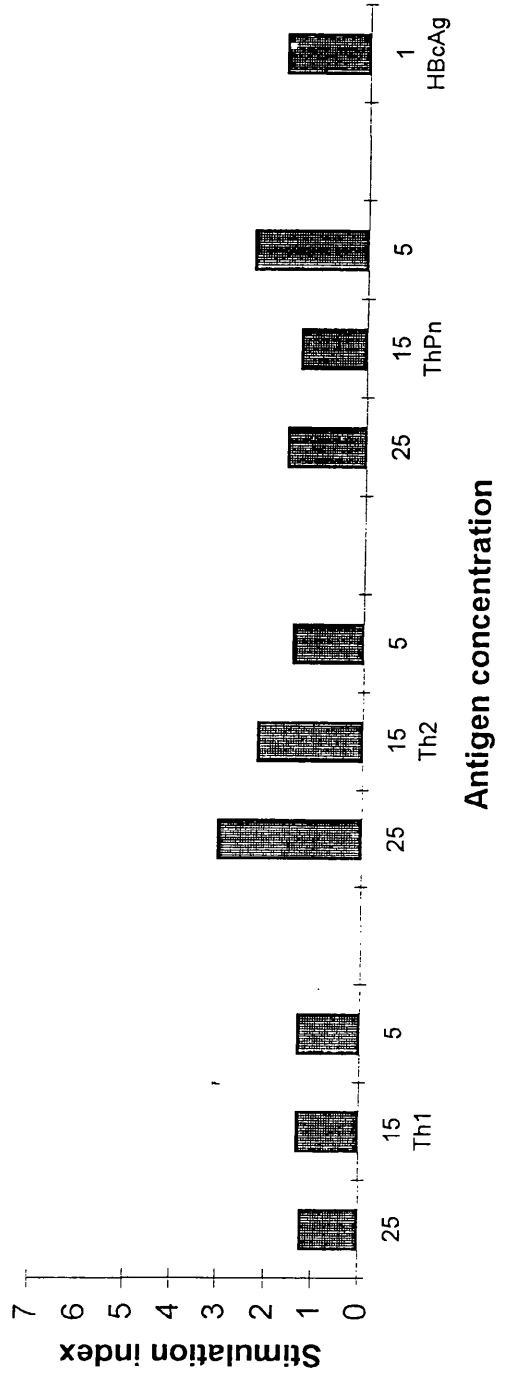
Patient 2



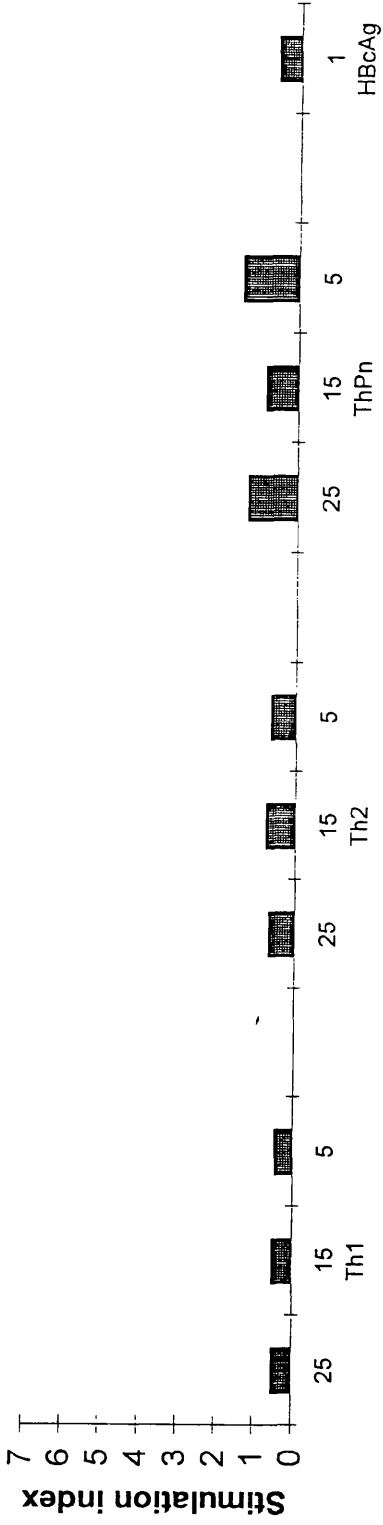
Patient 3



Patient 4



Patient 5



Control

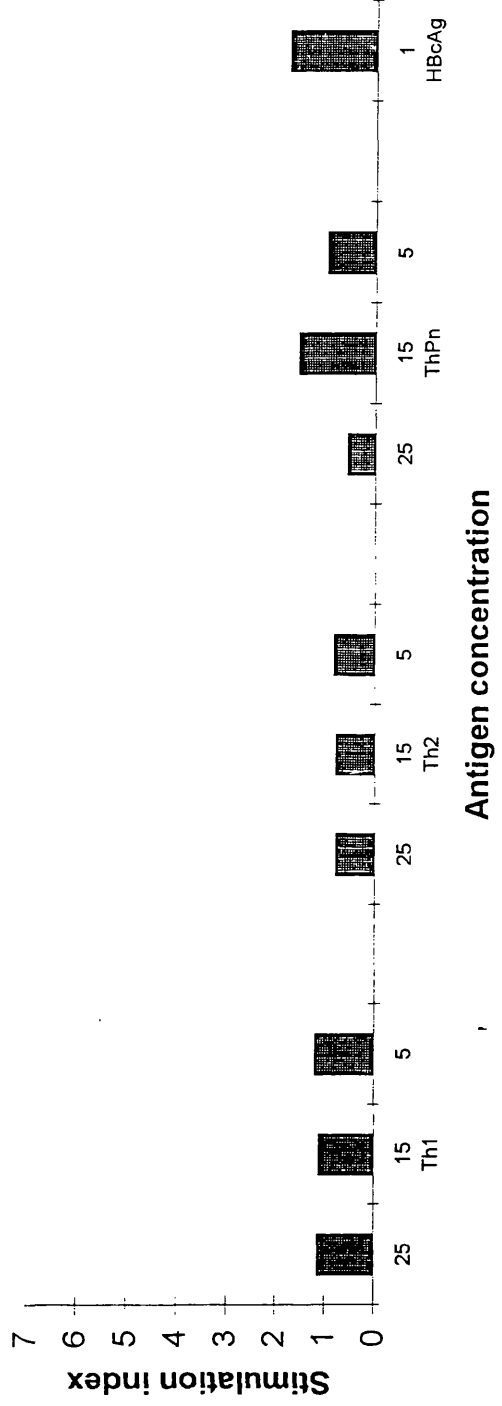
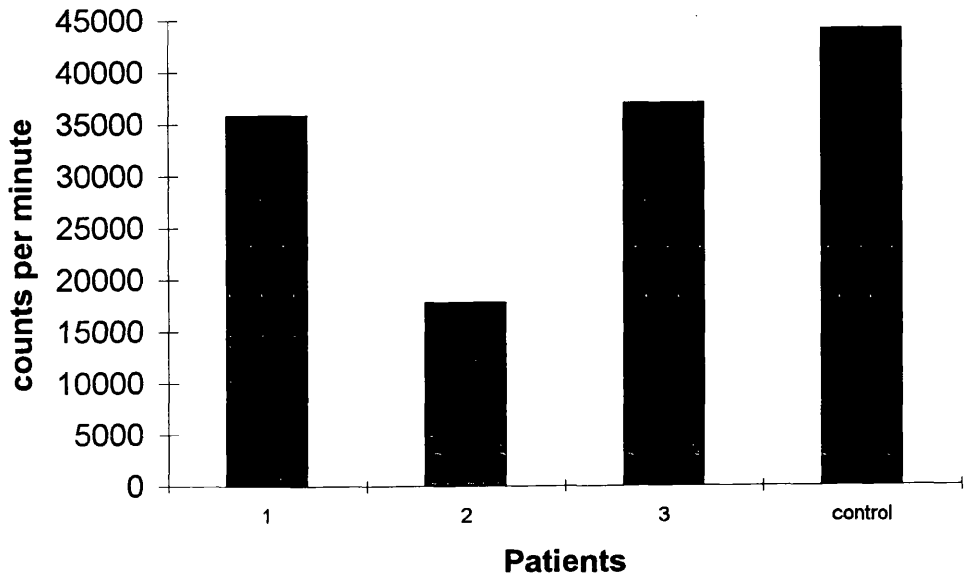


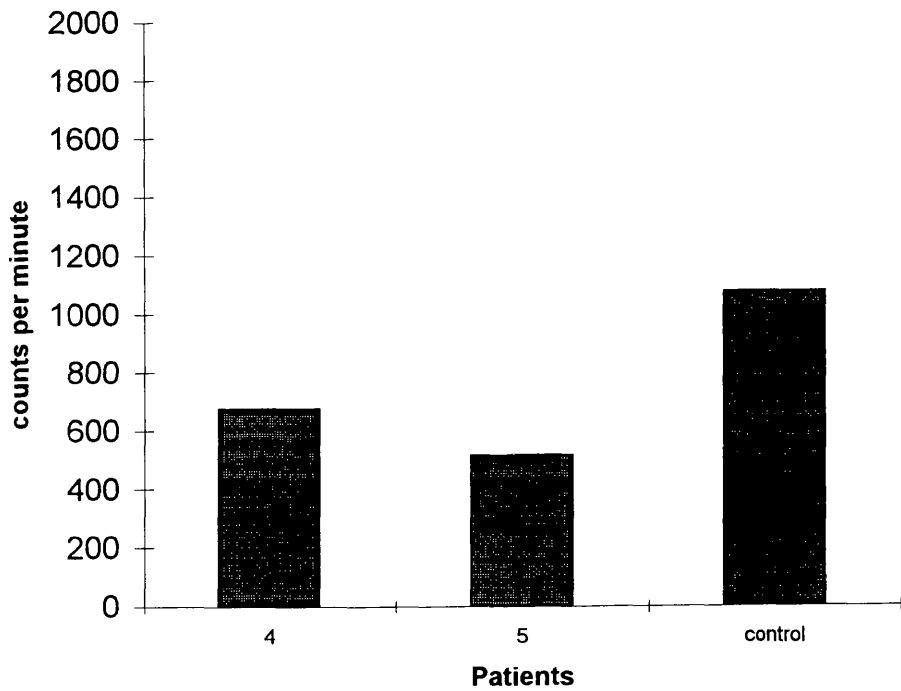
Figure 7.4 Whole blood assay responses to HBcAg peptides (Th₁, Th₂ and ThP_n) and HBcAg in five non-symptomatic chronic carriers and a control patient (anti-HBc negative). SI= Stimulation Index.

Figure 7.5 Whole blood assay responses to PHA in five non-symptomatic chronic carriers and a control patient.

