MOLECULAR BIOLOGY OF FULMINANT HEPATITIS B VIRUSES

MAHMUDA YASMIN

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789 East Eisenhower Parkway
P.O. Box 1346
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SUMMARY

Fulminant hepatitis B (FHBV) is a rare but severe outcome of acute hepatitis B virus infection. The severe liver injury is attributed to an exaggerated immune response directed at clearing virus by causing lysis of infected hepatocytes. Multiple factors could contribute to severity of the disease. Our study has shown that although there are no specific variant(s) linked to severity, there are clusters of variants in the *cis*-acting regulatory elements which can upregulate replication of FHBV strains. The most novel concept to come out of this study is that, most of these variants are not unique to FHBV sequences but they are significant when they occur in the form of 'motifs' or combinations; we showed that these multiple motifs are associated with a common functional effect.

We have sequenced: full genomes from 8 patients (5 FHB, 2 chronic contacts and 1 acute contact case), from beginning of enhancer-I to the end of core gene from 11 patients, and the core gene only from 3 patients. Clusters of nucleotide substitutions were found particularly in Enh-I (nts 1050, 1059, 1249 and 1250), the BCP/Enh-II complex and in the X gene. Aberrant cysteine and methionine residues in X gene were associated with FHBV sequences. No specific clustering was found in the pre-S/S or polymerase genes. Our study found that A_{1896} has an association with FHBV, but other variants elsewhere in the genome distinguishes FHBV-A_{1896} from other A_{1896}. A_{1896} is strongly linked with HBeAg negative/anti-HBe positive infection and associated with early clearance of viraemia.

We performed phylogenetic analysis on 30 core gene and 26 X gene sequences from patients with FHB and 161 core gene and 88 X gene sequences from non-FHBV case in order to identify any common patterns of genetic variation. Sequences from epidemiologically unlinked FHB patients cluster together on phylogenetic trees, indicating that particular strains are linked to FHB. The second novel concept is that the cluster identity was closely related to clinical outcome and progression of FHBV disease. There were significantly higher rates of evolution in FHBV sequences, consistent with an elevated rate of replication. Substitution of nucleotides in the *cis*-acting elements and amino acid residues in the X protein, the pre-core variant A_{1896}, are linked to FHBV pathogenesis in the form of particular combinations, or motifs which are predominantly or uniquely associated with FHBV sequences.

In functional analysis, We than showed that variants motifs affect the functional properties *in vitro*. We tested sequences from 11 FHB and 7 chronic carrier controls, not associated with FHB in contacts, and 2 subtype controls. Luciferase expression in hepatocytoma cell line (HuH7), driven from cloned BCP/Enh-II sequences, was high (>7 arbitrary units) in 7 FHB and intermediate (2-7 arbitrary units) in two others. Two of 9

controls had intermediate, and the others low(<2) expression. Specific variant motifs were associated with raised luciferase expression, in particular at nt 1766/1768, 1762/1826 and 1727/1740. High luciferase expression correlated with rapid progression of disease, clearance of viraemia and presence of A_{1896}; controls with A_{1896} had normal expression. Thus multiple sequences have had a common phenotype. Finally, a series of radiolabelled oligonucleotides with control and FHBV sequences, encompassing the major binding sites of transcriptional factors in the BCP, were bound to nuclear extract and shift assays performed. For all variant oligonucleotides, two complexes were missing compared to control sequences. This led to a unified theory to account for most FHBV sequences: that failure of inhibitory transcription factors to bind to variant BCP sequences led to increased replication.

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CONTENTS

LIST OF ABBREVIATIONS

aa	amino acid
Ag	antigen
ALT	alanine amino transferase
anti-HBc	antibody to hepatitis B core antigen
anti-HBe	antibody to hepatitis B e antigen
anti-HBs	antibody to hepatitis B surface antigen
APC	antigen presenting cell
BCP	basal core promoter
CREB	CRE (cAMP response element) binding protein
CP	core promoter
CTL	cytotoxic T lymphocyte
CURS	core upstream regulatory sequence
DNA	deoxyribonucleic acid
DR	direct repeat
Enh	enhancer
Enh-I/XP	enhancer I/X promoter
Enh-II/CP	enhancer-II/core promoter (includes enhancer-II, BCP and CURS)
FHBV	fulminant hepatitis B virus
FHF	fulminant hepatic failure
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HLA	human leukocyte antigen
IFN	interferon
LHBs	hepatitis B large surface protein
MHBs	hepatitis B middle surface protein
NRE	negative regulatory element
nt	nucleotide (numbered from Eco R1 site of the genome)
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
Pre-S1	pre-surface 1
Pre-S2	pre-surface 2

RNA	ribonucleic acid
S	surface
SHBs	hepatitis B small surface protein
TCR	T cell recognition
Th	T helper cell
TP	terminal protein

ONE AND THREE LETTER ABBREVIATIONS FOR AMINO ACID RESIDUES

Amino acid	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cystine	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

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CHAPTER-1

INTRODUCTION

1.1 HEPATITIS B VIRUS (HBV): MOLECULAR BIOLOGY

1.1.1 BRIEF BACKGROUND

Before the discovery of etiologic agents, two types of viral hepatitis were known on the basis of epidemiological studies. Type A was considered to be predominantly transmitted by a faecal-oral route, whereas type B was transmitted parenterally by human serum. Thus, hepatitis B was also known as 'serum hepatitis'. In 1963, Blumberg first discovered an antigen in the blood of an Australian aborigine which, after several years of investigation, was found to be associated with acute hepatitis B (Blumberg *et al.,* 1967). It was then named hepatitis associated antigen (HAA) and currently is designated hepatitis B surface antigen (HBsAg). HBsAg in blood has proved a convenient marker for hepatitis B infection providing a wealth of information about the epidemiology and course of infection. Early investigations of HBsAg led to the recognition of the description of persistence or chronic HBV infection. Persistent infection is defined by circulation of a relatively high concentration of HBsAg particles for years. The blood of persistently infected patients, also known as 'carriers', provides a good source of viral antigens for physical and chemical characterisation. Most of the HBsAg in blood is in the form of particles; they are pleomorphic or round, approximately 22 nm diameter and devoid of nucleic acid (Bayer *et al.,* 1968). However, larger, double-shelled particles (Dane particles) were detected in the serum of a carrier (Dane *et al.,* 1970). These particles were the only antigenic form known to contain nucleic acid and it was suggested that these were the complete form of HBV. Almeida *et al.* (1971) removed the outer shell of Dane particles using a non-ionic detergent, revealing a new specificity which was termed hepatitis B core antigen (HBcAg). Hirschman *et al.* (1971) first detected small amounts of DNA polymerase activity in crude pellets obtained from HBsAg positive serum by high-speed centrifugation and found endogenous template for the polymerase. Mistakenly, they postulated that the template of the enzyme preparation was RNA, as the polymerase reaction was interrupted by RNase treatment. Kaplan *et al.,* (1973) found DNA polymerase activity in a high-speed centrifugation pellet from HBsAg positive serum; the reaction product had a sedimentation coefficient of 15S. The reaction was inhibited by actinomycin D suggesting that the template was DNA, not RNA. The enzyme, the template and the reaction product appeared to be internal components of the core particle. Soon after this, the circular, partially double-stranded DNA was characterised (Robinson *et al.,* 1974).

The discovery of HBV led the way for the identification of further hepatitis viruses. Currently five definite hepatitis viruses are known, hepatitis A-E; there is some doubt over the relationship between 'hepatitis G' and hepatitis. These letters do not

indicate taxonomic relatedness, but only to the fact that these very different viruses may all cause clinical hepatitis.

Patients infected with HBV develop either an acute self limiting infection or persistence or may not cause disease. It was postulated that this virus might be the cause of liver cancer, leading to the search for an HBV-like agent in woodchucks (*Marmota monax*), which had been observed to develop liver cancer. Woodchuck hepatitis virus (WHV) was subsequently discovered (Summers *et al.,* 1978), with a morphology indistinguishable from HBV and a genome with approximately 60% nucleotide sequence identity with its human counterpart (Galibert *et al.,* 1982). A series of similar viruses has now been recovered from a variety of animal species, including the ground squirrel hepatitis virus (GSHV) in Beechy ground squirrels (Marion and Robinson, 1980), a species that is distantly related to marmots, and duck hepatitis B virus (DHBV) in Peking ducks (*Anas domesticus*), which also sometimes develop liver cancer (Zhou *et al.,* 1980). Less well characterised viruses have been recovered from grey herons living in their natural habitat, domestic geese, marsupials and other hosts (Sprengel *et al.,* 1988; Tenant *et al.,* 1992). HBV and its relatives constitute the family Hepadnaviridae, the term derived from their hepatotropism and DNA genome (Marion *et al.,* 1983). All of them have common properties: being enveloped with 3-3.3 kb partially double stranded DNA; having a virion associated polymerase; production of excess subviral particles; narrow host range; and development of persistent infection. However, there are also a number of biological differences between them including variation in host range, tissue tropism, pathogenic spectrum and routes of transmission.

1.1.2 VIRION AND GENOME ORGANISATION

Electron microscopic studies on serum revealed that HBV infected cells produce multiple types of virus-related particles. Among these, the 42 nm diameter double shelled Dane particles are the infectious HBV virions. There are, in addition, 20 nm spheres and 20 nm diameter filaments of various lengths. The outer shell of a Dane particle, also known as the envelope, is a lipoprotein membrane containing the HBV surface proteins (HBs). The Dane particle comprises a 27 nm inner body, the nucleocapsid or core particle, surrounded by a 7 nm outer coat. Core particles are composed of core proteins or HBcAg and enclose the viral DNA, viral DNA polymerase, the terminal protein covalently bound to the minus DNA strand and a protein kinase.

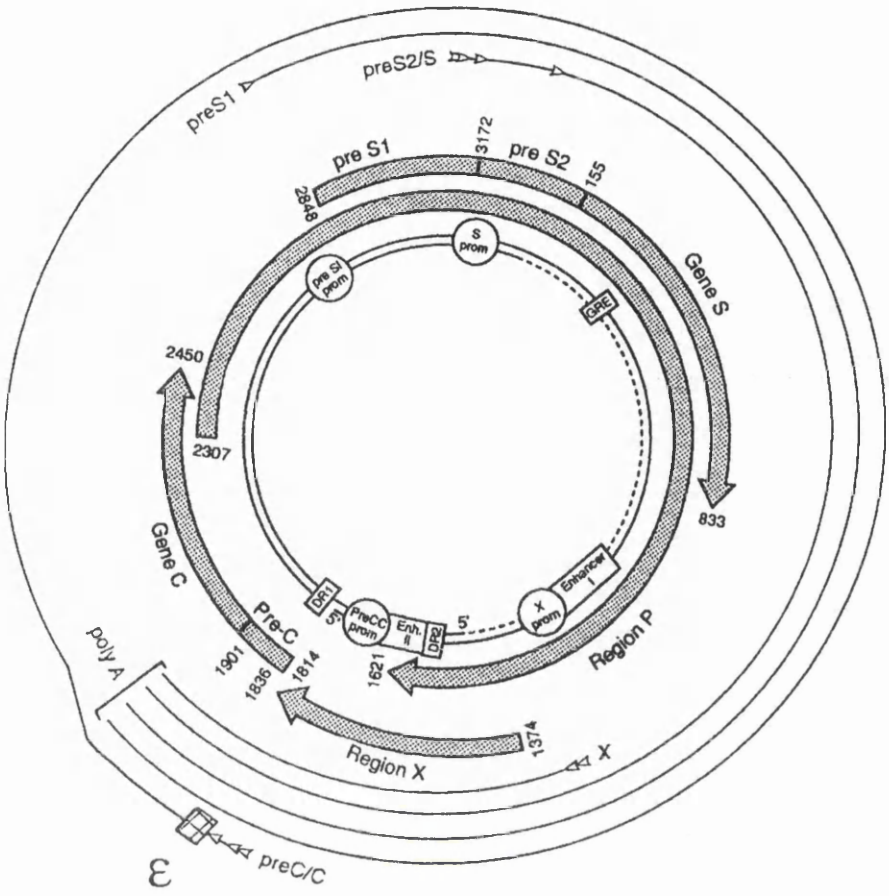


Figure 1.1 Genome organisation of HBV. The outer lines represent the different classes of RNA transcripts. The four major ORFs, pre-core (Pre-C)/core (C), pre-S1/pre-S2/S, P and X, are indicated in the inner shaded arrows. The lines in centre represent the partially double stranded DNA genome showing *cis*-acting regulatory elements. GRE = glucocorticoid-responsive element, prom = promoter, Enh = enhancer, ε = encapsidation signal.

The HBV genome is 3.2 kb long, relaxed circular, partially double stranded DNA and contains four open reading frames (ORFs). The coding organisation is highly compact, i.e. every nucleotide in the genome is within a coding region, and over half of the sequence is translated in more than one frame. As shown in Figure 1.1 there are four ORFs which encode the viral 'envelope' or surface, nucleocapsid, polymerase and X proteins (Ganem *et al.,* 1987). The largest ORF (P-ORF) encodes the viral polymerase, covers some 80% of the genome and is translated from the 3.5 kb pregenomic RNA (see below). The polymerase protein is also an indispensable component of the viral RNA encapsidation process. It contains four functional domains: reverse transcriptase, DNA polymerase, RNaseH and a 5' terminal binding protein (TP), which serves as a primer for reverse transcription of the pregenomic RNA (Wang and Seeger, 1992).

The second ORF contains two in-phase start codons defining two overlapping polypeptides. The larger translation product is the precore/core 25kD protein which is

translocated, via a signal polypeptide at its extreme amino-terminal end, into the endoplasmic reticulum (ER). Here it undergoes truncation of amino and carboxy terminal residues and is secreted into the blood as hepatitis B e antigen (HBeAg). This protein is not a structural component of the virus but probably plays a role in modification of the host immune defence against HBV. When translation initiates at the second ATG, the 21kD core protein is produced, which self-assembles in the cytoplasm to form nucleocapsid particles which package the viral polymerase and pregenomic RNA, thereby facilitating viral replication. Both pre-core and core proteins are translated from full length terminally redundant 3.5 kb transcripts with slightly different 5' ends. The longer pre-core mRNAs contain the pre-core initiation codon and code for HBeAg; the shorter pregenomic mRNA lacks this.

The third ORF encodes X protein (HBx) which is a transcriptional transactivator for the transcription of HBV as well as viral and cellular promoters (*in vitro*).

The fourth open reading frame, the surface gene, contains three in-phase translational start codons which define the amino termini of three overlapping polypeptides, designated large, middle and small surface proteins. All share the same carboxy terminus, HBsAg or SHBs. The middle (MHBs) and large proteins (LHBs) have additional pre-surface regions, known as pre-S1 and pre-S2, at the amino terminus, which are involved in binding to hepatocyte receptor(s) during viral entry to the cell.

1.1.3 VIRAL LIFE CYCLE

The life cycle of all hepadnaviruses can be divided into several steps: i) attachment of the virus to the host cell, ii) viral penetration and release of its genome, iii) expression of viral genes and replication of viral genome and iv) virion formation and release (Figure 1.2)

i) Attachment to the host cell

Virus attachment is one of the crucial steps that determines the host range and organ tropism of viruses. So far, little is known about the mechanism of attachment of the HBV virion to the cell membrane of the human hepatocyte, the natural host of HBV. Viral surface proteins are crucial molecules in recognising a possible receptor on the membrane of hepatocytes. Studies on the attachment of HBV surface proteins have been hampered by the lack of a susceptible tissue culture system. Much work has been done on the viral surface proteins that may be involved in host cell interaction. For HBV and DHBV several lines of evidence indicate that pre-S proteins participate in cellular receptor binding. For HBV, the pre-S1 domain of LHBs and two elements of the pre-S2 domain in the MHBs have attachment sites for hepatocyte membranes or hepatoma cells. Pre-S1 mediated attachment was found by Neurath *et al.* (1986) using HepG2 hepatoblastoma cells, but not using animal liver cells or human carcinoma cells of non-hepatic origin.