(A)



(B)



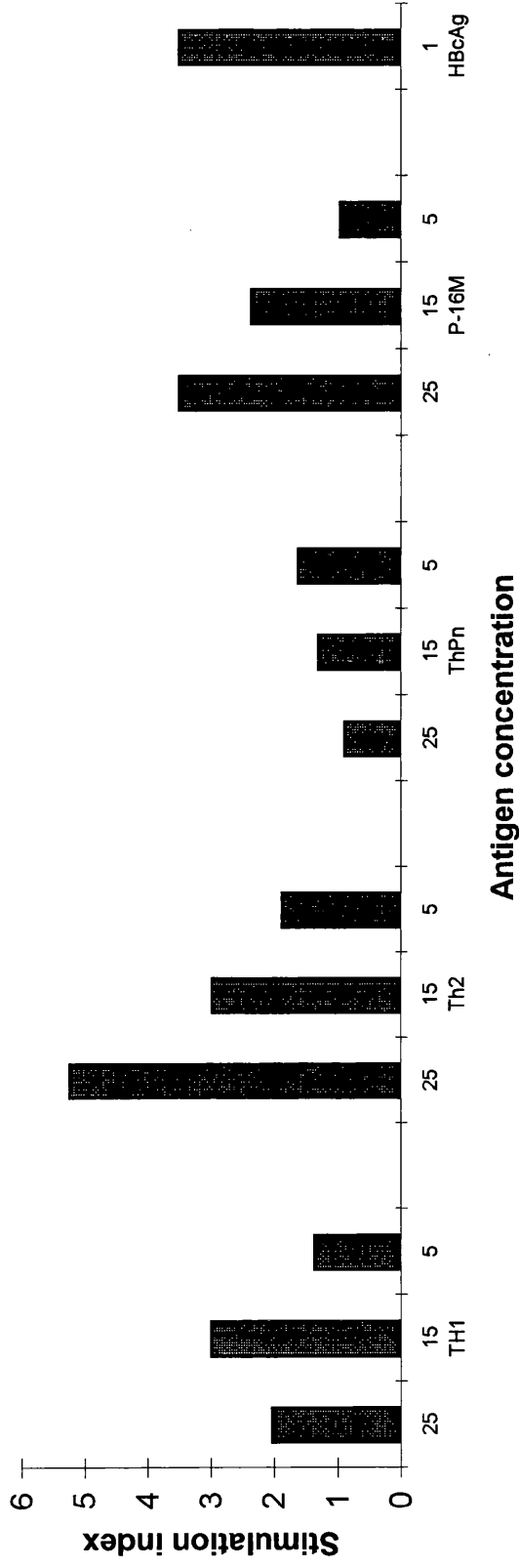


Figure 7.6 PBMC response to peptides (Th₁, Th₂, ThP_n and P22-16M) and HBcAg in a patient with acute self-limiting hepatitis.

Study 2 (Athens) The overlapping peptide series was tested in lymphoproliferation assays using PBMCs from patients with a variety of disease profiles: acute hepatitis, fulminant hepatitis, chronic HBV and HCC. Patient details are given in Table 7.7. Lymphoproliferation was observed (Figure 7.7).

Acute HBV patients: those with acute hepatitis (PBMC10 and PBMC20) did not respond to peptides P22-4, -5 and -6, which contain either all or part of the Th epitope between amino acids 1 and 20. In fact, PBMC20 showed little response to any peptide, whereas PBMC10 responded to P22-9, which contains the major Th epitope (amino acids 50 to 69) and also to P22-16M, which contains the Th epitope at amino acids 117 to 131.

Fulminant HBV patients: PBMC1 and PBMC15 responded similarly to all the peptides. They responded well to P22-4 with stimulation indices of 6, and also showed a response to peptides P22-11, -12, -13, -15, -16 and -19.

Chronic HBV patients: a definite response to peptides P22-4, -5 and -6 was observed in 5 out of 18 patients with chronic hepatitis. Unfortunately, PBMC11 and PBMC12, who responded well to the Th epitope amino acid 1 to 20, were not tested against peptides P22-6 to P22-20. Only one of the remaining three (PBMC3) responded to other peptides (P22-10M, -12, -15 and -16M). The asymptomatic chronic carrier PBMC16, surprisingly, showed a response to several peptides (P22-17, -18, -19 and -20), all of which are at the C-terminus of HBcAg.

HCC patients: PBMC18 and PBMC22 responded to P22-4 and PBMC18 responded to additional peptides at the carboxy-terminal of HBcAg.

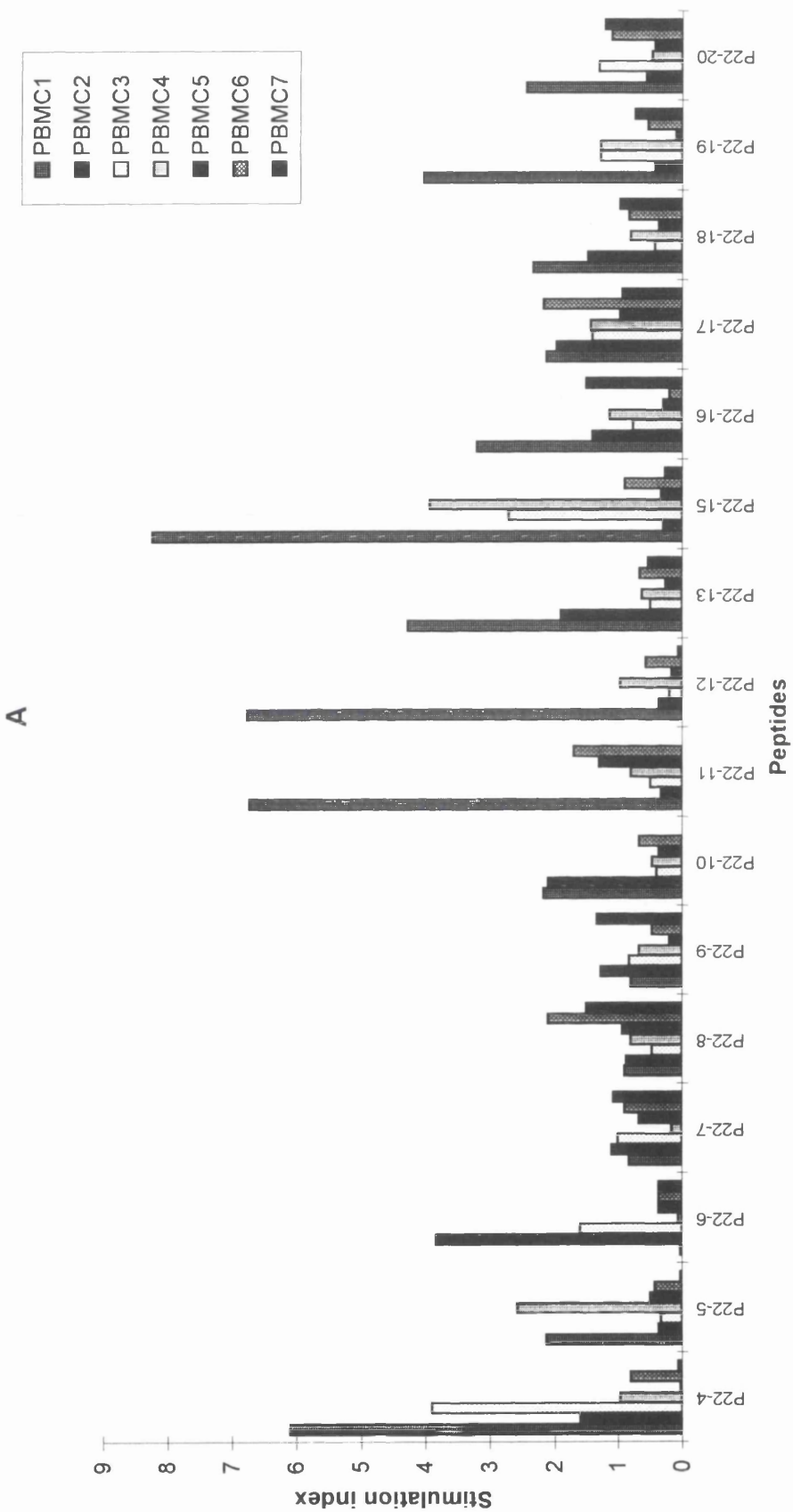
Table 7.7 Clinical details of patients tested in the lymphoproliferation assay

Patient	Diagnosis	HBeAg	Anti-HBe	ALT
PBMC1	Fulminant	-	+	>2000
PBMC2	Chronic	-	+	305
PBMC3	Chronic	+	-	48
PBMC4	Chronic	-	+	491
PBMC5	Chronic	+	-	57
PBMC6	Chronic	-	+	158
PBMC7	Chronic	+	-	121
PBMC8	Chronic	+	-	214
PBMC9	Chronic	+	+	118
PBMC10	Acute	-	+	850
PBMC11	Chronic	-	+	988
PBMC12	Chronic	+	+	661
PBMC13	Chronic	-	+	700
PBMC14	Chronic	+	-	534
PBMC15	Fulminant	-	+	>2000
PBMC16	Carrier	-	+	19
PBMC17	Chronic	+	-	78
PBMC18	HCC	-	+	67
PBMC19	Chronic	-	+	42
PBMC20	Acute	-	+	119
PBMC21	Chronic	-	+	49
PBMC22	HCC	-	+	5
PBMC23	Chronic	-	+	59
PBMC24	Chronic	-	+	33
PBMC25	Chronic	-	+	43

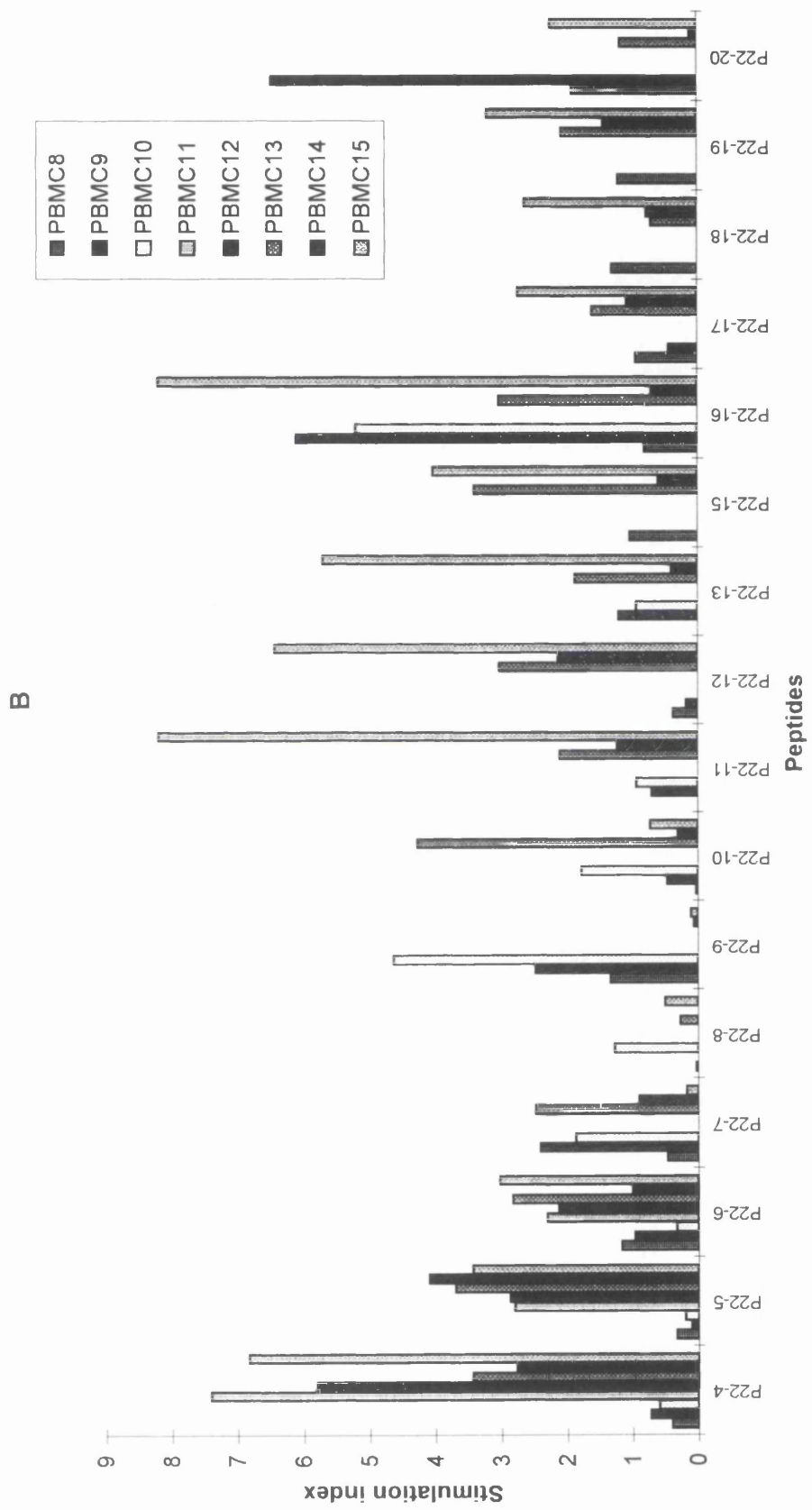
HCC= hepatocellular carcinoma.