Binding could be blocked by antibodies to amino acids 21 to 47 of the pre-S1 protein, neutralising infectivity of HBV (Neurath *et al.,* 1989). However, no liver protein has been identified that specifically binds to the pre-S1 peptide. The pre-S1 sequence is also believed to bind to a novel form of membrane bound interleukin-6 (Neurath *et al.,* 1992).

There are three hepatocyte attachment sites in the pre-S2 domain. The pre-S2 linked glycan of MHBs has a weak but distinct affinity for HepG2 cells, but not for mouse hepatocyte culture or the human carcinoma HeLa cell line. Pre-S2 attachment to cells can be mediated by modified human serum albumin (pHSA) pre-treatment of liver cell membranes, which strongly increases the binding of HBV (Pontisso *et al.,* 1989, Korne *et al.,* 1990). However, recent studies suggested that the role of MHBs is dispensable for both morphogenesis and infectivity, as patients with chronic HBV can harbour variants devoid of MHBs which are infectious *in vivo* (Kann *et al.*, 1995).

The SHBs is not only indispensable for virion morphogenesis, but also for infectivity. Human hepatocyte membranes seem to bind SHBs via membrane-bound endonexin II (E-II), a protein which specifically binds to HBsAg (Hertogs *et al.,* 1993). Human E-II is a 34kD protein and is also known as annexin-V (Walker *et al,.* 1992). Anti-HBs antibody developed spontaneously in rabbits immunised with human liver E-II and also in chickens immunised with the $F(ab')_2$ fragments of anti-human liver E-II immunoglobulin G (Hertogs *et al.,* 1994). These antibodies are also able to compete with E-II for the binding of HBsAg, indicating that the anti-idiotypic antibodies mimic a region of E-II that interacts with small HBsAg. Later, HBsAg conjugated to gold particles was shown to bind to human hepatocyte and were internalised (Bruin de *et al.,* 1995). Inhibition by anti-E-II or anti-idiotypic antibodies of HBsAg-gold particle binding to hepatocytes supports the idea of specific attachment through the SHBs.

ii) Viral penetration and genome release

The mechanism of HBV entry into the hepatocyte is not well understood. Some data suggest that the entry of the nucleocapsid into the cytoplasm may occur by pH independent mechanisms (Rigg and Schaller, 1992), because there is evidence that uptake of DHBV into hepatocyte does not require proteolysis and acid pH.

Nothing is known about the delivery of the genome into the nucleus. In the DHBV system, DNA is found in the nucleus 24 hrs after infection, and is converted to covalently closed circular (ccc) molecules (Tuttleman *et al.,* 1986). However, it is not yet known whether the free DNA or the core particles are transported to the nucleus. Some speculate that core particles themselves are transported to the nucleus since the core protein contains a nuclear localisation signal at its carboxy terminal end (Yeh *et al.,* 1990) and the diameter of the core particle is at the limit of the functional nuclear pore size, as determined by experimental measurements in *Xenopus* oocytes (Feldherr *et al.,* 1984).

However, the possibility that core particles disassemble in the cytoplasm and deliver their DNA to the nucleus has not been excluded.

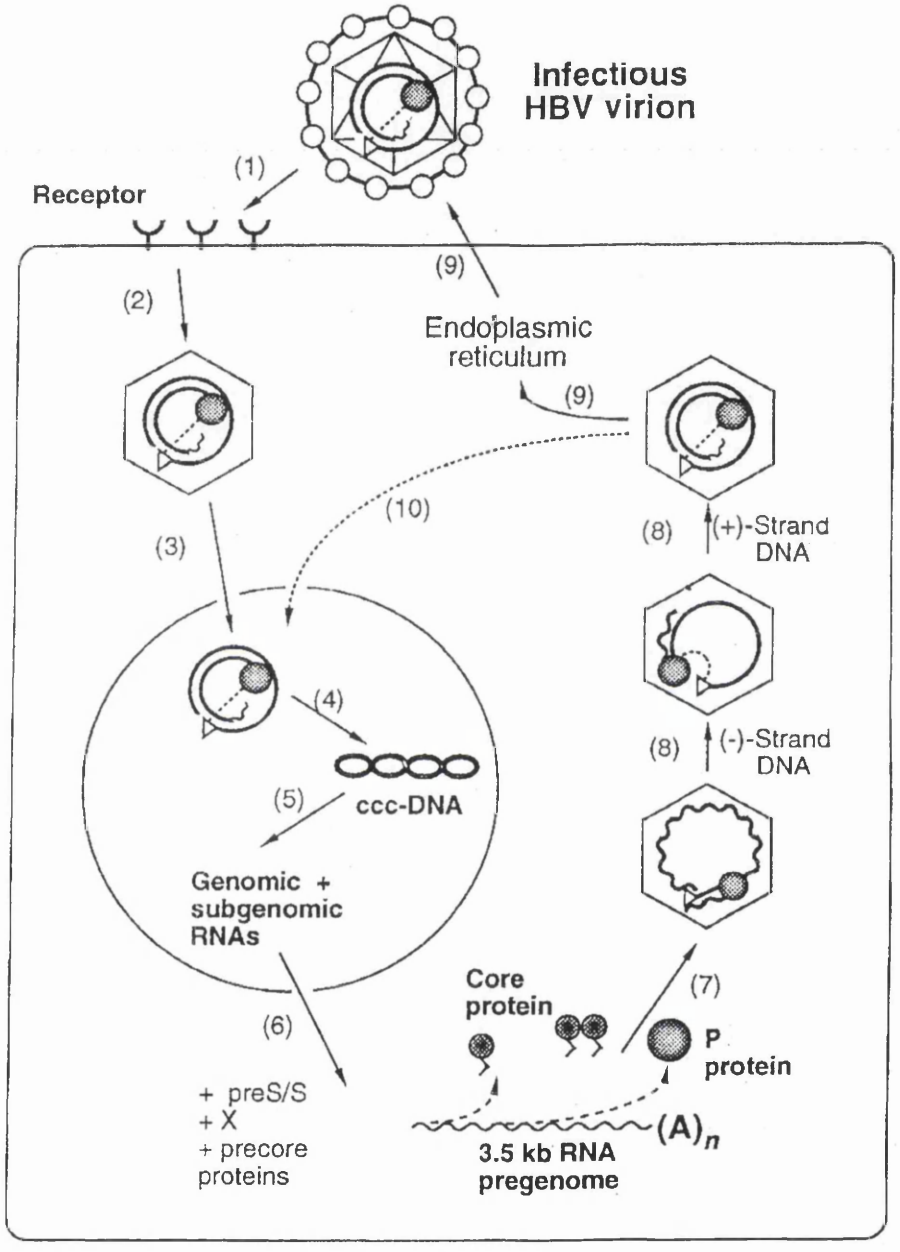


Figure 1.2 Life cycle of HBV in simplified diagram: (1) attachment to and (2) penetration of the host cell; (3) uncoating and transport of viral genome to the nucleus; (4) conversion of ccc DNA and (5) RNA transcription; (6) transport and translation of RNA; (7) assembly and encapsidation into core particle; (8) reverse transcription of pregenomic RNA and plus strand DNA synthesis; (9) envelopment and export from the cell; (10) core particle may transport to nucleus for amplification of ccc DNA. (This picture is taken from Nassal and Schaller, 1993)

iii) Genome replication

The main steps of genome replication occur in the nucleus. and there are four fundamental steps, as reviewed by Ganem and Varmus, (1987). Firstly, the partially double-stranded virion DNA is converted into ccc DNA which is used as the template for the synthesis of viral RNAs (Mason *et al.,* 1982; Miller *et al.,* 1984). Formation of ccc DNA from relaxed circular DNA requires; i) dissociation of terminal protein (TP) from the 5' end of the minus strand DNA and removal of a terminally redundant segment from the same strand, ii) ligation of the 5' and 3' ends of minus strand DNA and iii) the removal of the RNA primer from the 5' end of the plus strand DNA and completion of plus strand DNA synthesis. The ccc DNA has an extremely long half life as this form is found in the liver cells of chronically infected animals. This indicates that infected cells may maintain their ccc DNA pool by reinfection of the hepatocyte (Tuttleman *et al.,* 1986).

Once inside the host cell nucleus, ccc DNA serves as the template for the synthesis of viral RNAs using host RNA polymerase II. The ccc DNA preparations from cloned DNAs are infectious *in vivo* (Will *et al.,* 1982; Segeer *et al.,* 1984). Transcription of the genome results in both genomic and subgenomic mRNAs. 3.5 kb, 2.1 kb and 2.4 kb transcripts have all been isolated from infected liver cells. The 2.1 and 2.4 kb transcripts are the mRNAs for surface proteins (Cattaneo *et al.,* 1983; Standring *et al.;* 1984 and Will *et al.,* 1987). The pregenomic RNAs serve as both mRNAs for core and polymerase proteins and also as a template for the viral reverse transcriptase (Summers and Mason, 1982, Enders *et al.,* 1985, Buscher *et al.,* 1985 and Will *et al.,* 1987). The mRNAs are transported to, and translated in, the cytoplasm. Virion assembly occurs only when sufficient quantities of core, polymerase protein and pregenomic RNAs accumulate in the cytoplasm. Core proteins self assemble and encapsidate pregenomic RNA into the nucleocapsid along with the viral polymerase.

The core protein alone is able to self assemble via a dimeric intermediate (Nassal, 1992a; Zhou and Standring, 1992). However, for efficient encapsidation of RNA and polymerase, hepadnaviruses have evolved a strategy to package pregenomic RNA along with the polymerase into core particles. The polymerase protein binds directly to a *cis* acting recognition site, the encapsidation signal (ε) consisting of some 94 nucleotides (nt) close to the 5' end of pregenomic RNA (Junker-Niepman *et al.,* 1990). ε is a hairpin-like secondary structure consisting of two stems, a bulge, a loop and a non-paired U. This moderately stable structure is sufficient for encapsidation. Packaging of polymerase protein into the core particle occurs via an RNA-protein interaction with the encapsidation signal rather than by a covalent bond to the core protein. Mutational analyses revealed that the sequence in the loop and first two of the six nucleotides in the bulge are important for productive interaction with polymerase protein (Rieger and Nassal, 1995). ε is also present in pre-core mRNA but due to movement of the ribosome during protein synthesis,

this RNA can not fold to form the secondary structure (Nassal *et al.,* 1990). ε sequence is also present in the 3' end of pregenomic RNA, but this copy is not involved in encapsidation (Nassal and Schaller, 1993). The 3.5 kb pregenomic RNA is greater than genome length and bears a terminal redundancy (R), which includes one copy of the encapsidation signal and one copy of direct repeat 1 (DR1). DR1 is an 11 to 12 nt sequence located at both the 5' end and 3' end (DR1*) of the pregenomic RNA. A third copy of the repeat, called direct repeat 2 (DR2), is located upstream of the DR1*. DR1 and DR2 are the sequences required for the initiation of minus and plus strand DNA synthesis respectively.

As encapsidation proceeds, or after it is completed, reverse transcription of the pregenomic RNA begins, generating the minus (-) strand DNA. The pregenomic RNA serves as the template for minus strand synthesis. In the woodchuck system, it was found that reverse transcription initiates at the 3' end direct repeat 1 (DR1*) on the pregenomic RNA, which contains a short sequence (UUCA), essential for initiation (Seeger *et al.,* 1990). A similar mechanism was observed in HBV where the 3' end DR1* was believed to initiate the synthesis of minus strand and, as the synthesis progressed, the RNA template was simultaneously degraded by RNase H. It was assumed that minus strand DNA synthesis was a continuous process starting *de novo* from within the 3' proximal DR1* and then proceeding continuously along the RNA template (Seeger *et al.,* 1991a). However, recent studies suggest that the polymerase protein binds to the bulge of ε at the 5' end of the pregenomic RNA, copies a part of it (Rieger and Nassal, 1996) which is complementary to UUCA (Nassal and Rieger, 1996) and this protein-oligonucleotide complex is subsequently efficiently translocated to 3' DR1*. This short oligonucleotide, covalently linked to the polymerase protein, serves as a short primer for the reverse transcriptase (Nassal and Rieger 1996). Two complementary nucleotides at its 3' end are sufficient to direct the oligonucleotide to the correct target site (Nassal and Schaller, 1996). The minus strand DNA remains covalently attached to the TP domain of the polymerase protein through a phosphodiester linkage (Gerlich *et al.,* 1980; Wimmer, 1982). As elongation proceeds, the presence of heterogeneous length DNA of less then 3200 bases is not uncommon. However, in a full cycle of DNA synthesis, reverse transcription continues until the pregenome is completely transcribed. This generates a 9 nucleotide redundancy (r) in minus strand DNA (Seeger *et al.,* 1991b).

The last step in the viral replication process is plus (+) strand synthesis using minus strand DNA as the template. Plus strand DNA synthesis is primed by an RNA oligomer originating from the 5' end of the pregenomic RNA, bearing a copy of DR1 (Seeger *et al.,* 1986; Will *et al.,* 1987). This DR1 oligoribonucleotide is generated by RNaseH activity which degrades the pregenomic RNA. Following completion of minus strand DNA synthesis, the RNA primer is translocated to DR2 sequences on the minus strand where it acts as a primer for plus strand DNA synthesis (Seeger *et al.,* 1986).

During plus strand elongation the genome is circularised but, in a majority of cases, polymerisation ceases before the plus strand is complete. Thus the mature virus particle contains relaxed circular, partially double-stranded DNA (Summers and Mason, 1982, Lien *et al.,* 1986). The reason for incomplete plus strand synthesis remains unknown. The envelopment of core particles may result in a shortage of nucleotides, consequently leading to premature termination of plus strand elongation

iv) Envelopment of core particles and virion release

Pregenomic RNA is translated to produce core and polymerase proteins in the cytoplasm; core particle assembly and genome maturation also occur in the cytoplasm. Thereafter, core particles can follow one of the three alternative paths (reviewed by Kann *et al.,* 1995): i) they acquire the virion envelope and are released into the blood-stream, ii) remain in the cytoplasm and are degraded, or iii) are transported to the nucleus by a still unknown mechanism where they maintain persistent infection of the cell.

As with the core and polymerase proteins, translation of LHBs also initiates in the cytoplasm as pre-S domains do not contain signal peptide (Gallina *et al.,* 1992). However, as peptide synthesis continues, the signal peptides I and II in the S domain insert the growing LHBs into the ER. Signal peptide I rapidly translocates the growing MHBs to the ER lumen. After assembly, core proteins exhibit an affinity for ER membranes containing inserted LHBs molecules (Bruss and Ganem, 1991). The virion envelope is composed of mixed aggregates of LHBs, MHBs and SHBs. During secretion, virions and accompanying HBs particles are moved from the ER via the Golgi apparatus to the cell surface (Ganem, 1991). During transport of the virion, the glycoside chains of HBs proteins are modified and covalent disulphide bridges are formed within and between HBc and HBs subunits. Release of HBs particles and virions does not require any specific signal (Gerlich, 1993a).