Anti-HBe= antibody to HBeAg.

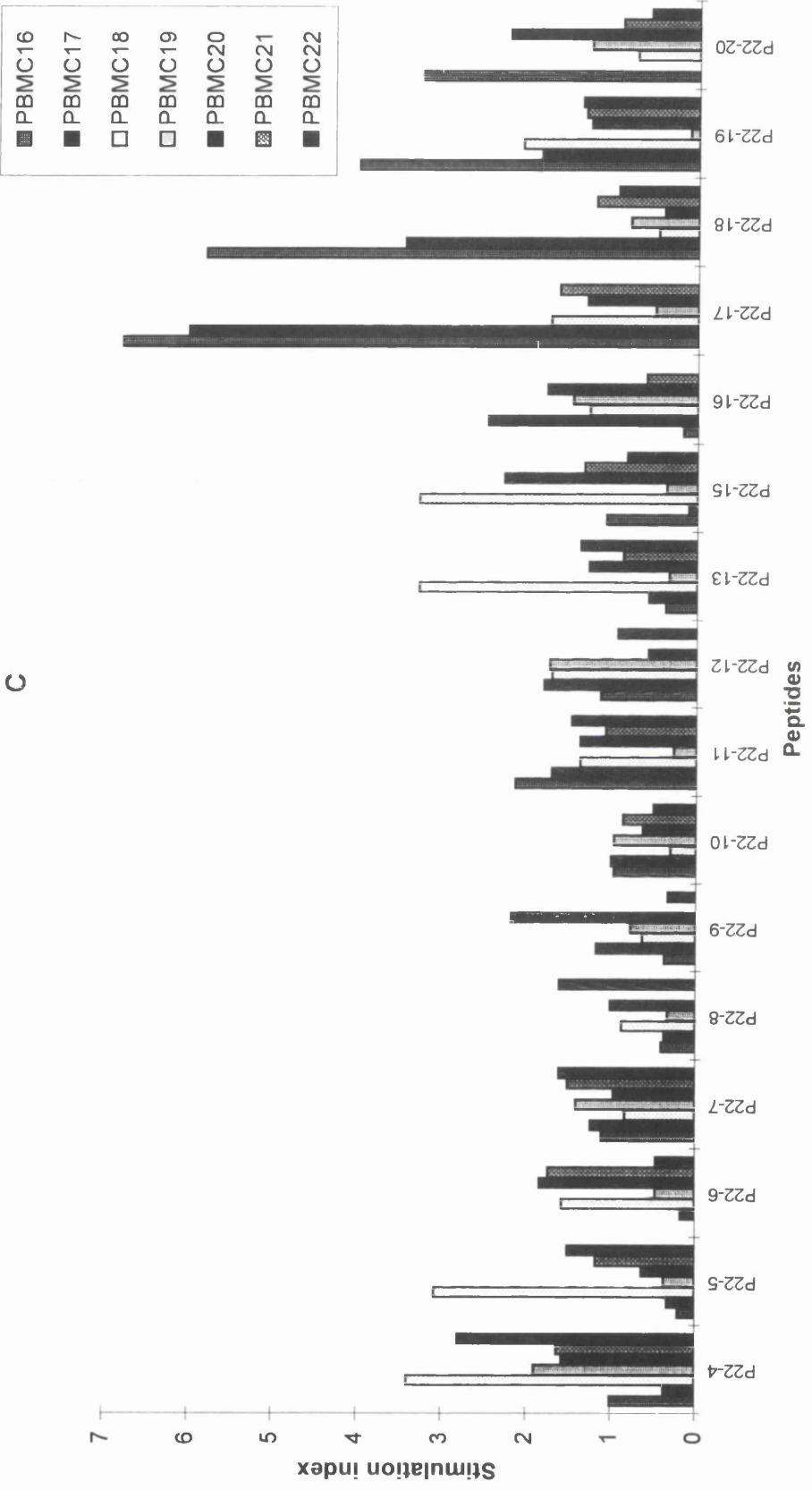
ALT= alanine aminotransferase (N<40iu/l).



A



B



C

Figure 7.7 PBMC response to the overlapping peptide series in patients with fulminant, acute or chronic hepatitis or HCC.

A - Reactivities to the overlapping peptide series for patients PBMC1 to PBMC7. B - Reactivities to the overlapping peptide series for patients PBMC8 to PBMC15. C - Reactivities to the overlapping peptide series for patients PBMC16 to PBMC22. Fulminant cases = PBMC1 and PBMC15, acute cases = PBMC10 and PBMC20, chronic cases = PBMC2 to 9, 11 to 14, 16, 17, 19, and 21 and those with HCC = PBMC18 and 22. A stimulation index >3 is significant.

Study 3 (Athens) To determine whether lack of response was due to a mutated sequence, amino acids 1 to 30 of HBcAg was subsequently sequenced for all the above patients (Table 7.8). Branched chain peptides were constructed (by Dr. A. Oswianka, Institute of Virology, University of Glasgow) that contained the variant amino acids (Table 7.9). Unfortunately, as yet, only one patient (PBMC13) has been tested against their variant peptide (7.4.2, study 6). This study is still in progress.

7.4.2 B-cell studies: results

Study 4 (Athens) Three patients were tested by IgM ELISA (3.20) against the overlapping peptide series. Their diagnoses were as follows:

BC1	Fulminant HBV infection.
BC2	Chronic anti-HBe-positive, with severe exacerbations of hepatitis.
BC3	Chronic HBeAg-positive at phase of seroconversion.
Positive control	Anti-HBe-positive, with very high level anti-HBc IgM.
Negative control	Chronic HDV infection, HBsAg-positive, HBV-DNA negative.
Control	Negative for HBV infection.

The results are given in Figure 7.8. The antibody class which was being measured was IgM, therefore a patient with HDV infection was used as the negative control.

The positive control sample, when compared to the negative control, reacted to all the peptides but with varying intensity. The fulminant hepatitis (BC1) case reacted similarly to the positive control, indicating a strong anti-HBc response in this patient. The reactivities of BC2 and BC3

Table 7.8 Sequence of aa 1-30 of HBcAg from patients analysed by lymphoproliferation assays

Patient	Sequence
PBMC1	MDIDPYKEFGATVELLSFLPSDFFPSVRDL
PBMC2	MDIDPYKEFGATVELLSFXPSDFFPSVRDL
PBMC3	MDIDPYKEFGATVELLSFLPSDFFPSVRDL
PBMC4	MDIDPYKEFGASVELLSFLPSDFFPSVRDL
PBMC5	MDIDPYKEFGATVELLSFLPSDFFPSVQDL
PBMC6	MDXDPYKEFGATVELLSFLPSDFFPSVRDL
PBMC7	MDIDPYKEFGATVELLSFLPSDFLSSVRDL
PBMC8	MDIDPCKEFGATVELLSFLPSDFFPSVRDL
PBMC9	MDIDPYKEFGATVELLSFLPSDFFPSVRDL
PBMC10	MDIDPYKEFGASVELLSFLPSDFFPSVRDL
PBMC12	MDIDPYKEFGATKELLSFLPSDFFPSVRDL
PBMC13	MDIDPYKEFGASVEILSFLPHDFFPSVRDL
PBMC14	MDIDPYKEFGATVELLSFLPSDFFPSVRDL
PBMC15	MDIDPYKEFGATVELLSFLPADFFPSVRDL
PBMC17	MDIDPYKEFGATVELLSFLPSDFFPSVRDL
PBMC18	MDIDPYKEFGATVELLSFLPSDFFPSVGD
PBMC19	MDIDPYKEFGATVELLSFLPSDFFPSVRDL
PBMC20	MDIDPYKEFGATVELLSFLPXDFPSVRDL
PBMC21	MDIDPYKEFGASVELLSFLPXDFPSVRDL
PBMC22	MDIDPYKEFGATVELLSFLPSDFFPSVRDL
PBMC23	MDIDPYKEFGATVELLSFLPSDFFPSVRDL

Single letter codes for aa are given in Appendix 2

Table 7.9 Sequences of the variant peptides derived from the PBMC series of patients

Peptide	Sequence
S1	A T V E L L S F L P S D F F P S V R D L
S2	A S V E L L S F L P P D F F P S V R D L
S3	A S V E L L S F L P H D F F P P V R D L
S4	A T V E L L S F L P A D F F P S V R D L
S5	A T V E L L S F L P L D F F P S V R D L
S6	A T V E L L S F L P S D F F P S V Q D L
S7	A T V E L L S F L P S D F F P S V G D L
S8	A T V E L L S F L P S D F L S S V R D L

Peptides span aa 11-30 of HBcAg

Single letter aa codes are given in Appendix 2

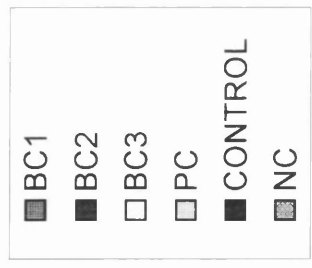
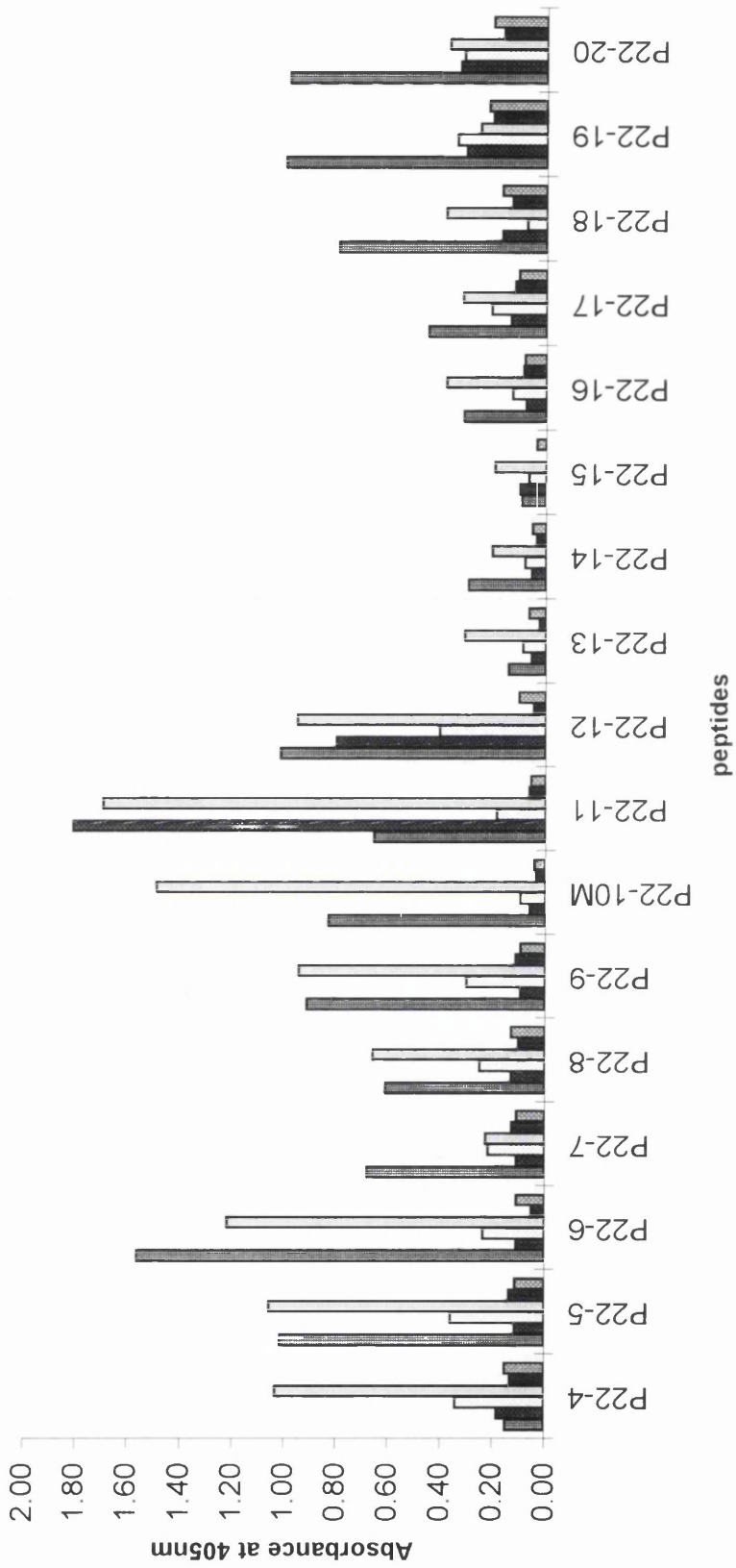


Figure 7.8 Reactivity of anti-HBc positive and negative serum tested against the overlapping peptide series. BC1 = patient with fulminant HBV infection. BC2 = patient with chronic anti-HBe-positive infection, with severe exacerbations of hepatitis. BC3 = patient with chronic HBeAg-positive infection, at the phase of seroconversion to anti-HBe. PC = the positive control, an anti-HBe-positive patient with very high level anti-HBc IgM. NC = the negative control, a patient with chronic HDV infection, who is HBsAg-positive and HBV-DNA negative. Control = An individual who has no markers of HBV infection.