The half life of the core particle within the cell is not known. Many infected liver cells contain core particles within the nucleus but most of them appear to be empty due to their low density within the nucleus. The entire core particle cannot cross the nuclear membrane in either direction (Guidotti *et al.,* 1994a). Thus, one possibility is that core particles may be trapped within the nucleus when the nuclear membrane dissolves during cell division. Another possibility is that core particles assemble within the nucleus, requiring a certain concentration of core protein dimers (Seifer *et al,.* 1993). This may be possible as the core protein contains nuclear localisation signals at its C-terminal end (Yeh *et al.*, 1990; Eckhardt *et al.,* 1991). Any viruses with deletions or mutations in these signals fail to accumulate core proteins in the nucleus (Eckhardt *et al.* 1991).

1.1.4 TRANSCRIPTION AND ITS REGULATION

i) HBV transcripts

Viral transcripts are produced from a low number of ccc DNA molecules in the nucleus using host transcription factors to produce a set of unidirectional and overlapping transcripts (Schaller and Fischer, 1991). HBV has four classes of mRNAs which are transcribed under the control of two enhancers (Schaller and Fischer, 1991; Siddiqui, 1991). RNAs initiate from four sets of promoters and terminate at a single polyadenylation site. All mRNAs are unspliced and therefore the shorter RNAs are fully overlapped by the larger species.

3.5 kb RNAs: The 3.5 kb RNAs are the major transcription products comprising the entire genome and including a short terminal redundancy which duplicates sequence elements essential for genome replication and circularisation (Seeger *et al.,* 1991). The core promoter, which is liver cell specific, controls the synthesis of two closely related, but functionally unrelated subsets of genomic transcripts, which function as pre-core and pregenomic RNAs. Of these, the shorter pregenomic RNA (initiating at nt 1818) is the most abundant, serving both as both the template for reverse transcription and as mRNA for core and polymerase proteins (Schaller and Fischer, 1991). The pre-core mRNAs initiate 20 to 30 bases upstream of pre-core ORF (1783/84, 1790±1) and serve as mRNA for the synthesis of a pre-core gene product (Yaginuma *et al.,* 1987).

2.4 kb RNA: The 2.4 kb RNA or pre-S1 transcript, specifies the synthesis of LHBs which spans the pre-S1, pre-S2 and S domains. In contrast to most other HBV transcripts, the pre-S1 transcript is characterised by possessing a unique 5' end, probably specified by a TATA box positioned at an appropriate distance of 30 nt upstream of the cap site (Will *et al.,* 1987). Transcription of the mRNA is controlled by a pre-S1 promoter element (SP-1) which is highly liver specific and is the least active promoter element in HBV.

2.1 kb RNAs: The second major family is the 2.1 kb RNAs or preS2/S transcripts, having heterogeneous 5' ends which synthesise two gene products from the same ORF. Several shorter RNAs serve for the synthesis of the SHBs whereas the longer major species allows the translation of MHBs. Synthesis of these RNAs is controlled by a promoter element called pre-S2/S promoter (SP-II), which is not stringently liver specific. This promoter lacks TATA-like signals and is similar to the GC rich elements of the TATA-less SV-40 late promoter (Cattaneo *et al.,* 1983).

0.9 kb RNA: A transcript of approximately 0.9 kb serves as a separate RNA for X-gene expression. The product of X-mRNA is repressed under conditions supporting viral replication which reflects their low abundance among the transcripts.

Like other eukaryotic transcripts, all hepadnaviruses' mRNAs terminate in a regulated process. Genomes of hepadnaviruses contain one common stop signal,

TATAAA, shortly after the start site of pregenomic RNA (Ganem and Varmus, 1987). This stop signal is active only when the RNA initiation site is more than 400 bases distant (Cherrington *et al.,* 1992). Thus, this signal is ignored during the first pass of RNA polymerase II after initiation at the core promoter. After transcription, all RNAs are modified by the addition of a methylated nucleotide to the 5' end, known as the cap, and addition of a poly-A tail at the 3' end. All known HBV RNAs are unspliced although numerous potential splice signals are present in RNAs. Spliced derivatives of HBV genomic RNAs have been observed in HCCs, in infected liver and in transfected cells (Su *et al.,* 1989; Chen *et al.*, 1989; Terre *et al.*, 1991). Their role in the life cycle remains uncertain, since mutational inactivation of the splice sites does not impair HBV replication in these cells.

ii) Transcriptional regulation

a)*Cis*-acting elements regulating transcription: Promoters are usually composed of modular elements consisting of 6 to 10 bases, sometimes palindromic or direct repeats. These elements bind single or dimerized transcription factors which co-operate for the activation or suppression of transcription. A typical eukaryotic promoter contains a TATA box approximately 30 nt upstream of the RNA initiation site, to which binds a TATA binding protein (TBP), also known as transcription factor IID (TFIID); this complex is recognised by RNA polymerase II. In HBV, transcription of the four major classes of transcripts is controlled by four functionally defined promoters. These promoters direct initiation of RNA synthesis upstream of the core/precore genes, large surface gene (pre-S1), middle /small surface genes (pre-S2/S) and X gene. The pre-S1 gene promoter (SP-I) has a typical TATA box and a sharply defined 5' end. As with other TATA-less promoters, the mRNAs for preS2/S, core/precore and X-gene have multiple initiation sites (Schaller and Fischer, 1991).

All promoter elements and their constituent sequence motifs have been studied in detail using conventional techniques, such as chloramphenicol acetyl transferase (CAT) assays and their specificity has been examined in cell lines of various origins. In addition, the size limit required for maximum activity has been determined by deletion analysis. These promoter elements are of varying length and their activity increases in a tissue specific manner. In HepG2 cells, the core promoter was found to be stronger than SP-II and SP-I in the absence of homologous enhancer. However, when coupled with enhancer-I, the core and the X promoters showed similar strengths whereas SP-I and SP-II had decreased activity (Antonicci and Rutter *et al.,* 1989). All promoters were more active in liver cells than in non-liver cells and the SP-II and X promoters were active in all mammalian cell types (Seifer *et al.,* 1990). The pre-S2 promoter requires transcription factor SP-1 (Raney *et al.,* 1992) and the large surface gene promoter requires hepatocyte

nuclear factor-I (HNF-I) (Shaul *et al.,* 1986; Chang *et al.,* 1989). The pre-core/core promoter, on the other hand, requires binding to the liver-specific transcription factors HNF-III (hepatocyte nuclear factor-III) and C/EBP (CCAAT/enhancer binding protein) for its activation (Yuh *et al.,* 1992).

Promoter activities are often regulated by enhancer elements. These regulate transcriptional activity of a given promoter in an orientation- and position-independent manner. Two enhancer regions with liver-specific characteristics have been detected in HBV genome. Enhancer I (Enh-I) is located between the open reading frames of the surface and X genes and partially overlaps the X promoter (Shaul *et al.,* 1985; Tognoni *et al.,* 1985). The other lies in the X-ORF, upstream of the core promoter and is designated enhancer II (Enh-II) (Yee, 1989).

Enhancer I was first mapped at approximately 450 nt upstream of the core promoter (nt 1080-1234). It is necessary for core gene expression (Shaul *et al.,* 1985), and is activated in both a host- and tissue-specific manner (Siddiqui *et al.,* 1986). Later studies found that the enhancer I element significantly increased the activity of the core, surface and X promoters (Chang *et al.,* 1987; Roossink and Siddiqui, 1986). The region between nt 964-1217 was found to be essential for X promoter activity (Treinin *et al.,* 1987). The tissue specificity of the surface promoter was markedly enhanced when coupled with enhancer I elements (Bulla and Siddiqui, 1988), however, the X promoter displayed considerably reduced tissue specificity, although it contains the same enhancer element (Raney *et al.,* 1990; Antonucci and Rutter, 1989). There is however debate concerning the liver cell specificity of Enh-I. Studies found that Enh-I stimulated heterologous promoters in non-liver cell lines (Vannice and Levinson, 1988), although it showed preferential activity for hepatoma cell lines when combined with the core promoter (Honigwachs *et al.,* 1989). This may be due to the combined effect of Enh-I and core promoter, as binding of transcription factors to the core promoter in hepatoma cells allows high transcriptional activity (Yen *et al.,* 1993).

A number of transcriptional factors, both liver cell specific and ubiquitous, bind to Enh-I (Dikstein *et al.,* 1990; Trujillo *et al.,* 1991) (Figure 1.3). Only two of them are hepatocyte specific: C/EBP, which binds to several sites of Enh-I and shows concentration dependent activity (Dikstein *et al.,* 1990), and an unidentified factor called hepatitis B liver factor (HBLF), also known as 2c binding factor (Guo *et al.,* 1991). HBLF was shown to play a key role in the overall enhancer function by interacting with the ubiquitous factor EF-C (Trujillo *et al.,* 1991), and was later named as HNF-III (Chen *et al.,* 1994). The EF-C recognition site, also known as the EP element, is a 20 bp sequence which requires the adjacent GB element for efficient Enh-I function (Garcia *et al.,* 1993; Gustin *et al.,* 1993). The GB element is the binding site of HNF-IV, RXRα (retinoid X receptor) and also COUP-TF (chicken ovalbumin upstream promoter transcription factor). The EP element interacts with transcription factors MIBP1 and

RFX1, which associate *in vivo* to form a complex which binds to the MIF-1 element of the c-*myc* gene (Blake *et al.,* 1996). Finally, Enh-I contains a site that binds AP-1 and CREB, which appears to mediate transactivation of Enh-I by X-protein (Maguire *et al.,* 1991).

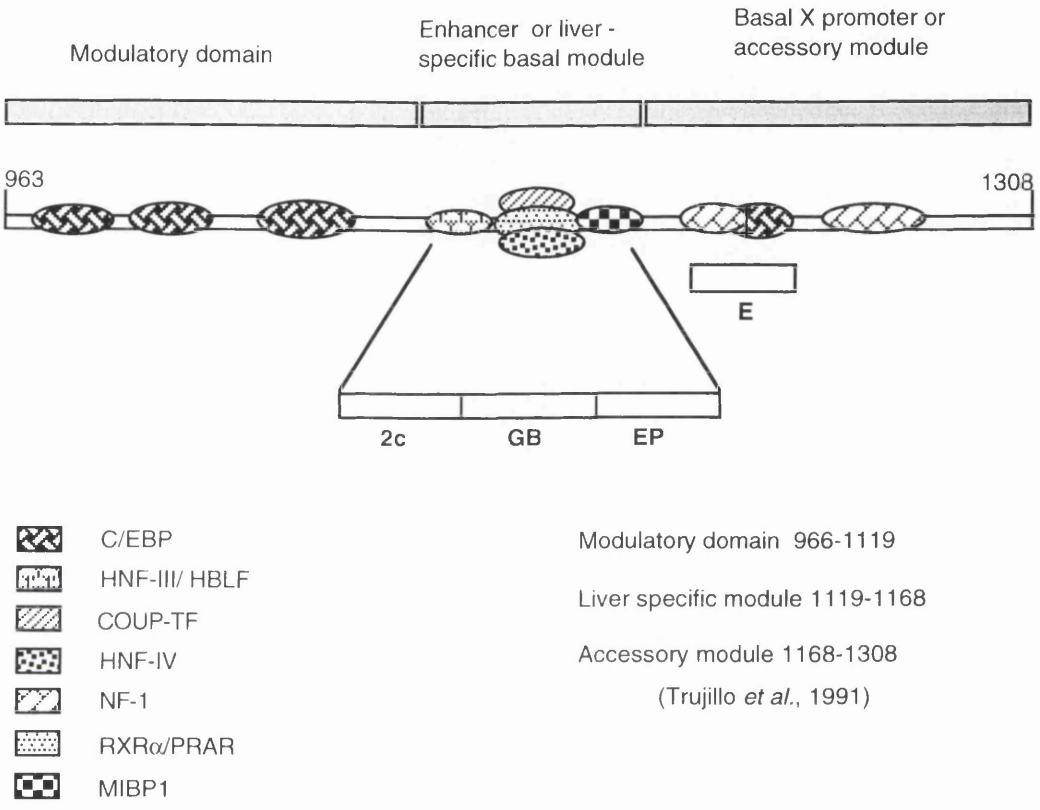


Figure 1.3 Schematic representation of the functional domains, individual cis-elements and their binding factors in HBV Enh I (nt 966-1308). HNF-III/HBLF binds to 2c site; GB element binds to HNF-IV, RXRα/PRAR and COUP-TF factors; and EP element or EF-C site interacts with MIBP/RFX1. The E element is the binding site for NF-1, C/EBP, CREB/ATF and AP-1.

Yee (1989) identified a second enhancer (Enh-II) located in a 92 base pair HBV DNA fragment spanning nt 1685-1773, overlapping the core promoter region. It is highly tissue specific since it functions only in some highly differentiated human hepatoma cell lines (HepG2, Huh6). Further studies showed that either enhancer could activate major HBV promoters in several hepatoma cell lines (Su and Yee, 1992) and both together affect the overall level of HBV gene expression. A 20 base pair sequence motif was found to be essential for Enh-II activity (Chen *et al.,* 1993) and any deletion in the region between nt 1704-1734 reduced the enhancer activity significantly. Similarly, by analysis of a series of substitution mutations, it was found that the region between nt 1707-1718

represents an essential functional motif of HBV Enh-II. However, the region between nt 1721-1735 also has an effect on overall enhancer activity. The region between nt 1708-1719 shows a similarity with the enhancer element of rat tyrosine aminotransferase (TAT) gene and two different enhancer elements of the human transferrin gene, suggesting the involvement of common transcription factors.

In parallel, a group in China (Yuh and Ting, 1990), identified Enh-II between nts 1636 and 1741 which activated the transcription activity of both Sp-I and Sp-II promoters in a liver specific manner. The same group (in 1992) identified a region between nt 1744-1851 as the basic core promoter (BCP) which directed the initiation of both pre-core and pregenomic messages. Sequence upstream from nt 1744 (nt 1636-1743) was referred to as the core upstream regulatory sequence (CURS). It has a strong stimulatory effect on the BCP. The latter has sufficient promoter activity to allow synthesis of the 3.5 kb pregenomic transcripts and virion production, but the addition of the CURS allows increased virion production. CURS stimulated BCP by 1900-fold in HepG2 cells and 180-fold in Huh7 cells. The minimal constituents of Enh-II displaying liver cell specificity were identified as box α and box β (Yuh and Ting, 1993). Box α is a 23 nt sequence (nt 1645-1669) which showed very strong BCP activation in HepG2 and Huh7 cells (120- and 14-fold respectively), whereas box β (nt 1705-1721) moderately activated the BCP in these cells. Gel shift experiments revealed a liver-specific box α binding protein. A negative regulatory element (NRE) upstream of the Enh-II (nt 1613-1636) was identified which can repress both the enhancer and the upstream regulatory functions of Enh-II sequences (Lo and Ting, 1994). Mutational analysis revealed that the sequence from nt 1616-1621 was required for repression of enhancer activity by the NRE. The presence of the NRE indicates that HBV gene transcription is controlled by the combined efforts of both positive and negative regulation.

So far, no canonical TATA or CAAT elements have been found in the core promoter. It is therefore not clear exactly what sequence elements regulate the transcription of either transcripts. Three AT rich regions have been mapped on BCP which function as a TATA box (Okamoto *et al.,* 1994). The first AT rich region, ATA (nt 1752-1755), and the second TTAAA (nt 1758-1762) are located 23 to 28 bp upstream of the initiation site of two pre core mRNAs (nt 1783±1, and 1789±1). The third AT rich region, ATAAATT (nt 1789-1795), is the initiation site for the shorter pregenomic RNA, which is located 23 bp upstream of the start site (nt 1818). Occasionally in eukaryotic systems, a second kind of minimal promoter element, called an initiator, can function as a TATA element. An initiator can occupy a position that directly overlaps the start site or can also co-operate with a TATA box placed approximately 35 bp upstream, to enhance its activity (Smale *et al.,* 1989, Zenzie *et al.,* 1992). A 15 bp sequence in the BCP has been identified which directs the precise initiation of both pre-core and pregenomic

transcripts (Chen *et al.*, 1995). This sequence fragment (nt 1790-1804) functions either as an initiator element for pre-core or a TATA box for pregenomic RNA.

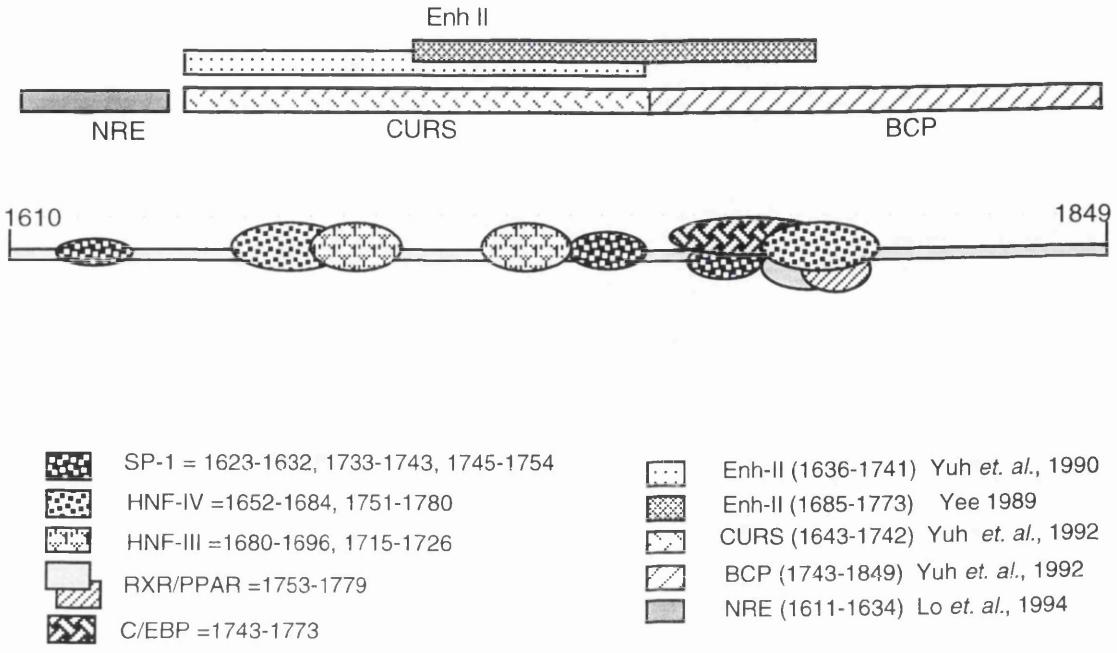


Figure 1.4 Schematic representation of Enh-II/core promoter complex and nuclear factor binding in different regions.

Like Enh-I, a number of transcription factors bind to the core promoter/Enh-II complex (Figure 1.4). One of the principal liver-specific factors that binds to Enh-II is C/EBP (Lopez-Cabrera *et al.*, 1990, 1991). Other liver-enriched factors, HNF-III and HNF-IV, have also been shown to transactivate by binding to the upstream sequence (Yuh *et al.*, 1992; Johnson *et al.*, 1995; Gou *et al.*, 1993). It binds to the transcription factor TF-IID and also to SP-1. Recently, three binding sites for transcription factor SP-1 have also been mapped in the core promoter (Zhang *et al.*, 1993). A very recent study showed that a member of the nuclear receptor superfamily which includes RXR, PRAR (peroxisome proliferator-activated receptor), COUPTF1 and ARP1 bind to the BCP and can modulate the level of transcription (Raney *et al.*, 1997).

The two surface promoters are only approximately 400 bp apart, but are regulated quite differently. The pre S1 promoter (SP-I) has a classic TATA box with a YY1 initiator motif at the transcriptional initiation site (Seto *et al.*, 1991). The transcription factor HNF-I binding site lies approximately 75 bp upstream of this motif (Chang *et al.*, 1989). Although HNF-I is a liver specific factor, a ubiquitous factor, Oct-1, is required for its function (Zohu *et al.*, 1991a). Another binding site for the transcription factor HNF-III

has been mapped 54 to 65 nt upstream of the TATA box. The pre-S2/S promoter (SP-II) is TATA-less, but contains at least two regions that can individually initiate transcription (Zohu *et al.,* 1991b). The upstream initiator transcribes mRNA for MHBs, while the downstream one transcribes SHBs.

b)Transcription regulation by viral gene product: When the HBV genome was first sequenced, an ORF coding for a polypeptide of approximately 17kD was identified. Since it did not correspond to any known HBV proteins nor showed any homology to other proteins it was named 'X'. Nowadays X protein continues to remain an enigma. Based on its position at the 3' end of the viral transcripts and the evolutionary relatedness of HBV with retroviruses, it was postulated that X protein may be a regulator of gene expression, similar to the HTLV-1 *tax* protein (Miller and Robinson, 1986).

In most viruses, the first gene products to be expressed are transcription factors which activate promoters of other viral genes in a temporally ordered manner. Such products are called immediate early (IE) genes. For HBV, the time order of gene expression has not yet been analysed, but it has been speculated that HBV X protein (HBx) is the first protein to be expressed (Wu *et al.,* 1991), as peak levels of X transcripts were observed in the early stages after transfection; however, these transcripts disappeared quickly.

The X gene product is capable of stimulating activity of a variety of viral and cellular promoters including both the HBV enhancers and the core and surface gene promoters. The X protein transactivates transcription from a wide range of viral promoters coupled to HBV enhancers, including the long terminal repeat of HIV-1 (Seto *et al.,* 1988), Rous sarcoma virus and SV-40 enhancer/early promoters in CV-1 cells (Spandau and Lee, 1988) and the herpes simplex virus thymidine kinase (HSV-TK) promoter (Spandau and Lee, 1988). The cellular promoters include the c-*fos* and c-*myc* promoters, the β interferon promoter and also HLA-DR and MHC class-1 promoters (Balsano *et al.,* 1991; Zhou *et al.,* 1990). In addition the list also includes RNA polymerase III promoters (Aufiero and Schneider, 1990). An 8 to 10 fold stimulation of the synthesis of HBsAg, HBcAg and genomic transcripts by X-transactivation of HBV genomes lacking a functional X gene has been observed in human hepatoma cells during transient expression of X gene (Colgrove *et al.,* 1989). An increased level of activity has been reported for the corresponding promoters when coupled individually to reporter genes such as *cat* (chloramphenicol acetyl transferase) or *luc* (luciferase) (Colgrove *et al.,* 1989, Raney *et al.,* 1990). The mechanism responsible for activation by the X protein is not clear, but its interaction with many targets seems unlikely in view of the low concentration in HBV infected liver cells (Pfaff *et al.,* 1987). X protein does not directly interact with *cis*-acting DNA elements, but some data suggest it activates the formation of diacylglycerol, a protein kinase C activator which phosphorylates the transcription factors AP-1 (Kekule *et al.,* 1993), AP-2 (Seto *et al.,* 1990), ATF-2, CREB (Maguire *et al.,*

1991) and possibly SP-1 (Lucito and Schneider, 1992). These factors act on many promoter/enhancers including that of HBV. The X protein also interacts with the TATA binding protein (TBP), suggesting that it plays an important role in modulating the tanscriptional regulatory pathway (Qadri *et al.*, 1995).

However, there is much debate regarding the role of X protein in the viral life cycle. There is no X gene in duck hepatitis B virus (DHBV), raising the question whether the X protein is essential for viral gene expression and replication. Blum *et al.* (1992) showed that a nonsense mutation in the X gene in an HBV genome still allows the synthesis of wild type levels of viral proteins, replicative intermediates and virion export when transfected into either hepatoma cells or primary rat hepatocyte cultures. In contrast, Colgrove *et al.*, (1989) had previously shown, in almost identical experiments, that an HBV genome with a mutated X gene initiation codon expressed approximately 10 fold lower amounts of viral transcripts than wild type HBV upon transfection into hepatoma cells. This study also demonstrated that transactivation of HBV and heterologous genes occurs when X protein is expressed in its native state. The contradiction of these studies may be explained due to the use of a dimer form of the genome in the plasmid by Blum *et al.*, while the latter group used recircularised DNA.

Nevertheless, since the X gene is conserved in all mammalian hepadnaviruses, and as the X protein has a transactivating function, it is likely that this transactivation is important in the viral life cycle. In woodchuck, WHV X protein deficient mutants were viable for replication *in vitro*, but levels of viral DNA were reduced in transfected cells. The mutants, however, did not initiate a detectable infection in susceptible woodchucks (Zoulim *et al.*, 1994). Therefore, X protein is indispensable for viral infection *in vivo*. Furthermore, the X protein plays a key role in HBV-associated carcinogenesis, since X protein can transform cells in culture (Hohne *et al.*, 1990) and some strains of transgenic mice expressing high levels of the protein develop HCC (Kim *et al.*, 1991).

1.1.5 HBV: PROTEINS

i) Core

The HBV core protein (HBcAg) is a 183 or 185 amino acid (aa) polypeptide with a molecular weight of approximately 21,399 daltons (p21). Translation of HBV core protein initiates at the second ATG, 29 codons downstream of the core ORF (Ou *et al.*, 1986). Core protein contains many hydrophilic, charged amino acids and is phosphorylated *in vivo* and *in vitro* when expressed from either heterologous or homologous promoters (Roosinck and Siddiqui, 1987). Phosphorylation of core protein occurs at serine 170 or 172 and phosphorylated core protein can assemble into particles in the absence of viral genomes (Roosinck and Siddiqui, 1987). Core protein is translated in the cytoplasm of infected cells. It packages the pregenomic RNA and the polymerase protein as it assembles into core particles or nucleocapsids. A cellular protein kinase C is

also packaged within the particle (Albin and Robinson, 1980). Expression of core protein *in vitro* also leads to the formation of empty core particles with a diameter of 27 nm (Pasek *et al.*, 1979).

Mutational studies have provided convincing evidence that the core polypeptide contains two functionally distinct domains. The first 147 or 149 residues of core polypeptide form a hydrophobic domain which is resistant to proteolytic digestion (Seifer *et al.*, 1994). This domain enables core proteins to self assemble into core particles (Gallina *et al.*, 1989). In contrast, the last 36 residues of core polypeptide are extremely arginine-rich and display a protamine-like structure which is referred to as the protamine domain (Gallina *et al.*, 1989). This domain is highly protease-sensitive and is involved in nucleic acid interactions (Nassal *et al.*, 1992b) during packaging of the pregenome. The arginine residues are clustered in four blocks, three of which contain the motif SPRRR(R). By deletion analysis, it was found that HBV core proteins truncated up to aa 149 from the C terminus are assembly-competent for capsid formation (Gallina *et al.*, 1989; Stahl *et al.*, 1989). Birnbaum *et al.* (1990) showed that core protein up to 144 aa long can form core particles in bacteria whereas deletion beyond residue 139 abrogated capsid assembly. Effective encapsidation of viral genomes requires at least 164 amino acids from the amino-terminal (N-terminal) end; variants ending at aa 164 have a drastically reduced capacity to complete positive strand DNA synthesis, which makes the C-terminal domain an essential component in HBV replication (Nassal *et al.*, 1992b). Deletions at the N-terminus are more deleterious, as truncation up to residue 10 prevents capsid formation (Salfeld *et al.*, 1989). However, chimeric core polypeptides bearing inserted foreign sequences in the epitope regions and at the C-terminal regions still produce particles (Seifer and Standring, 1995). A minimal assembly domain of HBV core polypeptide thus requires approximately 130 aa residues (10-144).

The concentration of HBc polypeptide is also important for assembly. First the core protein dimerises (Zhou and Standring, 1992), requiring a minimal concentration of 0.8 μM (Seifer *et al.*, 1993). These dimers then assemble to icosahedral particles of T=3 symmetry; 180 subunits of core protein monomers forming one particle (Birnbaum *et al.*, 1990). Once core particles are assembled their structure is stabilised by disulphide bonds (Zhou and Standring, 1992; Nassal *et al.*, 1992b).

ii) HBeAg

All hepadnaviruses have evolved the ability to express a secretory form of core protein known as HBeAg or e antigen. As HBeAg is translated from the first ATG of the core ORF, it contains 29 amino acids upstream of the core start codon (Ou *et al.*, 1986), known as precore sequence. The first 19 aa of this amino-terminal extension act as a signal peptide which translocates the protein into the endoplasmic reticulum (ER) as p25e (Bruss and Gerlich 1988; Standring *et al.*, 1988). During the secretory pathway, 19 of

these 29 amino acids are cleaved off by the signal peptidase which generates an intermediate protein product that remains in the ER or is released back to the cytoplasm as p23e (Garcia *et al.,* 1988) The remaining 10 aa of pre-core sequence prevent the assembly of HBeAg into core particles by interacting with the HBV core protein sequence (Wasenauer *et al.,* 1992). p23e is either secreted from HBeAg-producing cells or incorporated into the outer cell membrane for expression (Schlicht and Schaller, 1989). This membrane-expressed p23e may play a role in the antibody-mediated elimination of infected cells. The p23e in the ER is further cleaved at the arginine rich C-terminal domain by a Golgi protease and is secreted from the cell as p16e, p18e or p20e; all three of these share identical N-termini but have different carboxy ends (Wang *et al.,* 1991). Sometimes, part of the HBe protein does not reach the ER but remains in the cytoplasm as the uncleaved form p25e. Like core protein, p25e contains a nuclear transport signal at the arginine rich carboxy domain (Yeh *et al.,* 1990). Thus, HBe proteins of variable length are found in all compartments of the cell. However, HBe protein has not been detected in any of the compartments of hepatocytes in transgenic mice, using immuno-histochemical techniques (Guidotti *et al.* 1994).

HBeAg is not essential for the viral life cycle and its exact function in the biology of HBV infection is still unclear. HBeAg is found in the serum of HBV infected individuals where it generally correlates with a high level of viremia. Elimination of HBeAg is generally accompanied by detectable levels of anti-HBe immune response and a decrease in viremia. HBeAg non-producing variants of woodchuck hepatitis virus (WHV) were found to be infectious for new born woodchucks but it could not induce persistent infection (Chen *et al.,* 1992). This suggested that HBeAg suppresses the immune elimination of HBeAg-expressing cells, possibly by blocking cytotoxic T cell activity against HBV core associated epitopes (Milich *et al.,* 1990). Infants born to HBeAg positive mothers become chronic carriers in more than 90% of the cases (Stevens *et al.,* 1975). In contrast, HBV infection in adult age leads to chronicity in about 5-10% of the cases (Aldershvile *et al.,* 1980). This may be due to immunologic tolerance in neonates induced by viral proteins to which they are exposed in *utero* (Thomas *et al.,* 1988), shown in transgenic mice (Milich *et al.*, 1990). The HBe/HBcAg-specific T cell tolerance may be due to the secreted HBeAg gaining access to the thymus and leading to functional deletion of HLA class-II restricted HBe/HBcAg-specific T helper (Th) cells (Milich *et al.*, 1991).