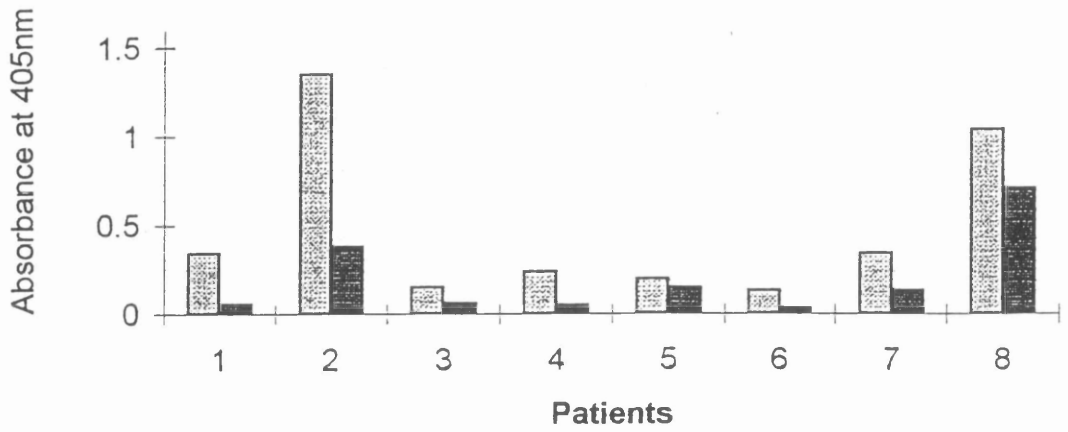
were generally lower. Patients BC1 and BC3 reacted to the N-terminal of HBcAg, peptides P22-4, -5 and -6.

A very strong response was observed to the major B-cell epitope (amino acids 74 to 83); peptides P22-11 and -12 by the positive control serum and also by patients BC1 and BC2. Patient BC3 did respond, with a 3-fold and 10-fold increased response over the negative control, but not to the same extent as the other two patients. Interestingly, patient BC1 and the positive control serum reacted to P22-10, a monomeric peptide; the intensity of this reaction was unexpected. Patient BC1 also reacted to the C-terminal of HBcAg, peptides P22-18, -19, and -20.

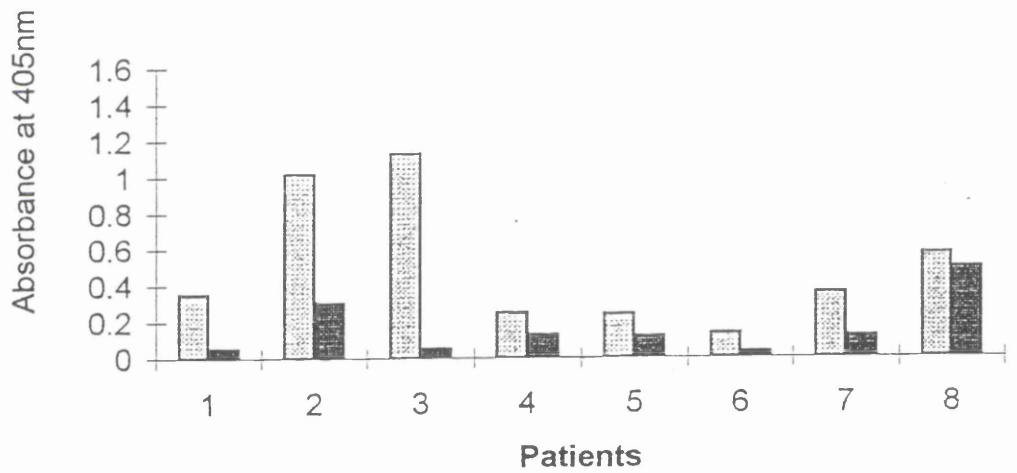
Study 5 (Athens) Peptides P22-4, -5 and -6 were also constructed as monomers. They were compared to their branched counterpart in an ELISA (3.20) against various patients' sera regardless of diagnosis (information on the patients was not supplied by Professor Hadziyannis despite a number of requests) (Figure 7.9). In the preliminary study we had shown that branched chain peptides were more sensitive when used in an ELISA. Professor Hadziyannis repeated this experiment, using different peptides, confirming that the branched chain peptides are superior in the detection of anti-HBc.

Study 6 (Athens) Patient PBMC13's response to the overlapping peptide series was also tested by ELISA, for both IgG (Figure 7.10) and IgM (Figure 7.11). Clearly, patient PBMC13 has a stronger IgG than IgM response. There does not appear to be a particularly strong response to the major B-cell epitope but he has, like the earlier patients, responded to peptides P22-4 and -5. A variant peptide (S3) (Table 7.9) was produced because of PBMC13 lack of reactivity in the lymphoproliferation assay to peptides P22-4, -5 and -6 (Figure 7.7). This peptide (and the other variant peptides) were tested by ELISA, both IgG and IgM. The reactivities

Peptides P22-4 and P22-4M



Peptides P22-5 and P22-5M



Peptides P22-6 and P22-6M

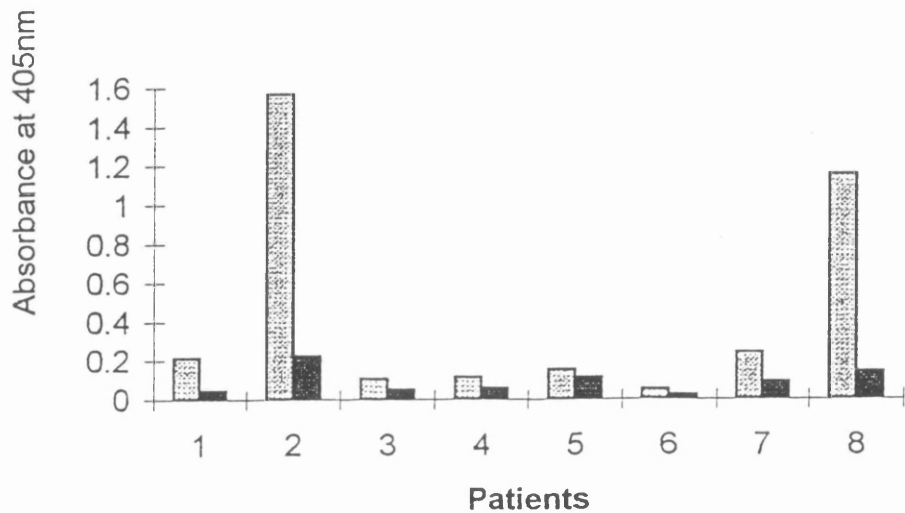


Figure 7.9 Comparison of reactivity of branched and monomeric peptides P22-4, -5 and -6 tested against anti-HBc positive sera. Peptides were tested at 5 μ g/well.

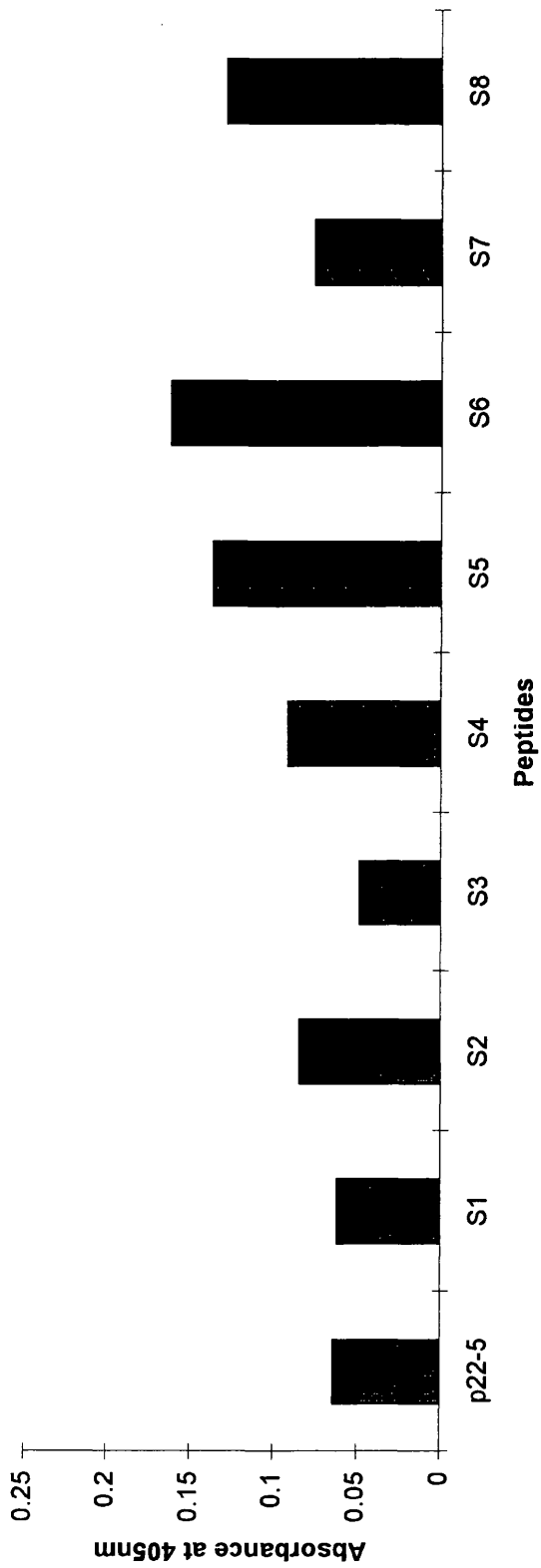


Figure 7.10 IgG ELISA results of patient PBMC13 tested against the variant peptides S1 to S8