In similarity with the duck system (Chang *et al.*, 1987), HBeAg seems to be non-essential for viral replication in human. HBeAg negative HBV strains appeared to be highly replicative than HBeAg producing strains when transfected into HCC cells (Lambert *et al.,* 1993). Transient over-expression of pre-core gene resulted in striking inhibition of HBV replication (Scaglioni *et al.,* 1997). This was due to interaction of non-

secreted p23e with core protein (p21) forming hybrid core particles devoid of pregenomic RNA.

iii) Surface

All three HBV surface proteins are encoded by a single open reading frame of 389 or 400 codons depending upon the subtype. Three polypeptides are produced from three in-phase start codons in the S open reading frame. The three proteins are termed LHBs, MHBs and SHBs. The LHBs is a 39 kD protein translated from first ATG of the S ORF (Heerman *et al.,* 1984); initiation at the second ATG generates the 31 kD MHBs (Machida *et al.,* 1983). Both share the common C-terminal S domain and are quantitatively minor components of the circulating pool of surface related antigens. MHBs accounts for approximately 5-15% of the total, and the LHBs represents only 1-2% of the total (Ganem, 1991) HBs proteins, while SHBs is the most abundant. All three proteins are found either glycosylated or unglycosylated at Asn 146 of the SHBs sequence. The MHBs is additionally glycosylated at Asn 4 (Heerman and Gerlich, 1991). Extensive production of surface proteins reveals a large number of non-infectious particles in the sera of highly viremic carriers. Most abundant are spheres of 17-25 nm; less abundant are filaments of 22 nm diameters of variable length.

a) Small surface protein: SHBs is synthesised on membrane bound ribosomes of the rough ER and consists of a 226 amino acid (aa) long polypeptide chain. Expression of SHBs in cell culture results in the secretion of 22 nm HBs particles, suggesting that no other viral factor is necessary for sub-viral particle formation (Liu *et al.,* 1982). This is in contrast to the 42 nm virion, for which SHBs alone is not sufficient (Bruss and Ganem, 1991). SHBs appears as both unglycosylated 24 kD or glycosylated 27 kD forms, and its sequence contains three hydrophobic regions separated by two moderately hydrophilic domains. The hydrophobic regions are folded into α helices; the amino terminal helix from aa 11-29 constitutes the signal polypeptide I and helps to translocate the protein into the ER (Eble *et al.,* 1990). The second hydrophobic helix, aa 80-90, constitutes signal II, and allows translocation of downstream sequence (Eble *et al.,* 1987). Between the first two hydrophobic domains is the first hydrophilic region (aa 29-79), and is exposed on the cytoplasmic side of the ER membrane. This domain lies on the interior of the virion particle and may facilitate attachment of the virions as well as the envelopment of the core particles (Prange and Streeck, 1995).

The second hydrophilic loop, consisting of aa 99-168, faces into the ER lumen where it acquires carbohydrate modification (Eble *et al.,* 1987; Stirk *et al.,* 1992). This hydrophilic region carries the major group- and subtype-specific epitopes and forms the outer surface of SHBs spheres, filaments and virions. This region contains clusters of cysteine residues that form inter- and intramolecular disulphide bridges (Guerrero *et al.,* 1988). Eight of the 14 cysteine residues present in SHBs are located in this domain.

These are highly conserved among the HBV subtypes (Prange *et al.,* 1995) and when cross-linked, result in a very compact protease sensitive structure. Sequence between aa 124-147 is widely known as the 'a' determinant which is common to all subtypes (Gavilanes *et al.,* 1982). This very important conformational determinant was proposed to form a double loop structure by cross linking between cysteine residues (Ashton-Rickardt and Murray, 1989). Antibodies raised against this determinant are important in inhibiting virus binding to hepatocytes (Petit *et al.,* 1991). However, recent evidence from natural variants and the complexity of the epitopes suggest that this epitope cluster could include the entire hydrophilic region and is presently termed the major hydrophilic region (MHR) (reviewed in Wallace and Carman, 1997).

Almost all characterised antigenic subtypes have sequence variations in the area between aa 120-160. The most common subtype determinants are *d* or *y* (Le Bouvier, 1971) and *w* or *r* (Bancroft *et al.,* 1972). These specificities have allowed identification of four subtypes, *adw, ayw, adr* and *ayr*, where *a* is common to all subtypes. Determinant *d* contains a lysine at position 122, whereas *y* has an arginine (Peterson *et al.,* 1984). Similarly, determinant *w* has a leucine at position 160, whereas *r* has an arginine (Okamoto *et al.,* 1988). Replacement of these basic amino acids by other amino acids causes total loss of subtype determinants (Okamoto *et al.,* 1988). Recently a further putative subtype allele that has either isoleucine or threonine at position 126 of SHBs has been identified (Ohnuma *et al.,* 1993). Other aa exchanges are also important for the formation of subtype specificity (Gerin *et al.,* 1983). Subtyping by monoclonal antibodies or by sequencing gives a much larger number of subtypes than classical serology. Allele *w* can be subdivided into further subspecificities *w*1 to *w*4 (Heermann and Gerlich, 1991). These subdivisions make a total of 8 subtypes, two *adw*, four *ayw*, *adr* and *ayr.* With the identification of the *q* determinant (Maginus *et al.,* 1975), the number increased from eight to nine. This subdivides *adr* into *adrq*$^+$ or *adrq*$^-$. The occurrence of subtypes depends on ethnic group or epidemiologic relation. Among the four main subtypes, *adw* and *ayw* are predominantly found in Western Europe (Schmidt *et al.,* 1972), while *adr* and *ayr* are confined to the East Asian population (Courouce *et al.,* 1974). The subtype *adw*4 is found in Western Amazonia and in Polynesia, suggesting a common origin of these populations. Six genotypes A-F have been identified, which differ by more than 8% in their protein sequence (Okamoto *et al.,* 1988; Norder *et al.,* 1992). Genomes encoding subtype *adw* were found in the genomic groups A, B and C, while genomes encoding *ayw* were all in genotype D. Subtypes *adr* and *ayr* occurred in genotype C alongside *adw* (Okamoto *et al.,* 1988). In a later study from Indonesia, genomes encoding *ayw* were also encountered in genotype B (Sastrosoewignjo *et al.,* 1991). S genes of subtype *ayw*4 and *adw*4 differed by 4% or more from all previously defined genotypes and from each other, which eventually classified them as two new genotypes, E and F respectively (Norder *et al.,* 1992).

b)Middle surface protein: MHBs is a minor component of the virion and contains the S domain and the 55 aa long pre-S2 domain (Stibbe and Gerlich, 1983). The pre-S2 domain is hydrophilic, protease sensitive and contains no cysteine residues. It is exposed on the surface of the virion of HBs particles and sometimes partially covers the S domain of MHBs. Treatment of HBs spheres with trypsin cleaves MHBs at arginine 47 (Stibbe and Gerlich 1983) and increases the HBs reactivity of that particle (Heermann *et al.,* 1984). The pre-S2 domain is cotranslationally translocated into the ER lumen by signal-I in its downstream S domain (Eble *et al.,* 1990). MHBs is glycosylated at Asn 4, therefore the 30 kD unglycosylated form appears as a 34 kD N-glycosylated form in polyacrylamide gel analysis. Additional N-glycosylation in the S domain results in a 36 kD protein.

Pre-S2 domains bind to artificially polymerised human serum albumin (pHSA) (Machida *et al.,* 1984; Krone *et al.,* 1990). A correlation between high level viremia and the presence of pHSA receptor was observed in many studies. Only pHSA of primate origin bound to pre-S2 domain of MHBs (Machida *et al.* 1989; Neurath *et al.,* 1988). Furthermore, pHSA binds to liver cells, although its binding is not species-specific (Trevison *et al.,* 1982). Antibody against the pre-S2 domain (aa 1-24) was able to neutralise HBV in experimentally infected chimpanzees (Neurath *et al.,* 1986) and immunisation of chimpanzees with a conjugated pre-S2 peptide (aa 14-32) or the whole pre-S2 domain protected them against HBV challenge (Itoh *et al.,* 1986).

HBV particles from carrier plasma bind to HepG2 cells in an organ and species specific manner after prolonged incubation (Heermann and Gerlich, 1991), and this is mediated by a glycoside structure linked to pre-S2. Recombinant MHBs from mouse fibroblasts that contains a simple mannose rich glycan in the pre-S2 domain does not bind to HepG2 cells, while recombinant MHBs from Chinese hamster ovary (CHO) cells containing a mixed type of glycan does bind (Gerlich *et al.*, 1993b). The receptor for this site is probably a liver cell lectin specific for mixed types of N-linked glycan. Lectin is absent or has a very low activity in fresh liver membrane, suggesting that it may not play an important role in natural infection process (Pontisso *et al.,* 1989). *In vivo* HBV infection is highly efficient while hepatoma cell lines can barely be infected, although they can be transfected efficiently (Sells *et al.,* 1987). This raises the question as to whether the postulated lectin is related to a certain state of differentiation or dedifferentiation of the liver cell.

The pre-S2 domain is more immunogenic then HBsAg (Neurath *et al.,* 1984). The epitopes are not conformational and can be generated easily by synthetic peptides (Heermann *et al.,* 1987). Several subtype-specific epitopes are present in the pre-S2 domain (Neurath *et al.,* 1990). The pre-S2 sequence is conserved between subtypes in the N-terminal portion in contrast to the highly variable C-terminal portion.

c) Large surface protein: LHBs consists of three domains: pre-S1, pre-S2 and S. In the mature virion or HBs particles, the pre-S domains are accessible to antibodies (Heermann *et al.,* 1984), receptors (Neurath *et al.,* 1992) and proteases (Heermann *et al.,* 1987). During translation, the entire pre-S1 domain of LHBs faces the cytoplasmic side of the ER membrane (Bruss *et al.,* 1994; Prange and Streeck, 1995). Like the pre-S2, pre-S1 does not contain any signal sequence or hydrophobic region, however, signal-II of the S domain directs the LHBs to the ER (Bruss *et al.,* 1996). Asn 4 of the pre-S2 domain and Asn 15 of the pre-S1 domain are unglycosylated, probably due to their cytoplasmic location (Heermann *et al.,* 1984). The pre-S1 domain contains no cysteine residues and the protein is myristylated at Gly 2 (Persing *et al.,* 1987). During virion or HBs particle maturation, the pre-S1 domain is reconfigured and translocated to the surface of the particles (Gerlich, 1993a). Over-expression of LHBs was found to prevent the secretion of virions (Persing *et al.,* 1986), but formation of 20 nm filaments occurs within the ER (Chisari *et al.,* 1987). The accumulation of LHBs in the ER results in the formation of 'ground glass' cells viewed using light microscopy (Chisari *et al.,* 1987); retention of LHBs in the ER is due to a short peptide from aa 6-19 (Kuraki *et al.,* 1989). Morphological change from HBs spheres to HBs filaments results when there is an increased proportion of LHBs produced in transfected cells (Marquardt *et al.,* 1987), suggesting that the LHBs is responsible for the morphology of HBs filaments.

The pre-S1 domain of LHBs functions as an attachment site of HBV to liver cells (Neurath *et al.,* 1986). The peptide from aa 27-48 of pre-S1 domain is believed to recognise a cellular receptor since antibody against this peptide resulted in inhibition of attachment. A direct correlation was found between the degree of binding and the proportion of LHBs in natural HBs particles. Virions bound better than HBs filaments, which bound much better than spheres (Pontisso *et al.,* 1989). The pre-S1 attachment site was also found to recognise monocytes and lymphocytes (Pontisso *et al.,* 1992), membrane bound interleukin-6 (Neurath *et al.,* 1992) and competed with an IgA receptor on the liver cell membrane (Pontisso *et al.,* 1992).

The pre-S1 domain is one of the most variable regions of the HBV genome. This may be due to its overlap with the polymerase ORF in an area not essential for replication (Gerlich, 1993a). Alternatively, it may be that the surface structure is more intensively selected for by immune pressure. However, within the chronically infected person, or within a defined chain of infection, pre-S1 is not usually mutated (Uy *et al.,* 1992).

iv) X protein

The transactivation function of the X protein is discussed earlier. The smallest of HBV ORFs was initially designated as 'X' because its function was not known at that time (Galibert *et al.,* 1979). The X ORF partially overlaps with Enh-II/CP, DR2, DR1,

pre-core and pregenomic RNA start sites and also the 5' end of the polymerase gene. HBV X protein (HBx) consists of 154 aa residues and its exact function is still unknown. Expression of X ORF in prokaryotic and eukaryotic cells resulted in the formation of a 16.5 kD polypeptide that reacted with serum samples from HBV infected individuals (Meyer *et al.,* 1986; Pfaff *et al.,* 1987). Anti-HBx antibodies have been detected in acute infection (Vitvitski-Trepo *et al.,* 1990), but more frequently in chronic carriers with active disease (Haruna *et al.,* 1991). *In vivo*, HBx is localised to the cytoplasm, nucleus or cell membrane (Wang *et al.,* 1991) and *in vitro* , to the nucleus (Hohne *et al.,* 1990).

Apart from transcriptional transactivation, HBx may play a key role in HBV associated carcinogenesis. High level expression of X gene was found to induce malignant transformation of certain culture cells, like NIH3T3 cell line (Shirakata *et al.,* 1989). Such activity was also observed in immortalised hepatocytes expressing SV-40 large tumour antigens (Hohne *et al.,* 1990). Integration of HBV DNA and overexpression of HBx was evident in hepatoma cell lines and in human hepatocellular carcinoma (HCC). Recently, it has been found that X protein inhibits p53 gene function *in vitro* (Wang *et al.,* 1994); in transgenic mice, X protein induces liver cancer (Kim *et al.,* 1991).

HBx has no direct DNA binding activity. Therefore, its *trans*-activating and oncogenic function is probably mediated by protein-protein interactions with a number of cellular proteins. The hypothesis is that HBx may bind to form a stable complex with cellular factors. HBx binds to CREB or ATF-2, two factors through which it apparently can effect transactivation *in vitro* and alter their DNA binding specificity (Maguire *et al.,* 1991). HBx was also found to act by inhibiting the digestion of cellular factors, since it has a sequence similarity to pancreatic trypsin inhibitor (Takada and Koike, 1990). Amino acid substitution in this region can affect transactivation function.

It was suggested that HBx may enzymatically modify a host factor by phosphorylation as it has protein kinase activity (Wu *et al.,* 1990; Schek *et al.,* 1991). It also has dinucleotide kinase (Shaul, 1991) and protease inhibitor (Arii *et al.,* 1992) (or squelching factor) activity (Shaul, 1991). It was found that X protein has an amino acid sequence homologous to the functionally essential region of the kunitz-type serine protease inhibitor known as the kunitz domain (Takada and Koike, 1990). This kunitz domain-like region of X protein was indispensable for its transactivating function. Three separate sites of HBx, aa 65-72, aa 105-115, and aa 131-142, also known as U22, X1 and Z44 site respectively were found to interact with cellular proteins (Takada and Koike, 1994). Furthermore, these interaction sites also coincide with the structures necessary for serine protease inhibitor activity.

v) Polymerase

The longest ORF in the HBV genome encodes the viral polymerase protein, which consists of four domains (Schlicht *et al.,* 1991). The N-terminal domain encodes for

terminal protein, necessary for priming of minus strand DNA synthesis, also known as primase. The next domain functions as a spacer. The third domain encodes for reverse transcriptase, and the last carboxy domain is RNaseH domain. Like retroviral reverse transcriptase, HBV polymerase protein also contains amino acid motif YMDD at aa 535-539 and sequence comparisons with known proteins with RNaseH activity revealed a weak homology at a position located downstream to the YMDD sequence (Johnson *et al.*, 1986). Mutational analyses of polymerase gene revealed that the YMDD motif is essential for reverse transcriptase activity and mutations in the RNase H homology region can result in the production of polymerase protein that still produces minus strand DNA but devoid of plus strand synthesis (Radziwill *et al.*, 1990). Polymerase protein is also indispensable for its encapsidation function. Several point mutations, deletions and insertions throughout the gene revealed that all functional domains in the gene product are an absolute requirement for the encapsidation of pregenomic RNA and that the packaging function of the polymerase protein is separated genetically from its enzymatic activity (Bartenschlager *et al.*, 1990).

1.1.6 MOLECULAR VARIANTS OF HBV

RNA viruses and retroviruses have a tendency to high rates of nucleotide substitution in their genomes (Holland *et al.*, 1982), because of polymerase errors associated with the lack of a proof-reading function of reverse transcriptase (Steinhauer and Holland, 1987). Thus, multiple variants can be generated by replication competent strains constituting the 'quasispecies'. Variants without any phenotypic effect, known as 'silent mutants', become incorporated into the mixture of strains by random sampling and others are selected by host immune pressure, antiviral agents or differences in cell biology (Carman *et al.*, 1993a). In HBV the genome is more constrained than in retroviruses, however, these processes are clearly common (Carman *et al.*, 1993a; 1993b).

i) Pre-core gene

The pre-core and core proteins are translated from the common ORF and are initiated from two different start codons but have a common stop codon. The pre-core region, which is the 87 nt upstream of the core ORF, is indispensable as it contains the 5' end of the encapsidation signal (Junker-Niepmann *et al.*, 1990), DR1 and the start site of pregenomic RNA. Variation in the pre-core region affects the secondary structure of the RNA encapsidation signal. There are certain epitopes for B and T cells which are common between the two proteins, others are peculiar to one polypeptide. The difference in the antigenic epitopes between the two proteins may be because of conformational differences related to the 10 aa pre-core peptide at the N-terminal end of HBeAg and the C-terminal arginine rich domain of the HBcAg (Carman *et al.*, 1993b). HBeAg is not essential for the viral life cycle as it is not a structural component of the virion (Chen *et al.*, 1992).

Nevertheless, HBeAg is important as it is believed to play a role in modulating the host immune defence against HBV (Thomas *et al.,* 1988; Carman *et al.,* 1992a). Variations in the pre-core gene can interfere with HBeAg production and thus affect host immune response. Clearance of virus is accompanied by the elimination of infected hepatocytes by cellular and humoral immune responses. There is little evidence that B cells play a major role in the clearance of virus during acute infection but a number of studies suggest T cell epitopes are recognised by cytotoxic T lymphocytes (CTLs), at least in the acute phase (Mondelli *et al.,* 1982; Bertoletti *et al.,* 1991). Although the exact mechanism is unknown, during chronic hepatitis the initial target is probably HBeAg, presented on the hepatocyte membrane (Schlicht and Schaller, 1989; Yamada *et al.,* 1990), which may result in the elimination of HBeAg producing cells. Thus, the HBeAg non-producing virus may escape from the host immune response and be 'selected' from HBeAg producing strains.

The most common variant in the pre-core region is a G to A variation at nt 1896 (A_{1896}), resulting in the premature termination of pre-core protein (Carman *et al.,* 1989; Brunetto *et al.,* 1989; Tong *et al.,* 1990; Okamoto *et al,.* 1990). This mutation converts the TGG, a tryptophan at codon 28 to TAG, a stop codon. Since it occurs upstream of the core start codon, and as the core protein is produced from a separate mRNA, core particle production is not affected (Figure 1.5). Other mutations have also been described in the pre-core region. These are either an insertion/deletion leading to a frame shift (Santantonio *et al.,* 1991), appearance of an in-frame stop codon or a mutation within the pre-core start codon (Foirdalisi *et al.,* 1990) (Figure 1.6). A G to A mutation at nt 1897 (A_{1897}) results in the stop codon TGA. A mutation at the pre-core start codon (ATG to ACT or to ATA), deletion in any of the four Gs at nt 1896-1899 leading to frame shift of codons, also results in an HBeAg negative phenotype (Fiordalisi *et al.,* 1990; Okamoto *et al.,* 1990). All these interfere with the synthesis of HBeAg. Variants associated with pre-core amino acid substitution have also been reported (Carman *et al.,* 1992b; Boner *et al.,* 1995); substitution of proline by serine at aa 15 was found in anti-HBe positive Chinese patients. This occurs due to a nt variation at 1856 (T_{1856}), which is never detected in patients containing A_{1896} variant. Fifty per cent of the patients with T_{1856} also shared a G to A substitution at 1898, where a glycine is converted to serine, but its functional significance is not clear. Another mutation from G to A at nt 1899 has been described in some studies (Carman *et al.,* 1989; Brunetto *et al.,* 1989; Naoumov *et al.,* 1992), mostly linked with A_{1896}. Again, its exact role in disease severity is still unknown however it was mostly found in severe cases and rarely found alone in HBeAg positive cases.

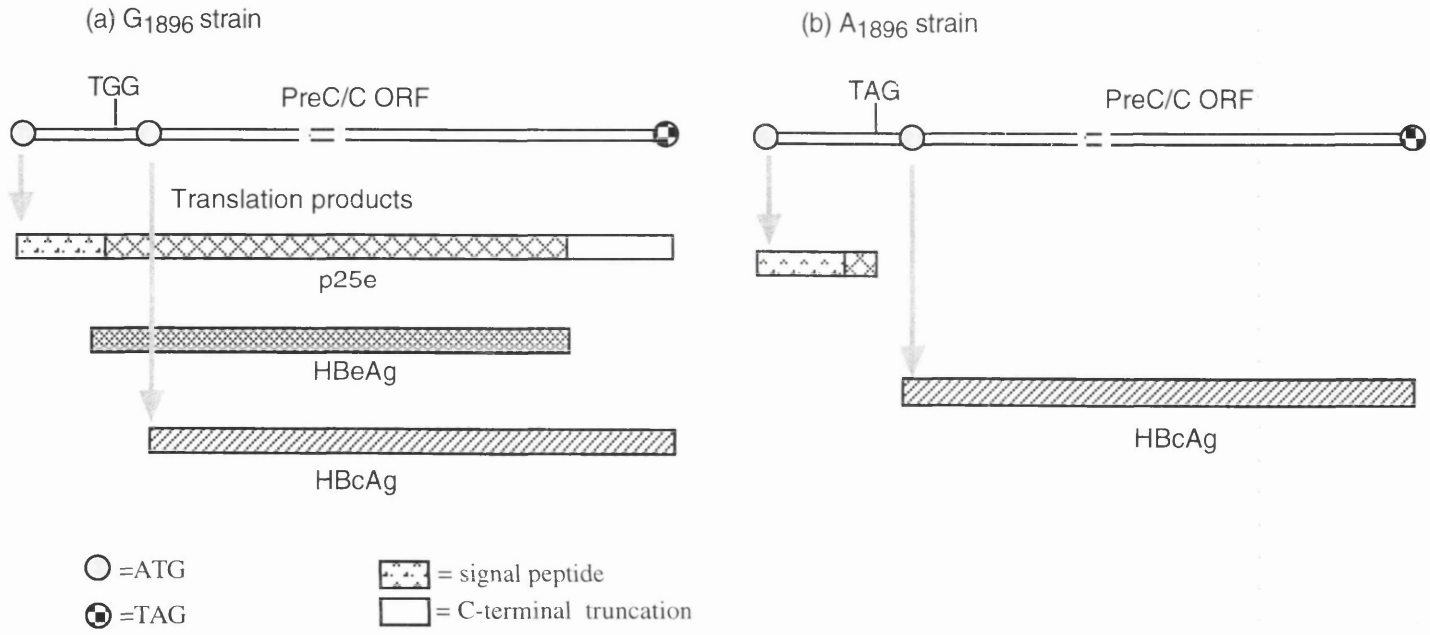


Figure 1.5 Translation products of pre-core/core ORF in G_{1896} and A_{1896} strains. (a) In G_{1896} infected cells, HBe and HBc proteins are initiated from two in-frame ATGs within the single ORF. p25e is the precursor of HBeAg, p22 or HBcAg is assembled into core particles. (b) The pre-core variant has a nt substitution at 1896 (A_{1896}) which generates a translational stop codon at amino acid position 28 in the pre-core region, stopping production of HBeAg.

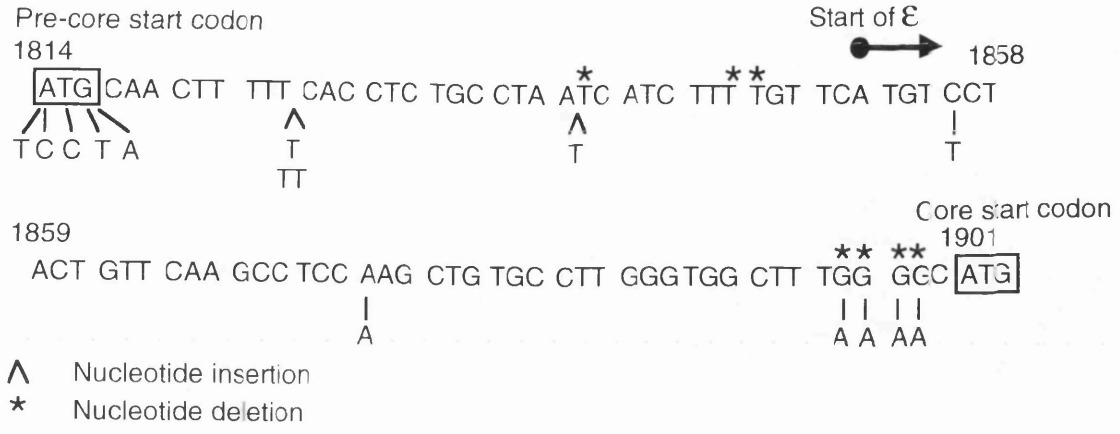


Figure 1.6 Some variants identified in the pre-core region. Pre-core and core start sites are shown in box. Start of encapsidation signal is shown by arrow.

A_{1896} frequently appear in chronic carriers in the Mediterranean (Carman *et al.*, 1989; Brunetto *et al.*, 1989) and Far Eastern countries (Okamoto *et al.*, 1990) during seroconversion to anti-HBe and is associated with high level viremia. It is less common in the USA and Northern Europe which is genotype dependent. In genotypes B, C, D and E, the HBV genome contains a T at nt 1858 (T_{1858}), which is opposite nt 1896 in the encapsidation stem structure making a wobble pairing with G_{1896}, whereas selection of A_{1896} creates a more stable pairing with T_{1858}. In Northern Europe and the USA, where genotype A is common, the genome contains a C at this position. The change from G to A at nt 1896 would interfere with the encapsidation signal stability and hence lead to very poor encapsidation (Laskus *et al.*, 1994a; Lok *et al.*, 1994). A_{1896} is rarely selected in the genotype A or F, but when selected, it is always in the presence of T_{1856}. However, HBeAg negative strains without A_{1896} seemed to cause more severe liver damage than the strains with A_{1896} (Lindh *et al.*, 1996). This was believed to be due to presence of C_{1858}. A_{1896} is commonly found in chronic and fulminant cases, but is less common in acute cases. This may be because a long term or an imbalanced immune pressure is required for its selection. Selection of A_{1896} from normal G_{1896} takes a long period, and during this stage a mixture of strains are found.

ii) Core gene

HBV core protein contains T helper (Ferrari *et al.*, 1991), CTL (Bertoletti *et al.*, 1991; Missale *et al.*, 1993) and B cell epitopes (Waters *et al.*, 1986; Salfeld *et al.*, 1989; Collucci *et al.*, 1988), some of which are common to HBeAg and are under strong immune pressure. Selection of HBeAg negative variants sometimes leads to loss of

tolerance against HBcAg epitopes and is accompanied by amino acid substitution in the core protein (Carman, 1995a) especially in the B cell and T helper (Th) cell epitopes. A higher degree of core variation is observed in anti-HBe positive patients with severe disease and in fulminant cases (Carman *et al.,* 1995b) compared to HBeAg positive patients, with variation occurring mostly in B and Th cell epitopes. Variants in Th cell epitopes have also been reported in patients with hepatoma (Hosono *et al.*, 1995). HBeAg positive cases with core variation have also been described (Ehata *et al.,* 1992); these tend to cluster in non-antigenic areas, particularly between aa 84-101. Amino-acid substitutions at specific positions have also been described. A substitution of threonine to serine at aa 12 was found in cases with severe disease, but always with A_{1896} (Carman *et al.,* 1994) and only in Mediterranean patients. This substitution is believed to play a role in escape from the CD4 restricted Th cell response, as aa 12 is in a Th cell epitope. Greater numbers of core variants are also found in anti-HBe positive patients who do not respond to interferon than those who do respond (Fattovich *et al.,* 1995). Similarly, in HBeAg positive patients, a lower response to interferon is found in cases with higher numbers of core variations (Naoumov *et al.,* 1995)

iii) Surface gene

Reports on variation in pre-S1 and pre-S2 are still few. Variation in the pre-S2 ATG has been reported a few times (Santantonio *et al.,* 1991), but it is not likely to be a result of cellular immune pressure since the large protein is still present and contains this domain.

Most variation in the S ORF is seen in the SHBs. Some of the variants described in the MHR are subtype related and others are selected during or after immune therapy, vaccination or in the course of chronic infections (reviewed in Carman, 1996).

The most common vaccine induced escape mutant is the glycine (Gly) to arginine (Arg) substitution at aa 145 described in a number of studies. The first described case was observed in a vaccinated infant born to HBeAg and HBsAg positive mother and developed chronic disease (Carman *et al.,* 1990). The infant was given hepatitis B immunoglobulin (HBIG) at birth and at 1 month and a course of vaccine at 3, 4 and 9 months. Monoclonal antibody against the 'a' determinant bound poorly, although sera from the mother had shown normal binding activity. Gly to Arg at aa 145 was responsible for the loss of binding with neutralising antibodies. However, the mother had the normal glycine at that position. Similar cases have been reported in other studies (Fujii *et al.,* 1991; Okamoto *et al.,* 1992). In all cases, the nucleotide sequences from both mother and child were identical apart from aa 145. Naturally occurring Arg 145 variants also occur; in a patient from Indonesia Arg 145 appeared along with a two amino acid insertion between aa 122-123 and resulted in fulminant hepatic failure (Carman *et al.,* 1995c). The patient was anti-HBc and HBeAg positive and HBV DNA positive (by dot-blot hybridisation).

Serum HBsAg was undetected using one commercial kit but was highly reactive with another.

Variants in other positions in the same area have also been described. In one study from Spain (Wallace *et al.,* 1994), 7 cases were reported where sera were untypable or typed very weakly as *ay* using monoclonal antibodies. Sequencing of this region associated the *y* determinant at aa 122, however variations were found in other sites at aa 120, 143 and 144, affecting reactivity either singly or in combination. Similar variants were also described by Moriyama *et al.,* (1989, 1991). Substitution of aspartate to asparagine at aa 144 along with other changes at aa 126, 131 and 133 were found. They may have affected the antigenicity of the MHR.