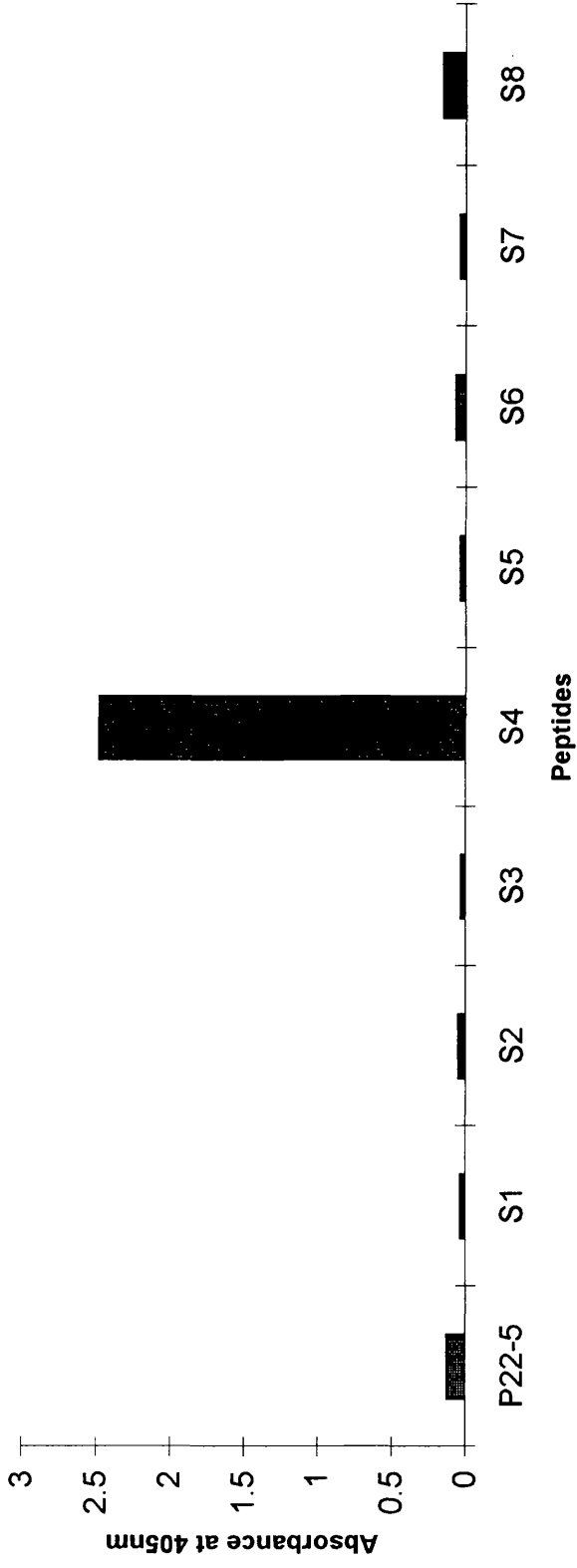


Figure 7.11 IgM ELISA results of patient PBMC13 tested against the variant peptides S1 to S8

against the variant peptides are shown in Figure 7.11. The OD values are generally low except for peptide S4 in the IgM assay.

7.4.3 Conclusions and discussion

Professor Hadziyannis's group were given the peptides in July 1994, despite this all of the above results are only preliminary, each experiment only having been completed once or twice. Therefore, it is difficult to make any conclusive statements concerning these results. What is clear is that the peptides I synthesised do work in these systems.

The non-uniform response observed when whole blood was tested (on several occasions one of the triplicate test wells gave background readings despite the other two wells giving positive readings) was considered to be an inherent problem with the whole blood assay and therefore it was decided to use PBMCs.

The patients in study 1 did not respond quite as well to the peptides or HBcAg as we may have hoped, probably because the patients were asymptomatic HBV carriers and not experiencing active disease during the period of testing their PBMCs. There was virtually no response to Th₁. The absolute failure of these patients to recognise Th₂ could be related to the HBcAg sequence in these patients. Unfortunately, the HBcAg sequences of the patients were not examined. However, in accordance with Ferrari *et al* (1991) the majority (3/5) of the patients did respond to the major Th cell epitope. This was not observed in study 2 where only 2/25 (PBMC10 and 13) responded to these peptides in the assays. The number of cells primed to react to HBcAg peptides is likely to be low therefore a greater number of replicates, at least six, would be required to give conclusive results.

In study 2, where the lymphoproliferative response of patients with a variety of disease profiles was tested, not all patients responded equally

well to the peptides indicating a patient-specific response. There was no apparent correlation between HBeAg/anti-HBe status or ALT level and response to peptides. The dominant response was to P22-4 (which contains the Th epitope amino acid 1 to 20). In all patients who responded to amino acids 1 to 20, a decrease in response was observed as the epitope was gradually omitted from the peptides in the overlapping series, thus indicating that the epitope lies between 14 and 23.

There was a good response to several peptides in those with fulminant hepatitis (with both cases responding to the same peptides), indicating an active Th response in these individuals. Interestingly, sequencing of the first 30 amino acids of HBcAg of the patients in study 2 showed that the most variant amino acid was amino acid 21(4/20). The Th epitope has been described to be between amino acids 1 to 20 but our results indicate that it may be between 14 and 23. However, it is possible that adjacent residues, not involved in binding can have an effect on binding of the peptide to the groove of the MHC, for this reason amino acids outside this region were included in the variant peptides. Several of the patients (PBMC4, 7, 9, 17, 22,) who failed to respond to peptides P22-4, -5 and -6 did not have variant sequences and PBMC13, who did respond to these peptides, had the most variant HBcAg sequences (from amino acid 1 to 30). Therefore, from the sequencing data, it seems unlikely that the lack of response is due to variation in the Th epitope.

Results were available for comparison only between branched and monomeric peptides for the B-cell studies. The branched peptides are clearly more reactive than the monomers. What is puzzling is what exactly these sera are reacting to. The peptides tested, P22 -4, -5, -6, span the region amino acids 1 to 43. No B-cell epitope has been described in this region which, of course, does not mean that there is not one! When patients' sera were tested against the overlapping peptide series, three of the patients (BC1, BC3 and PBMC13) and the positive control also

responded to these peptides, implying that there is something interesting happening in this region - an undescribed B-cell epitope? Although there was no direct comparative data on branched versus monomeric peptides for the lymphoproliferation assay, monomeric peptides were equally efficient at stimulating proliferation as branched chain peptides, indicating that there will be little difference between the two configurations when they are examined directly. Further work is still being carried out in this area.

A recent report from Italy has shown that biotinylation of monomeric peptides enhances the binding efficiency of the peptides (Professor Hadziyannis, personal communication).

As expected all patients reacted to the major B-cell epitope, providing good evidence that the peptides are specific for HBcAg. The fact that the positive control (IgM anti-HBc positive) reacted to all the peptides is of concern. The reactivities to peptides P22-13, -14, -15 and -16 were low but, as these peptides are monomeric, this was expected. Reactivity to peptides P22-17, -18, -19 and -20 (branched peptides) was also low (only 2 or 3 x background), though an epitope has been described in HBcAg in this area (between amino acids 150 and 159) by Machida *et al* (1989). Patient BC1 reacted well to this epitope (approximately 6 x background).

One of the original aims of this section of work was to test sera around the time of an acute exacerbation to determine whether a flare of hepatitis is caused by a 'new' virus or whether the flare results in appearance of a 'new' virus. Unfortunately, although a preliminary experiment has been completed, I was not provided with the data before presentation of this thesis but these studies are ongoing.

Patient PBMC13 was tested against the variant peptides in an ELISA. Although he did respond in the IgM assay to one of the variant peptides

(S4), this peptide did not contain the variant specific to him. His variant peptide was peptide S3, to which he failed to respond. Have these variants rendered this epitope unreactive? In the test for IgG he had weak reactivities to most of the variant peptides. Why he should respond to a variant which he does not have is not clear. Perhaps our assumption that mixtures below our level in detection (5%) by direct sequencing are unimportant was wrong; to determine whether or not PBMC13 has this variant we would have to sequence clones of HBcAg from this serum.

7.5 Future work

Due to the reduced sensitivity in antibody binding studies with monomeric peptides we propose the use of entire core proteins in future studies. Sequential cloned HBcAgs from patients experiencing acute exacerbations of hepatitis tested in an ELISA would determine whether or not antibody escape is occurring. These recombinant HBcAgs would have the advantage of having the correct epitopic conformation. Alternatively, biotinylation of our monomeric peptides may increase their sensitivity (as referred to earlier in this Chapter [7.4.3]) or could be used in conjunction with the recombinant HBcAgs to delineate any further epitopes .

Several patients reacted to the N-terminal peptides in the B-cell studies, indicating the presence of a B-cell epitope. Further investigation is required to determine whether this is a true B-cell epitope. If it does prove to be an epitope, then patient PBMC13 would be an excellent test patient, to look for B-cell escape here, as we have already demonstrated that he does not recognise his own peptide. To test whether there is ongoing B-cell escape subsequent or previous serum samples would have to be sequenced, the relevant peptides constructed and tested.

Chapter 8

Results: Section 5

Expression of HBcAg

8.1 Aim

The aim of this section of the thesis was to express HBcAg, from patients sequenced in Chapter 6, for use in antibody binding studies.

The peptides which we produced (Chapter 7), were also to be used for this purpose. We considered the possibility that the peptides may not form the correct conformation, despite their length. Thus, to overcome this potential drawback we decided to express the HBcAg of some of the Greek patients (sequenced in Chapter 6) and use these in conjunction with the peptides.

There was also the possibility that the mutated core proteins described in Chapters 5 and 6, may not have the same antibody binding efficiency as compared to the standard HBcAg. This has clearly been observed with HBsAg (Wallace *et al*, 1995), where changes in the a determinant have either reduced or completely abrogated binding efficiency in diagnostic assays. This raises the possibility of false negatives and, such variant HBcAg could be used in new generation anti-HBc diagnostic assays.

8.2 Selection of HBcAg sequences to be cloned and expressed

In parallel to this study, a separate study was being carried out (by Mr. E. Dornan) in our laboratory, to examine changes in the distribution of sequential HBcAg sequences from patients, in hepatocytes, during the course of infection. Therefore, the same HBcAg sequences were selected for cloning and expression in the antibody binding study. The hope was that, by collating the data from both studies, we may be able to determine whether the mutations observed did represent humoral escape or whether they affected the distribution and accumulation of HBcAg in the hepatocyte (subsequently having a detrimental effect on the hepatocyte). HBcAg from the first and last sample in the serum series for each patient were used. The pairs used were as follows:

Gr2 and Gr6, from patient H20

Gr22 and Gr25, from patient H27

Gr45 and Gr49, from patient H32

Gr50 and Gr54, from patient H33

Gr82 and Gr86, from patient H37

Patient details are given in Figure 6.2.

8.3 Strategies employed in the cloning of HBcAg: methods and results

Several strategies were employed in our attempt to express the above HBcAgs. They are outlined below.

Strategy 1

The C1/C2N PCR products of the above patients were amplified (3.3.2) using primers C5/C4N, producing a product with an *Eco* R1 site at the 5' end and a *Hin* dIII site at the 3' end. The products were digested (3.7) with *Eco* R1 and *Hin* dIII and subsequently electrophoresed on a 1%, 1xTBE agarose gel (3.5). The products were excised and purified by the

GeneClean method (3.4.2), ligated with the vector pRK5 (3.8) and transformed in *E.coli*. (3.10).

At this point the procedure was stopped since pRK5 was primarily used for expression in mammalian systems it was envisaged that it would not be suitable for expression in *E.coli*.

Strategy 2

C1/C2N products were amplified using primers CPK and C4N, thus producing a product with an EcoR1 site at the 5' end and a *Hin* dIII site at the 3' end. The amplified products were ligated (3.8) directly into the PCR cloning vector, pT7Blue, and transformed in *E.coli*. After 16h, the plates were examined and five white colonies were picked from each plate, transferred into a glass universal containing 3mls of selection medium (LB broth plus 100µg/ml of ampicillin and tetracycline) and grown overnight at 37°C in a shaking incubator. Cultures were subsequently mini-prepped, restriction enzyme digested (3.7), with *Eco* R1 and *Hin* dIII and then electrophoresed on a 1%, 1xTBE agarose gel (3.5). DNAs from the clones which contained inserts of the correct size were subcloned using *Eco* R1 and *Hin* dIII sites, and ligated with pKK223.3 and transformed in *E.coli*. (3.10). Colonies were picked, transferred into a glass universal containing 3mls of selection medium (LB broth plus ampicillin) and grown overnight at 37°C in a shaking incubator. Cultures were subsequently mini-prepped, digested (3.7), with *Eco*R1 and *Hin* dIII, and electrophoresed (3.5).