Insertion of amino acids has been described in several studies (Yamamoto *et al.,* 1994; Carman *et al.,* 1995c; Hou *et al.,* 1995). In one study, a patient was found to have two amino acids inserted between aa 122 and 123 and in another case a 3 amino acid insertion between aa123 and 124. Sera were positive for HBV DNA by dot-blot hybridisation or PCR, but commercial assays detected HBsAg poorly. A further patient who was HBeAg and anti-HBs positive had an insertion of 8 amino acid between aa 123-124. These reports suggest that this area is flexible (aa 122-124) and not functionally important in the viral life cycle.

iv) X-gene

X-gene is the smallest ORF in HBV, however this region is of great importance not only because the HBx protein is a transcriptional transactivator, but also because it overlaps with Enh-II/C-promoter, the C-terminal end of the polymerase gene and DR1. Many cases have been described with deletions, insertions, and point mutations in the X-gene (Laskus *et al.*, 1994; Okamoto *et al.*, 1994). Some of the point mutations are significant at the nucleotide level, especially when in Enh-II/CP region, and others at the amino acid level, affecting the X and/or polymerase protein. A number of variants in the X-gene are associated with fulminant cases and others have been found in chronic carriers. These changes are responsible for either upregulating or downregulating transcriptional activity. Variants at the 3' end of the X gene, resulting in higher replication and transcriptional activity, were reported from a fulminant case (Baumert *et al.,* 1995). A number of variants in the same region, believed to be associated with downregulation of pre-core mRNA transcription, have been described from asymptomatic carriers, patients with chronic persistent hepatitis (CPH) and fulminant cases. Most common amongst these are T_{1762} and A_{1764}, located in the BCP within the binding sites of nuclear factor. Okamoto *et al.* (1994) described these two variants in anti-HBe positive asymptomatic and CPH cases, as well as HBeAg positive CPH cases; however, they were not seen in HBeAg positive asymptomatic carriers. They were believed to downregulate HBeAg production as other studies reported a similar occurrence in HBeAg negative, anti-HBe positive

asymptomatic cases (Sato *et al.*, 1995; Kurosaki *et al.*, 1996). In two patients, these two variants appeared as they seroconverted to anti-HBe. However, they were subsequently described equally from both HBeAg positive and negative cases (Laskus *et al.*, 1995). Other variants in Enh-II/CP leading to amino acid changes in X protein are less common.

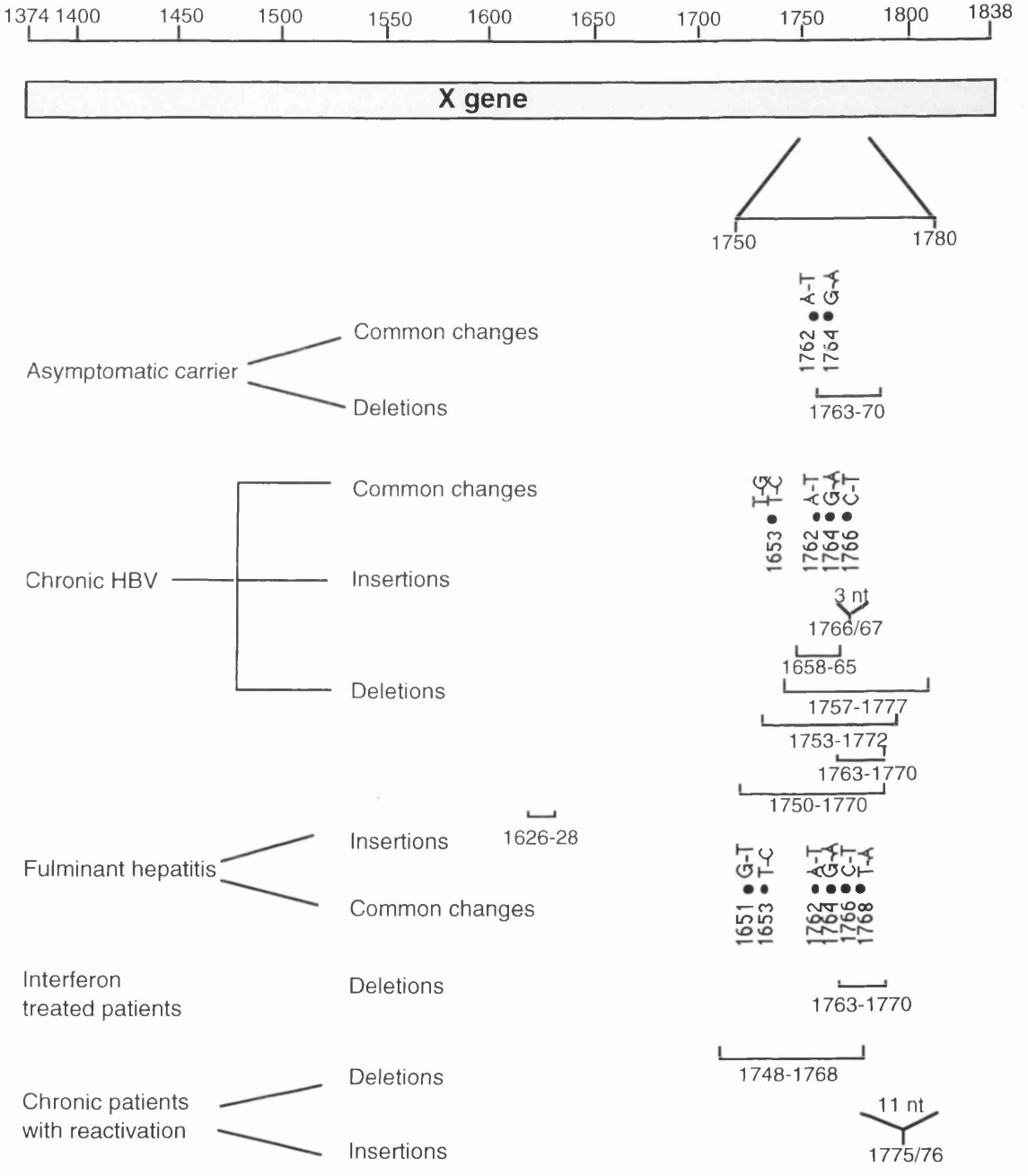


Figure 1.7 Clinical associations with variation in the X gene are shown simplistically.

Deletions, insertions, and point mutations are also found in patients with HCC (Takada and Koike, 1990), thalassaemia (Feitelson *et al*,. 1994), renal dialysis (Feitelson

et al., 1995) and also from chronic patients without the usual serological markers (Blum *et al.,* 1991; Liang *et al.,* 1990). Deletions overlapping the start codon and DR2, and deletions or insertions within the core promoter have also been observed in interferon α (IFNα) treated and chronic HBV patients (Laskus *et al.,* 1994). The X ORF was interrupted in all of the described variants (Figure 1.7), resulting in expected truncation of X protein. Deletions in the same region were described by Repp *et al.* (1992): an 8 nt deletion was detected in approximately 15% of the clones from 5 of 6 patients, resulting in truncation by 26 aa and insertion of 5 novel amino acids. Deletions, insertions, and/or point mutations affecting nuclear factor binding sites have also been described from immunosuppressed patients with severe liver disease (Gunther *et al.,* 1996). Most of these changes created HNF-I or HNF-III binding sites, and/or duplication of C/EBP and HNF-IV binding sites; these resulted in a drastic reduction in the level of pre-core mRNA and increase in replication. Truncated X gene have also been linked to a low serum DNA level. In one study (Horikita *et al.,* 1994), X gene deletions were seen in asymptomatic carriers with low serum DNA level. However, such changes were not seen after fulminant reactivation with high serum DNA level. Low levels of viral DNA due to deletions in the X gene in asymptomatic carriers but not in patients with chronic active hepatitis (CAH) has also been reported from other studies (Fukuda *et al.,* 1995). The HBx deleted strain can only survive in the presence of full length strain. Finally, two serologically non-A-E viral hepatitis cases (Uchida *et al.,* 1995), both negative for HBsAg and anti-HBc, had an 8 nucleotide deletion at nt 1770-1777, creating a truncated X protein; interestingly, no full length X gene was detected by direct sequencing. The deleted X gene and BCP may have suppressed HBV DNA replication and expression.

1.2 HBV: PATHOLOGY AND IMMUNOPATHOGENESIS

1.2.1 EPIDEMIOLOGY AND TRANSMISSION OF VIRUS

Infection with HBV results in a broad spectrum of liver disease, ranging from subclinical infection to acute, self limited hepatitis and often fatal fulminant hepatitis. Exposure to HBV particularly when it occurs early in life, may also result in an asypmtomatic carrier state that can progress to chronic active hepatitis, cirrhosis of the liver and eventually hepatocellular carcinoma. There are 400 to 500 million HBV carriers in the world today. The HBsAg carrier rate varies world-wide from 0.1-0.2% in Britain, the USA and Scandinavia, to 3 percent in Greece and Italy and up to 10-15% in Africa and the Far East. Carriage of HBV can be even higher in some isolated communities such as Alaskan Eskimos or Australian Aborigines. In endemic areas such as east Asia, sub-Saharan Africa and the Amazon basin, where carrier rates range from 8-25 percent,

prevalence of anti-HBs is from 60-85 percent. However, these numbers reduce with vaccination.

Percutaneous transmission is one of the principal modes of transmission of hepatitis B. Infection can occur with transfusion of blood or blood products, although the risk has reduced dramatically in recent years by routine blood screening. Persons at greatest risk of acquiring hepatitis by the percutaneous route are haemophiliacs, who receive clotting factor concentrates prepared from large pools of plasma, intravenous drug abusers and hemodialysis patients and staff. Despite the reduced risk of hepatitis B found among the recipients of blood, the hazard of parenteral exposure acquired by sharing contaminated needles by drug abusers continues to be responsible for many hepatitis B cases. Transmission of HBV by the percutaneous route may sometimes occur as a result of inapparent inoculation following the use of shared razors, toothbrushes, communal bathbrushes or towels, and by tattooing, acupuncture, or ear piercing.

In low prevalence countries, most adult infections occur by sexual contacts or blood contacts with chronically infected persons. In non-human primates, experimental infection with semen was successful, but the per-oral administration of HBV contaminated saliva failed to infect chimpanzees. Therefore kissing is not a significant means of transmission, although biting may be. HBV DNA has been detected in the saliva of HBsAg carriers, but the concentration is much lower than found in serum. Transmission by sexual contact among spouses and sexual partners is a substantial risk. This is particularly true among homosexual males. In the United States, as many as a half of hospitalised patients with hepatitis B are homosexual males.

Prenatal and transplacental transmission of HBV from infected mother to offspring is the most important mechanism for maintenance of an HBV carrier population in high carriage areas (such as Asia or Africa). Almost all women who are HBeAg positive transmit the virus to their newborn infants, most of whom become persistently infected. In contrast, transmission is less common from anti-HBe positive carriers. Eighty to ninety percent infants born to HBeAg positive mothers become carriers during the first 2-3 months of life. Infected babies born to anti-HBe positive mothers are much more likely to have a transient infection. When a child is infected before the age of 1 year, the risk of chronicity is high (80-90 percent); however, infection in adults gives rise to a carrier rate of only 1-2 percent.

Infection from mother to infants is not usually *in utero*, but at the time of birth and during close contact afterwards. The virus apparently does not cross the placenta easily, perhaps 5-10% of children born to HBsAg positive mother may be infected *in utero*.

Hospital staff in contact with patients and patients blood, usually have a higher carrier rate than the general community. This applies particularly to staff on renal dialysis or oncology units. The patient's attendant is infected through contact with blood from pricking or from skin abrasions.

1.2.2 PATHOLOGY AND CLINICAL FEATURES

i) Acute hepatitis

Acute hepatitis is characterised by diffuse inflammation of the liver. Liver cell necrosis involves single hepatocytes or small groups of cells, particularly in the centre of lobules. The most characteristic clinical feature is jaundice, also known as the icteric phase. However, some patients develop anicteric hepatitis, being development of clinical symptoms without jaundice. The non-icteric patient is more likely to become a chronic carrier than the icteric one. Sometimes a patient may have inapparent or sub-clinical hepatitis with neither symptoms nor signs.

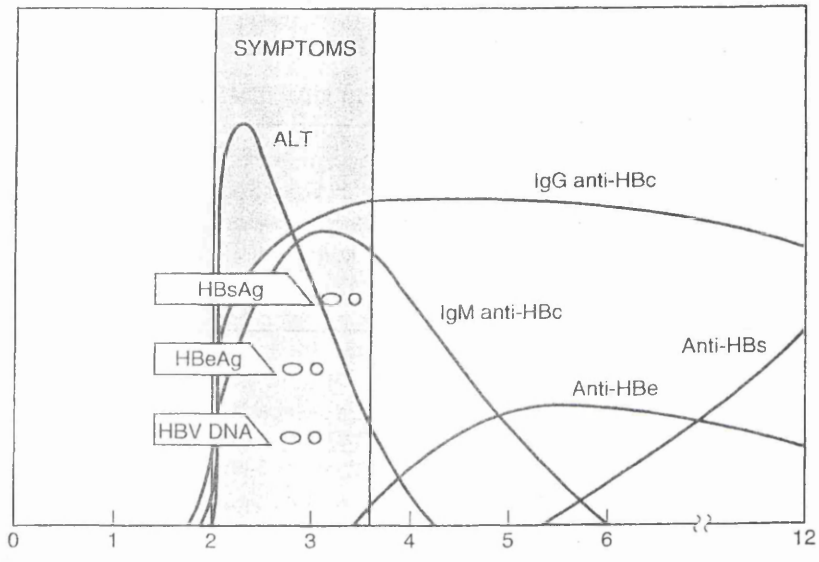


Fig 1.8 Profile of serological markers of hepatitis B during the course of infection and convalescence.

An acute hepatitis patient usually presents a spectrum of serum viral antigens, and antibodies as well as HBV DNA (Figure 1.8). The first marker is probably virus detectable by PCR and HBsAg which is followed by elevated levels of serum alanine aminotransferase (ALT). Anti-HBc IgM and IgG appear prior to clinical symptoms. HBeAg and HBV DNA by hybridisation appear at about the same time. Disappearance of these markers and seroconversion to anti-HBe precedes clearance of HBsAg; appearance of anti-HBs is associated with recovery.

ii) Chronic hepatitis

In adult cases with acute infection, serum HBsAg appears within 3-4 months after exposure. In about 5%, antigenemia is detected for more than 6 months, defining the carrier state. The major determinants of chronicity of HBV infection is age of exposure;

about 90 percent chance when exposed perinatally to <1 percent in adult stage. Every year, about 5-10 percent of these adult-onset carriers spontaneously lose HBsAg and seroconvert to anti-HBs. In contrast, 90% of babies infected perinatally or within the first five years of life become carriers and have a lesser chance of spontaneous recovery during their lifetime. Some chronically infected patients have little clinical, biochemical or histologic evidence of liver disease, these are known as asymptomatic carriers.

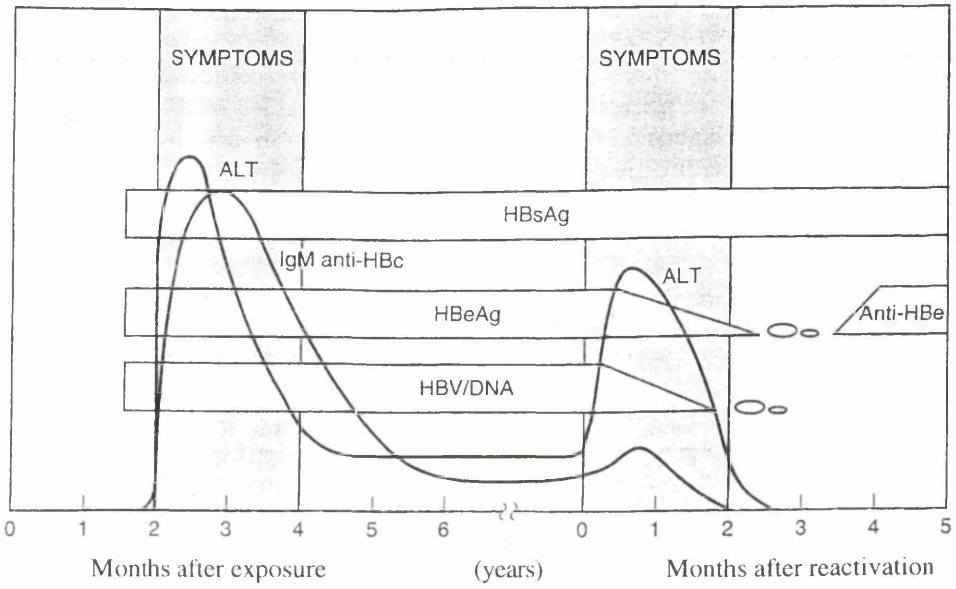


Fig 1.9 serological profile of hepatitis B progressing to chronicity, followed years later by termination of replicative phase.