When a large number of colonies were obtained, they were screened by PCR (3.3.4). Potentially positive clones were sequenced (3.6.1) using primer BC1 (Appendix 1) to ensure the correct insert had been cloned.

Restriction enzyme digestion with *Eco* R1 and *Hin* dIII produced a product that was shorter than expected (Figure 8.1). A mapplot analysis (Figure 8.2) revealed that Gr49 had an internal *Hin* dIII. A mapplot analysis was

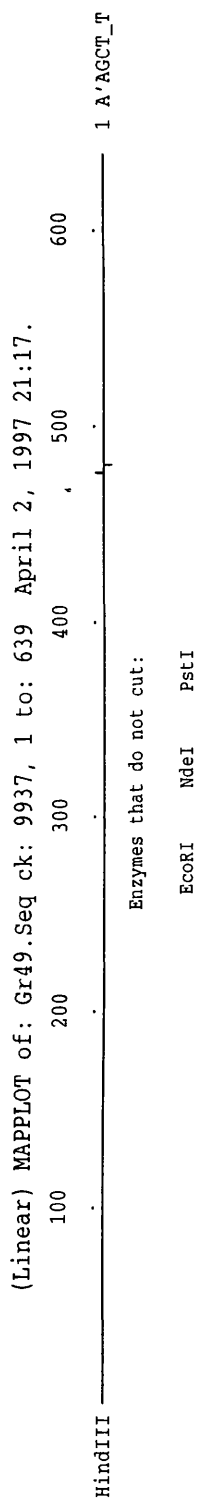


Figure 8.2 A mapplot analysis of Gr49. The mapplot shows that there is an internal *Hin* dIII site but no *Eco* R1, *Nde* 1 or *Pst* 1 sites.

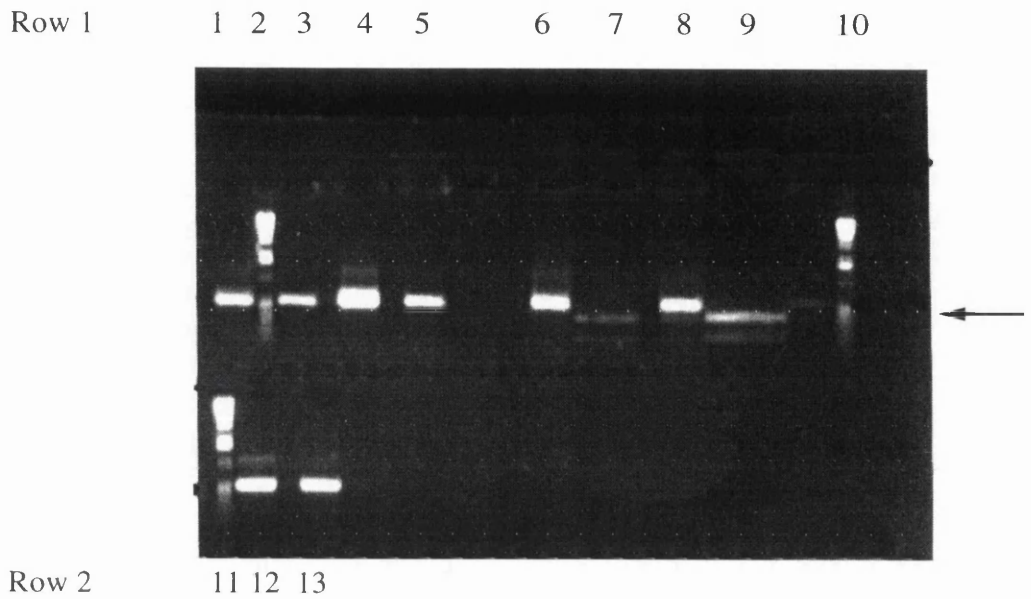


Figure 8.1 Eithidium bromide stained 1% (1xTBE) agarose gel showing the products of restriction enzyme digestion of pT7Blue/inserts with *Eco* R1 and *Hin* dIII. Row 1 (top wells): lane 1- Gr2, lane 2 - 1kb molecular weight marker, lane 3 - Gr6, lane 4 - Gr22, lane 5 - Gr25, lane 6 - Gr45, lane 7 - Gr49, lane 8 - Gr86, lane 9 - Gr82, lane 10 - 1kb molecular weight marker. Row 2 (bottom wells): lane 13, 1kb molecular weight marker, lane 14 - Gr50, lane 15 - Gr54. Arrow indicates shorter than expected band in lanes 7 (Gr49) and 9.(Gr82).

then performed on all the HBcAgs to be used in the study, revealing that Gr82 also had a *Hin* dIII site (Figure 8.3). A new primer was prepared replacing the *Hin* dIII site with a *Pst* 1 site; Gr 49 and Gr82 were then 'put through' the procedure again.

When the inserts were screened by the PCR (using primers CPK and C4N) method, positive inserts were obtained for Gr2, Gr45, Gr49, Gr60, Gr65, Gr82, Gr86 (Figure 8.4). However, when they were sequenced they did not have the correct insert. The sequence of the insert was that of the *Eco* R1 site with pT7 sequence

When these 'positive clones' were then digested with *Eco* R1 and *Hin* dIII or *Eco*R1 and *Pst* 1, only two of the plasmids showed an insert, Gr2 and Gr65 (Figure 8.5); there were no inserts evident in the others. On resequencing, even these proved to be incorrect.

Strategy 3

DNA was extracted from the serum samples for all patients (3.1.1) and amplified using primers CPET3 and CD3, if unsuccessful, CPET3 and Bamtag were tried (Appendix 1). On successful amplification the same methods as for strategy 2 using the vector pET3a were then employed.

There appeared to be some contamination in strategy 2 from the PCR cloning vector. Therefore it was decided to use original first round products as the starting product. The primers (CPET3 and CD3 [or Bamtag]) were designed to amplify the C ORF and not to the pre-C region because we did not want to express HBeAg. However, this time the contamination appeared to be from the first round product as the sequence obtained was that of the pre-C region. This may have been due to possible contamination by the original product amplified by C1/C2N - these primers amplify the entire pre-C/C ORF. It is difficult to explain how this product could have been carried through all the steps involved in the

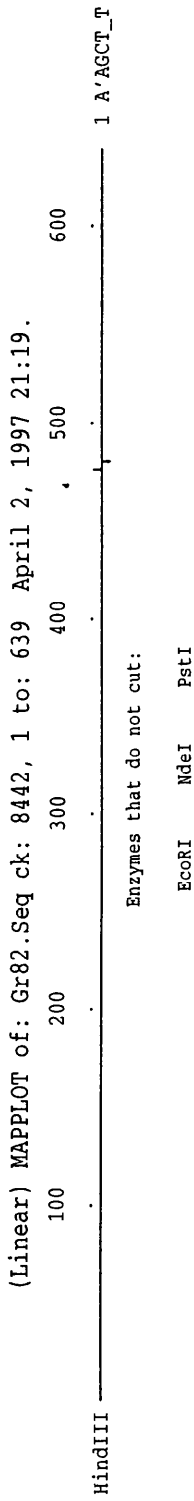


Figure 8.3 A mapplot analysis of Gr82. The mapplot shows that there is an internal *Hin* dIII site but no *Eco* R1, *Nde* 1 or *Pst* 1 sites.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

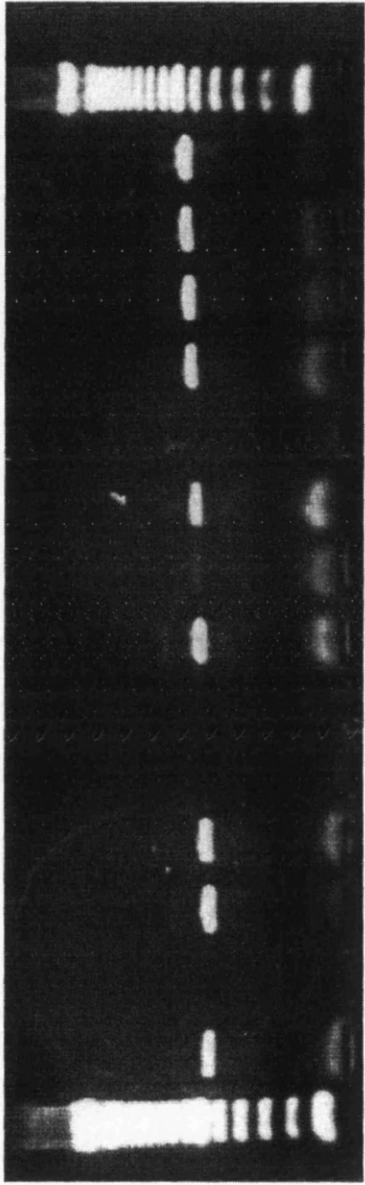


Figure 8.4 Eithidium bromide stained 1% (1xTBE) agarose gel showing the result of screening for inserts by PCR. Lanes 1 and 16 - 1kb molecular weight marker. Positive inserts were observed for Gr2 (lane 2), Gr60 (lane 4), Gr65 (lane 5), Gr49 (lanes 8 and 10), Gr45 (lane 9), Gr82 (lanes 12 and 14) and Gr86 (lanes 13 and 15). Negative PCR control are shown in lanes 6 and 11.

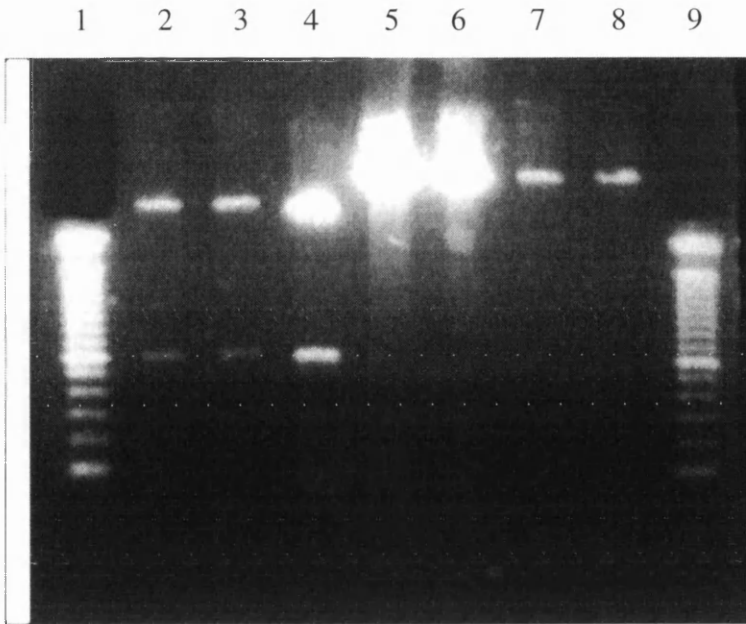


Figure 8.5 Eithidium bromide stained 1% (1xTBE) agarose gel showing the products of restriction enzyme digestion of pKK223.3 inserts with *Eco* R1 and *Hin* dIII. Lanes 1 and 9 - 1kb molecular weight marker. Lanes 2 and 3 show positives inserts for Gr2, lane 4 shows a positive insert for Gr 65, lanes 4 to 8 show positive clones by PCR of Gr45 (lane 5), Gr49 (lane 6) Gr82 (lane 7), Gr86 (lane 8) digested with *Eco* R1 and *Hin* dIII with no inserts evident.

cloning procedure and inserted into the pKK223.3, especially as C1 does not have an *Eco* R1 site but there was no other explanation forthcoming.

Strategy 4

As referred to earlier, several of the Greek samples were cloned for the hepatocyte expression study into the mammalian expression vector pRK5. Cloned pRK5/HBcAgs were used as a last attempt to clone into pET3a. HBcAg was amplified by PCR (3.3.1), using primers CPET3 and Bamtag (Appendix 1). 25µl of the amplified products were electrophoresed (3.5), excised and purified by the GeneClean method (3.4.2). The excised DNA was digested (with *Bam* H1 and *Nde* 1) as detailed in 3.7 and the DNA further purified (3.4.1). The DNA fragments were ligated with pET3a (3.8) and transformed in *E.coli*. (3.10). Plates were examined the following morning; colonies were picked, transferred into a glass universal containing 3mls of selection medium (LB broth plus 100µg/ml of ampicillin) and grown overnight at 37°C in a shaking incubator. Cultures were subsequently mini-prepped, restriction enzyme digested (3.7), with *Nde* 1 and *Bam* H1, and electrophoresed (3.5) to identify plasmid clones containing HBcAg.

There appeared to be some contamination in strategy 3 by the original amplified product, in this attempt we decided to re-extract the serum samples and amplify directly using the cloning primers in the hope that there would be no contamination from earlier products.

Despite having a low background level of re-ligation and many colonies on each test plate, all colonies failed to have an insert. Each colony was then tested by the PCR for inserts, again this proved to be negative.

8.5 Conclusions

For some unknown reason, but not for want of trying, I failed to successfully clone any HBcAgs from the Greek patients. The spectre of contamination was addressed at several times during the course of cloning and all the normal steps to prevent it were taken. It is unlikely that there was inherent contamination of the original PCR products as the same products were used in the study by Mr. E. Dornan. As indicated above, several different avenues were explored in attempt, often desperate, to successfully clone the HBcAgs. Unfortunately, time was against us and this section of the work remains incomplete.

At the time I was attempting to clone the HBcAgs several other people in the laboratory were successfully cloning HBcAg, and other areas of the HBV genome, why I had so much trouble still remains a mystery. As I will describe later in the general discussion, we remain committed to this approach as the best means of resolving the issue of potential antibody escape.

Chapter 9

General discussion and future work

The fundamental conclusions which can be made from the work carried out in this thesis are:

- that serine15 and A₁₈₉₆ are separate lineages.
- that HBV evolves within individuals.
- that selection of A₁₈₉₆ is temporally associated with substitutions in HBcAg
- that substitutions are associated with HBcAg B- and Th-cell epitopes.
- that HBcAg substitutions are not associated with multiple flares of hepatic activity.
- that the distribution of HBcAg amino acid substitutions are different between the Chinese and the Mediterranean groups.
- that amino acid substitution in HBcAg is dependant on selection of a pre-C variant.
- that branched chain peptides work in both antibody binding and T-cell studies.
- and that they are more reactive in ELISA when compared to monomeric peptides.

What is the selection pressure for pre-C stop mutants in chronic hepatitis?

Sequencing of HBcAg from patients has shown that A₁₈₉₆ arises during the course of chronic infection. As it does arise so frequently, we propose that there is a selection pressure that gives rise to this particular mutant. What this pressure could be is still unknown. If it is immune mediated then which particular arm of the immune system is responsible for this pressure

is still unresolved. However, as discussed in Chapter 1 (section 6.4) it is unlikely that CTL are involved. Primarily, this is because CTL are virtually non-existent in chronic hepatitis B infection and therefore are not vigorous enough to exert selection pressure. Also, as described CTL epitopes in HBcAg and HBsAg span critical areas (the nuclear localisation and genome encapsidation signals in HBcAg and an important topogenic sequence in the transmembrane domain in HBsAg) in these genes, mutations in these areas could be lethal for the virus. Rehermann *et al* (1996) have described CTL in chronic hepatitis patients, however the response was a latent one and required augmentation with IFN treatment to reactivate the CTL response, indicating once again that patients with chronic HBV infection do not have an active CTL response. It has been proposed that the Th-cell response to HBcAg/HBeAg may contribute to selection of pre-C stop variants.

Jung *et al* (1995) have shown that Th-cell responses are also often weaker, though present, in chronic hepatitis infection than in acute infection, yet the fine specificity of HBV nucleocapsid recognition by Th cells is similar in both acute and chronic HBV patients. However, those patients examined that had a pre-C variant failed to respond to HBcAg/HBeAg and derived peptides. Was this due to escape? It seems unlikely, as the pre-C region seldom induced T-cell proliferation, indicating the absence of a Th epitope in this area. Also, the sequencing results (from Chapter 7) of the PBMC patients showed that those who failed to respond to peptides that contained one of the HBcAg Th epitopes did not have variant amino acids in this region.

HBeAg is a secreted protein and, as pre-C mutants often appear around the time of seroconversion to anti-HBe, the possibility that the selection pressure is humoral is feasible. Lindh *et al* (1996) indicate that selection of the pre-C mutant is an escape mechanism that reduces the host's immune response (and therefore liver damage). In that study, the pre-C

mutant was associated with less liver damage than the HBeAg producing strain but, in the patients we have studied, we believe there is ongoing pressure as increased ALTs were still observed after appearance of a pre-C stop variant.

Pre-C mutants other than A₁₈₉₆ can be selected (Chapter 5), but A₁₈₉₆ is by far the commonest. A possible explanation for this could be stabilisation of ϵ . It has been proposed that stabilisation of ϵ and subsequently improved encapsidation (and so replication) may be a stronger reason, from an evolutionary perspective, for selection of these mutants than 'switching off' HBeAg to escape the immune system. If it is the immune pressure causing selection of A₁₈₉₆ then why are different pre-C stop mutants (or mutants which do not allow initiation of pre-C translation) not selected in those with serine15? Figure 5.5 shows that serine15 (UCC) also stabilises ϵ , so perhaps there is no need to select a stop mutant. It is possible that other pre-C mutants may interfere with other functions of the pre-C region. However, if A₁₈₉₆ is evolutionarily 'fitter', why is this not found to be the infecting virus in most cases?

HBeAg has been implicated in mediating inhibition of HBV replication (Lambert *et al*, 1993). Over expression of HBeAg in transgenic mice greatly reduces the cytoplasmic content of HBV nucleocapsid particles (Guidotti *et al*, 1996). As shown for DHBV *in vitro*, a high level of replication is cytopathic for the hepatocyte (Summers *et al*, 1990), thus the regulation of replication could represent a means of survival for the virus. Selection of a pre-C variant in this instance would remove the regulatory effect of HBeAg and subsequently increase viral replication. However, if the finding of Summers *et al* (1990) is correct, then it would not be in the interest of the virus to select pre-C variants as this would result in increased pathology in the host. The almost universal take-over by variant viruses with pre-C stop codons, observed in this thesis and many other studies, do not bear this finding out.

There are two ways to decipher whether the role of pre-C variants is to enhance replication. One is by using a cell line which is susceptible to HBV replication: a cell line which expresses the proposed viral receptor, annexin V, is now available. A second method would be to use an animal model: work on a SCID mouse model for hepatitis viruses is currently under investigation by our laboratory. In both instances, by infecting the cells or animal model with variant viruses either solely, to test their cytopathic effect, or as mixtures to assess replicative advantage, some of the above points could be answered experimentally. Unfortunately, such systems would not be useful for addressing the role of immune selection on HBV.

Are serine15 and A₁₈₉₆ truly separate lineages and is one 'taking-over'?

The phylogenetic analysis (Chapter 5) indicated that serine15 and A₁₈₉₆ are circulating independently in the Hong Kong Chinese community. This poses the question of whether both these mutants evolved together or whether serine15 is in the gradual process of becoming the dominant strain in this community.

A large retrospective study examining HBcAg sequences in the Hong Kong Chinese community over the past 50 years would document the distribution of these two variants and determine whether they are truly independent. If serine15 is evolutionarily 'fitter', then we would see a gradual change in their distribution. To assess this properly, multiple sequential samples from a large study group, in the region of one hundred individuals, would have to be examined. Careful selection of the clinical background (?and the HLA type) of the patients would be essential in order to ensure viral factors are being investigated and that there is not too much influence from host factors. Studies to search for shifts in the viral sequence over the last 50 years have not, to our knowledge, been done, but it would be interesting to see how many of these variants are

indeed viral dead ends and how many have become established in the population. There is some evidence that the A₁₈₉₆ variant is transmitted on a large scale (Bollyky *et al*, submitted); such a retrospective study would provide experimental evidence.

Why serine15 is particular to the Hong Kong Chinese community and is not observed in any other part of the world, remains unclear. Of course there are many areas of the world in which the pre-C sequence of HBV has not been extensively studied, for example South America, thus perhaps it is prevalent in other populations. There has been one report of HLA type influencing the outcome of infection (Thursz *et al*, 1995), where HLA-DRB1*1302 was found to be associated with protection against persistent HBV infection in children and adults in the Gambia, but whether the HLA background of an individual could have an influence on the infecting strain has yet to be demonstrated.

Is there a geographical difference in HBcAg variation?

Our results on Far Eastern patients are in agreement with Ehata *et al* (1993), in that there is a cluster of amino acid substitutions between amino acids 84 and 101; there were statistically more substitutions here than in the rest of HBcAg ($p=0.0001$). Mutations have also been observed for the region between amino acid 84 and 101 in studies by Bozkaya *et al* (1996), Hur *et al* (1996), Akarca and Lok (1995), and Hosono *et al* (1996) all of whom studied patients from the Far East. When this area is analysed according to the groupings, only group I is significant (group I, $p=0.001$; group II, $p=0.01$, group III, $p=0.18$), implying that the association may be with A₁₈₉₆. Interestingly, when this region is analysed in the patients from Chapter 6, who are of Mediterranean origin, there is no significant difference in the rate of amino acid substitution between this region and the rest of HBcAg ($p=0.94$; CI: 0.45-1.89; CR 0.98) in those

with A₁₈₉₆. This indicates that there is a geographical difference in regions of amino acid substitution that must be related to host.

Therefore, we have shown that the overall rate of core variation is not only dependent on geographical location, but also on selection of a pre-C stop codon, as these genomes have a higher rate of mutation compared to serine15.

The threonine to serine substitution (at amino acid 12 of HBcAg) often observed in Mediterranean patients after selection of A₁₈₉₆ is not observed in the Far East. Interestingly, groups which have analysed HBcAg sequences from the Far East quote the consensus amino acid at amino acid 12 as serine (Hosono *et al*, 1995; Lee *et al*, 1996). This, indicates a difference in the strains circulating in the Far East and Mediterranean countries.

As most investigators in this field examine patients from the Far East the difference in HBcAg substitution between these groups has not previously been demonstrated. It is an interesting finding but, if anything, it complicates the picture of HBcAg variation and its possible role in pathogenesis by increasing the parameters that may be involved. From now on, not only should individual and collective variants be assessed but so must also the genotype, (possibly subtype) and host factors.

Core variation: is it only due to random errors during reverse transcription of the pregenome?

A longitudinal study by Bozkaya *et al* (1996) followed HBeAg-positive patients for up to seven years. When their sequences were analysed, identical nucleotide and amino acid sequences were found despite high serum HBV DNA levels. In the same study, those who were HBeAg-positive and had raised ALT or seroconverted to anti-HBe had a much

higher mutation rate (1.9 ± 0.3 and 2.4 ± 0.4 compared with 0.4 ± 0.1 /nucleotide position/year) . This suggests that the former group are still in the immune tolerant phase of the disease and that random errors that occur during reverse transcription of the pregenome are uncommon. We interpret these results such that the background level of random mutations is 0.4 ± 0.1 /nucleotide position/year and a higher mutation rate therefore indicates positive selection.

Is HBcAg variation related to disease severity or does it represent immune escape?

The initial aim was to answer the above question. The clinical features of a group of anti-HBe positive, viraemic chronic patients, seen most commonly in the Mediterranean and the Far East, prompted this question. Why should such patients have continued disease after seroconversion to anti-HBe?

The appearance of replication-competent core protein variants during infection raises the probability that this protein is under immunological pressure. The size and organisation of the HBV genome may hinder the development of some mutations yet this study has confirmed that it can tolerate multiple substitutions (the highest number of described substitutions in one HBcAg was 16; [Chapter 6]) thus highlighting, I believe, its remarkable capacity for adapting to the changing immunological environment yet maintaining the integrity of its open reading frames.

As discussed earlier (Chapter 1, section 5.4) it is currently believed that HBeAg induces tolerance, thus explaining the high proportion of neonates that become chronically infected and why most HBeAg positive chronic carriers initially have minimal disease. Eventually, tolerance to HBeAg seems to be lost, the immune system is 'switched on' to HBeAg, with the

result that hepatitis is induced. If a particularly vigorous immune response was activated, this could lead to pressure on the virus to 'switch off' this response, providing a possible explanation for pre-C variants. If variants did not appear then, as HBeAg-producing hepatocytes are eliminated, so is the virus. Once the mutant has been selected there appears to be very little back selection; if an HBeAg-producing strain was reintroduced it would probably be quickly recognised and destroyed. If HBeAg is the only immune target, this would bring host and virus back to the *status quo*. However, the patients studied here had continued flares of hepatitis even after seroconversion to anti-HBe and development of a pre-C variant, implying that the immune system is then directed against another antigen.

As HBcAg/HBeAg have sequence homology, the immune response may target HBcAg only after HBeAg has been 'removed' from the equation by a pre-C variant; thus, my work focused on examining the nature and distribution of variation in HBcAg.

The sequential sequencing (Chapters 5 and 6) allowed us to show unequivocally that there is an initial burst of amino acid substitutions simultaneously or soon after the appearance of a pre-C stop codon. Statistical analysis of the observed substitutions proved them to be significant in B- and Th-cell epitopic areas, indicating that there is immune pressure in HBcAg. This was not observed in a sequential study by Asahina *et al* (1996) in which there were no observed core mutations even after selection of a pre-C stop codon. However, in this paper, only one patient selected a pre-C mutant. Simultaneous pre-C/C mutations were observed in 11 patients (from both Chapters 5 and 6). Bozkaya *et al* (1996) also observed appearance of HBcAg mutations at the time of, or shortly after, the appearance of pre-C stop codon.

As discussed in Chapter 1 (section 6.2, Table 1.11) there appears to be an association with substitutions in the C-terminus of HBcAg and severe

disease. Recent clinical data was available for some of the Greek patients studied in Chapter 6. When the C-terminus of these patients was examined for substitutions, five had substitutions in this area (H25, H27, H33, H35 and H36). An update on health status was available for: H27 - currently inactive; H33 - died of HCC in 1995; H35 - underwent a course of IFN treatment in 1994 and is currently in remission; and H36 - still experiencing episodes of severe disease activity. No further information was available for H25. Two of the patients (H33 and H36) help to substantiate our theory but the other two patients (H27 and H35) do not. Perhaps, once again, the difference in positions of substitutions within HBcAg is ethnically influenced. All the patients in Table 1.11 are of Far Eastern origin. Unfortunately, no recent clinical information was available for those patients studied in Chapter 5.

Why should these patients have continued disease if they no longer have the appropriate epitope? One possibility is that these mutations result in presentation of a 'new' epitope to which the immune system responds. Yet, as we have shown from the sequential study (Chapter 6) in which we sequenced patients over multiple flares of hepatitis, there is very little further mutation after the initial burst that is associated with the appearance of the pre-C variant. Therefore, the variation in HBcAg is associated with disease progression but is not the cause of the disease; the assumption is made that liver injury is caused by the immune response to the virus. This leads to another possibility, that the new variants observed cause the virus itself to be pathogenic. This was not experimentally assessed in this thesis but work in our laboratory has shown that certain HBcAg mutations can cause a shift in localisation of HBcAg in the cell. The differential accumulation of HBcAg may (perhaps) cause cell damage. Again, this possibility could be formally assessed with the use of the cell culture system or animal model discussed earlier.

Unfortunately, this question remains incompletely resolved. The former seems unlikely for three reasons. Firstly, if a substitution appears which

leads to a fresh immune attack, the prediction is that this virus population would be cleared. However, in all 12 of the cases studied in depth (Chapter 6), mutants selected between the initial and second samples remained until the final sample; this is unlikely to be a chance occurrence. Secondly, few mutations were seen subsequent to the initial burst even though there were intermittent flares of hepatic activity in these patients. Thirdly, sequencing complete core genes from four or five sequential sera from each of three patients did not disclose transient mutations which would be predicted to stimulate and be cleared by a new immune response, such that they are not present in the final sample. Taken together, this evidence indicates that the mutated virus strains are escaping from the immune response against HBcAg, rather than causing peaks of hepatitis. That mutations occur within B-cell epitopes indicates that selection may be antibody-mediated and casts doubt on the widely held notion that anti-HBc is innocuous and merely a marker of infection. Furthermore, anti-HBc has been shown in the chimpanzee to favour viral persistence (Iwarson *et al*, 1985). We had hoped to provide direct evidence for this from our T- and B-cell studies but unfortunately time was against us. These studies are ongoing and will hopefully help to elucidate what is happening in HBcAg. Comparison studies between monomeric and branched peptides have shown that the latter are more sensitive for anti-HBc binding studies (Chapter 7). We believe that the direction we were tried in Chapter 8, using recombinant HBcAg, is probably the best route for trying to determine whether there is antibody selection as several of the overlapping branched peptides were insoluble. Ideally, we would like to test sera from anti-HBe chronic patients with multiple flares of hepatitis, before, during and after these flares using recombinant HBcAg (rHBcAg) derived from their circulating HBcAg at each time point. If later rHBcAg failed to bind to earlier sera this would be an indication that a

'new' antibody response has been initiated against a variant virus. A simultaneous experiment testing PBMCs from the same time points using the overlapping peptide series (preliminary results have shown that the configuration of the peptide is not important in lymphoproliferation studies) would address whether Th escape is occurring. This would hopefully, once and for all, determine whether these flares are associated with new variants or not and whether they are immune mediated. It is possible that either substitutions appear in protein products of the other three genes of the virus or that HBcAg mutations in a strain which is present at less than 5% of the population, account for the flares of hepatic activity. This was not assessed in this study as it is logistically difficult and time-consuming if statistical significance is to be achieved.

There is some evidence that the sequence context of substitutions may be as important as the variation itself (Bollyky *et al*, submitted). Thus, in future studies, there will need to be consideration of whether there are several nucleotide or amino acid motifs associated with a particular disease pattern. These analyses have to take into account the natural genotype variability, as a substitution in a genotype in which it seldom occurs, may be significant. Also, it is unlikely that there will be perfect associations between variants and functional effects as that would not take any host factor into account. Fulminant hepatitis, regarded as an 'end-of -the-road' infection that is not in the interest of the viral persistence, has been linked to HLA type (DR-1302/2) (H. Thomas, personal communication). This is consistent with the observation that the isolate from a case of fulminant hepatitis had an identical nucleotide sequence to that observed in the patient with minimal disease who was the source of the infection (Alexopoulou *et al*, 1996). What is more difficult to reconcile to a simple host factor is the finding of outbreaks of fulminant disease and the preferential transmission of fulminant hepatitis from anti-HBe carriers; there have been several outbreaks of fulminant hepatitis

from a single source (Oren *et al*, 1989). As shown for the multiple mutations that can inhibit HBeAg production, there are probably multiple ways of causing a similar functional effect in a regulatory region. New evidence suggests that the pre-C mutant is not the sole cause of fulminant hepatitis but that it is caused by a combination of mutations, including those within pre-C and the X gene (Bollyky *et al*, submitted, 1997). Additionally, A₁₈₉₆ strains can be transmitted independently and cause typical acute hepatitis.

Finally, HBV, with its compact organisation, seems to be highly evolved but we have shown that it does have the potential for significant change within HBcAg, that it has important effects on immune recognition and that there is interplay between mutation events both in place and over time. We have shown that with the appearance of HBeAg negative variants, in particular G₁₈₉₆A, there is strong indirect evidence that selection pressure builds on HBcAg epitopes. Despite extensive studies on pre-C/C variation and the vast amount of data published from these studies, from our laboratory and others world-wide, there is still no clear evidence for a causal link between specific mutations or the degree of substitution in HBcAg and the outcome of infection with HBV as, unfortunately, there has not been any good published data on the functional effects of HBcAg variants. Several groups have now changed direction and are now focusing on HBxAg. Many important regulatory elements reside within HBxAg and it is likely that variants occurring in this region will affect either gene expression or viral replication to some degree. The eventual outcome, I believe, will be explained by host and viral interactions, with the consequence that variants of HBV will behave differently in each population. Future studies, described here and which were initiated from work in this thesis will decipher their role.

Appendix 1: Details of primer sequences used in the course of this thesis

Primer Name	Primer Sequence
<i>sense- 5' to 3'</i>	
C1	CGGGATCCGAGGAGTTGGGGGAGGAGATT
C3	CGGGATCCCTTTGTACTAGGAGGCTGTAG
C α	CATACAGCACTCAGGCAAGC
CPET3	GGAATTCCATATGGACATTGACCCT/GTATAAAG
19	AGACCACCAATGCCCCTATC
<i>antisense3' to 5'</i>	
Bamtag	GAGCCCTTAGAGTTACACTCGCGTTCTGCGGGG <u>CGCAGTTGCCGCCGATCCCTAGGGC</u>
BC1	GGAAAAGAACTCAGAAGGCAA
C2	GAGAATTCACCTTATGAGTCCAAGG
C2N	CCCCGTAAAGTTC/TCCG/CACCTT
C4	GAGAATTCTACTAACATTGAGATTCCCA
C4N	CCTTATGAGTCCAAGGG/AATA
C5	AGTCGAATTCCTTTGTACTAGGAGGCTGTAG
CD3	TGCTGGGTCTGCCAAATTACCACCCACC
CPK	GGAATTCATGGACATTGACCC
KC1	GCGAATCCACACTCCAAAAGA
Ser15	GGGGTCGACCGCACCATGCAACTTTTTTCACCT CTGCCTAATCATCTCTTGTTTCATGTTCCACTGTT
WB2	AACTGCAGCCCTTATGAGTCCAAGGG/AATA

Single letter amino acid codes are given in Appendix 2.

The underlined amino acids identify the tag sequence

Appendix 2 One and three letter abbreviations for amino acids

Amino acid code	Three letter code	One letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Appendix 3 Publications arising from this thesis

Boner, W., Dornan, E., Hadziyannis, S., Thursz, M., Fattovich, G., Colman, K., Carman, W.F. Core protein substitution, anti-HBe positive chronic hepatitis and B-cell epitopes. *European Association for the Study of the Liver*, Athens, August 1995.

Boner, W., Lok, A.S.F., Carman, W.F. Characterisation of two pre-C variant HBV isolates from Hong Kong. *Congress of Virology*, Glasgow, August 1993.

Boner, W., Schlicht, H.J., Hanrieder, K., Holmes, E.C., Carman, W.F. (1995): Further characterisation of 2 types of precore variant hepatitis B virus isolates from Hong Kong. *J. Infec. Dis.* **171**: 1461-1467.

Carman, W.F., Boner, W., Fattovich, G., Colman, K., Dornan, E., Thursz, M. and Hadziyannis, S. (1997): Mutation of the pre-core/core gene of hepatitis B virus during chronic infection and correlation with epitopes. *J. Infec. Dis.* May issue

Carman, W.F., Boner, W. and Thomas, H.C. (1997): The role of host factors and viral variation in hepatitis B infection. *Gastroenterology*, Submitted.

Dornan, E., Boner, W., Wakeling, M., Hadziyannis, S., Fattovich, G., Carman, W.F. Shift in cellular distribution by confocal microscopy of in vitro expressed HBcAg from sequential patient samples. *IXth triennial international symposium on viral hepatitis and liver disease*. Rome, May 1996.

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