In chronic HBV infection, HBeAg and HBV DNA remain detectable for some years (Figure 1.9). Anti-HBe makes an appearance in the form of immune complexes with HBeAg. Eventually, at a rate of 5 percent a year, anti-HBe becomes predominant. With the appearance of anti-HBe, a marked reduction of viral replication and reduction of circulatory HBV DNA to low levels usually occurs. Anti-HBs appears once HBsAg is cleared. However, in some patients, loss of HBeAg represents the selection of HBeAg negative variants (A_{1896}). Patients have progressive liver disease, elevated ALT levels, and high serum DNA and yet are HBeAg negative. Such patients appear to have poorer prognosis with a greater risk of developing an aggressive chronic hepatitis.

1.2.3 IMMUNOPATHOGENESIS

The pathogenic mechanisms of acute and chronic liver disease, involving both viral and host factors, and the reasons for the development of hepatocellular carcinoma in chronically infected patients, are only partially defined because of the lack of an infectible/susceptible cell culture system. Most studies suggest that HBV is not directly

cytopathic, or at least not highly cytopathic, and experimental evidence suggests that the humoral and cellular immune responses to viral antigens are required, not only for viral clearance, but also play a role in pathogenesis.

i) Acute HBV

In acute HBV infection, intense hepatocyte lysis occurs, apparently as a result of a polyclonal multispecific immune response. The increase in serum ALT levels in acute infection is one of the manifestations of this phenomenon. Serum bilirubin concentrations increase in proportion to the severity of the hepatic damage. The immune response in acute HBV infection is targeted to HBsAg, HBc/HBeAgs and to the non-structural polypeptides of HBV (Mondelli *et al.,* 1982; Mishra *et al.*, 1992). HBsAg, HBcAg and HBeAg have all been detected on the hepatocyte membrane (Mondelli *et al.,* 1982).

a) Humoral immune response: The role of the antibody response to HBc and HBeAgs in immunopathogenesis is not clear. A high titre of anti-HBc antibodies is found during both acute and chronic phases of the disease, suggesting that they do not contribute to neutralising viral infectivity (Milich *et al.,* 1986). In acute infection, anti-HBc IgG and IgM appear prior to symptoms. The presence of a strong IgM anti-HBc response is an indication of the acute phase, but also persists at low levels in chronic hepatitis. IgG is present in high titres in acute infections, but persists at lower levels during the lifetime of recovered patients. The high titres of anti-HBc may be related to T-cell independence; as athymic mice could produce IgG and IgM antibodies to HBcAg (Milich *et al.*, 1986). Mapping of B cell epitopes on core particles has been undertaken by many groups using panels of overlapping synthetic peptides, screened against monoclonal and polyclonal antibodies. Most of the early studies suggested a highly conformational and discontinuous structure to the HBc antigenic determinant (Ferns and Tedder, 1988; Salfeld *et al.*, 1989). HBcAg was found to be destroyed by SDS denaturation and lost its antigenic specificity (Ohori *et al.*, 1980) exposing HBeAg specificity. A major immunogenic determinant, recognised by four different monoclonal antibodies was found by Waters *et al.* (1988). A single conformation dependent epitope was identified in the hydrophilic region between aa 74-83 which overlapped with a linear HBe-related determinant (Salfeld *et al.*, 1989). However, other studies reported several linear epitopes of HBcAg arguing against its strict conformational dependence. Five linear antigenic epitopes were identified within the peptides covering residues 1-20, 61-85, 76-83, 121-140 and 141-161 (Salberg *et al.*, 1994). These were recognised by only a minority of the antisera examined, indicating that the major recognition epitope is not linear. A B cell epitope was found between aa 107-118, which was highly reactive against all polyclonal and monoclonal antibodies: it was thus suggested to be a linear epitope of a larger conformational determinant (Colucci *et al.,* 1989). Another was found in the arginine rich domain (aa 150-159), specific for HBcAg but not HBeAg (Machida *et al.,* 1989); a final

epitope (aa 74-123), recognised by the majority of anti-HBc containing antisera, was identified by Tordjeman *et al.* (1993). Presence of many linear epitopes throughout the entire length of the core protein suggested that antigenicity is due to many epitopes and not always conformation dependent. This is probably irrelevant to pathogenesis, as previous studies showed that HBcAg activates B cells only in the particulate form (Milich *et al.,* 1986).

Although most of the primary amino acid sequence is identical, both HBeAg and HBcAg display different antigenic properties that are used for diagnosis. In contrast to anti-HBc, antibodies to HBeAg become predominant at various intervals after the appearance of anti-HBc, (or may not develop) and are related to the clearance of virus in self limited acute infections. By deletion analysis and using a panel of monoclonal and polyclonal antibodies, Salfeld *et al.* (1989) identified two B cell epitopes in p17e (the secretory form of HBeAg) polypeptide. One is linear (HBe1), mapped between aa 76 to 89, and overlaps with the HBcAg epitope. The other is conformational (HBe2) and requires the intramolecular participation of amino acid sequence between 10-140. The HBe2 sequence is also present in HBcAg but this epitope is masked in the assembled core particle.

The antibody response to HBsAg (anti-HBs) is a T cell dependent process (Milich *et al.,* 1986). As the anti-HBs antibodies which are directed to the HBs epitope, are readily detectable in patients who clear the virus and recover from acute infections, and as they are undetectable in patients with chronic infections, these antibodies are thought to play an important role in virus clearance (Milich *et al.,* 1986). During natural infection, antibodies to pre-S1 and pre-S2 appear earlier than the antibodies to HBsAg and correlate with the disappearance of serological markers for HBV replication (Milich *et al.,* 1985). Both anti-pre-S and anti-HBs are neutralising antibodies and prevent virions binding to the hepatocyte. The pre-S2 region is significantly more immunogenic than the S region and its regulation is independent of S regulation. The pre-S2 region generates T helper cell activity that can induce B cells to produce antibodies to pre-S2 region as well as S region; this can circumvent S region non-responsiveness (Milich *et al.,* 1985). HBcAg specific T helper cells can induce anti-pre-S1 antibody production in the absence of anti-pre-S2 and anti-S production (Milich *et al.,* 1987).

Antibodies to the RNaseH domain of the HBV polymerase protein are found in the sera of both chimpanzees and humans early in the infection, shortly after the appearance of anti-HBc. They persist in chronic carriers with ongoing viral replication, but decline and disappear at the time of virus clearance from the serum (Weimer *et al.,* 1990).

b) Cell-mediated immune response: Experimental evidence suggests that the T cell response to HBV epitopes account for cellular injury and virus clearance in

acute hepatitis B. In addition, elimination of virus-infected hepatocytes is suggested to be dependent on the recognition of the viral determinants, in association with HLA (human leukocyte antigens), by T lymphocytes on the infected hepatocytes. Usually, class-II restricted $CD4^+$ cells can recognise exogenous antigen after internalisation and intracellular processing by antigen presenting cells (APC). On the other hand, $CD8^+$ HLA class-I restricted T cells are generally activated by antigens which are endogenously synthesised by infected cells and presented on the cell membrane. Although, as a general rule, HLA class-II restricted $CD4^+$ cells recognise exogenously synthesised peptides presented on the APC and exert their activity by releasing antiviral mediators (interleukins [ILs] and tumour necrosis factors [TNFs]), hepatocytes may be able to present HLA class-II determinants on their membrane following HBV infection (Van Den Oord *et al.,* 1986). This suggests that class-II restricted $CD4^+$ cells can participate in immune clearance of HBV infected cells. A study by Penna *et al.* (1992) reported that HBsAg specific CTLs recognised endogenously synthesised HBsAg presented by class-II molecules as well as that expressed by APC, suggesting a role in hepatocellular injury.

The majority of experiments have shown proliferation of HLA class-II restricted $CD4^+$ cells to HBs, HBc and HBe antigens indicating a strong polyclonal and multispecific immune response in patients with acute hepatitis B, but a lower T cell responsiveness to HBc/HBeAgs in patients with chronic infection (Ferrari *et al.,* 1990; Jung *et al.,* 1991; Tsai *et al.,* 1992). In all studies, the HBcAg-specific responses were stronger than HBsAg specific responses. Acutely infected patients were found to show a vigorous response to HBcAg in terms of proliferation of HLA class-II restricted $CD4^+$ cells and interferon γ production during the acute phase associated with clearance of HBsAg and HBeAg from serum, but this response was absent in chronic patients. A number of investigators have reported HBcAg peptides that are recognised by a majority of the patients with acute hepatitis B. Ferrari *et al.* (1992) investigated 23 patients with acute hepatitis; 22 of them showed a strong HLA class-II restricted $CD4^+$ response to aa 50-69. Two additional $CD4^+$ epitopes were recognised, at aa 1-20 and 117-131, identified by 60% and 73% patients respectively. Another group used different peptides and defined aa 1-25 and aa 61-85 as predominant T cell recognition sites (Jung *et al.,* 1993). The immunodominant epitopes are expressed in both HBc and HBe antigens. In fact, both antigens are cross-reactive at the T cell level. However, these two antigens show preference in Th subset induction. A recent study reported that HBcAg and HBeAg elicit different subsets of Th cells (Milich *et al.,* 1997). In transgenic mice, HBcAg was found to be preferentially but not exclusively elicited Th_1 subsets; on the other hand, HBeAg elicited Th_2 or Th_0 subsets. This difference was believed to be due to structural differences between the two immunogens, and recognition by two different HLA class-II determinants (Milich *et al.,* 1995)

It is widely acknowledged that class-II restricted HBc/HBeAg specific $CD4^+$ T cell response play a critical role in viral clearance. This could be achieved in several ways. Firstly, $CD4^+$ cells can induce virus specific CTL because the intensity of class-II restricted T cell response to HBc/HBeAg during acute infection parallels the HLA class-I restricted CTL response to all the viral antigens (Penna *et al.,* 1991; Nayersina *et al.,* 1993). Secondly, HBcAg specific T helper cells help HBsAg specific B cells to produce anti-HBs antibodies.

Since it has been found from murine experiments that T helper cell tolerance specific for HBc/HBeAgs may be the basic immunological defect in neonates born to HBeAg positive carrier mothers, an investigation was performed on cellular immunity to HBcAg in HBV carrier children in such group (Hsu *et al.,* 1992). A significant proliferative response of PBMC (peripheral blood mononuclear cell) to HBcAg was found in five of eleven children with an elevated ALT level but none of the nine HBeAg positive children with a normal ALT level. This could be due to maternal HBeAg inducing specific non-responsiveness to T helper cells against HBe and HBcAgs in the at-risk neonates.

The class-II restricted HBsAg specific response is much less vigorous in the same patients. The reason for relatively weaker HBsAg specific T cell response of patients who respond quite vigorously to the HBcAg is not well understood. An anti-HBs specific T cell response was reported during the preclinical incubation of the disease (Vento *et al.,* 1987). This could become either exhausted as a result of stimulation by high dose of virus or paralysed by the high concentration of surface antigens (Chisari *et al.,* 1995). Information about class-II restricted $CD4^+$ response to polymerase protein and X protein is sketchy.

Antiviral CTLs are believed to play a major role in eradication of infection by killing virus infected cells through recognition of viral peptides presented by HLA class-I molecules. Acutely infected patients develop a vigorous, polyclonal $CD8^+$ CTL response against multiple epitopes in the HBcAg, HBsAg and polymerase proteins (Penna *et al.,* 1991; Nayersina *et al.,* 1993). Most of the CTL epitopes defined so far are HLA A2 restricted and contain the predicted HLA A2 binding motif with leucine at position 2 and valine at the C terminus end (Bertoletti *et al.,* 1993; Nayersina *et al.,* 1993). Until now, a single HLA A2 restricted CTL epitope has been identified in HBcAg, located between aa 11-27 (Bertoletti *et al.,* 1991). This endogenously synthesised epitope is recognised by T cells from all HLA A2 positive patients with acute infection. Using a panel of truncated and overlapping peptides, the optimal stimulatory sequence is a 10 mer (aa 18-27) containing the HLA A2 motif. Another CTL epitope was reported in acutely infected patients, located at aa 141-151 of HBcAg. CTL recognition of this epitope is restricted by two independent class-I molecules, HLA A31 and HLA AW-68, both of which respond

to the same 11 aa residues. This epitope is completely overlapping with a functional domain of core protein that is essential for nuclear localisation and genome packaging.

Several HBsAg specific class-I restricted CTL epitopes have been identified in acutely infected patients (Nayersina *et al.,* 1993) and five epitopes have been found in the polymerase protein. Most of them are specifically restricted by the HLA A2 allele. This number is considerably higher than that found for HBcAg. The extreme hydrophobicity of the surface protein may be one of the reasons for high representation of HLA A2 restricted CTL epitopes (Falk *et al.,* 1991). The HBsAg specific CTL response is also polyclonal and multispecific in acutely infected patients.

CTL can also play a role in clearing virus without killing the infected cells. In transgenic mice, it has been found that CTL suppressed HBV gene expression by a non-cytolytic process (Guidotti *et al.,* 1994b) which is mediated by IFN-γ and tumour necrosis factor α (TNFα).

ii) Chronic HBV

A number of defects have been characterised in patients with chronic hepatitis B. The suppression of humoral and cellular immune response to clear the virus is one of the most important reasons. A deficient production of interferon and suppression of the ability of the host to respond to interferon are probably another major factor (Thomas *et al.,* 1988).

Antibodies against all three classes of surface antigens, which are detectable early in acute infection, are not detectable in the chronic state. Appearance of anti-pre-S/S antibodies is followed by the clearance of HBsAg. Failure to clear HBsAg from the blood is the hallmark of chronic HBV infection. Production of HBs and HBe antigens in excess of that required for the virion formation may impair or reduce the effectiveness of neutralising anti-HBs antibodies by preventing binding to the infectious virions (Thomas *et al.,* 1988) and thus, may induce immunotolerance. Production and secretion of surplus HBs particles of 22 nm size can divert anti-HBs away from intact whole virions, and thus reduce the chance of neutralisation by anti-HBs. HBeAg, an immune modulator can induce tolerance against HBe/HBcAg specific peptides displayed on hepatocyte membrane, and reduces the degree of inflammation. Antibodies to HBcAg may also interfere with the expression of HBcAg specific T cell epitopes on the hepatocyte membrane (Mondelli *et al.,* 1982). Anti-HBc and anti-HBe block the CTL activity probably by modulating the recognition of HBc/HBeAg specific peptides displayed on hepatocytes (Pignatelli *et al.,* 1987). In chronic infection, significantly higher level of HBcAg/anti-HBc and HBs/anti-HBs immunocomplexes were produced in the presence of excess antigens (Maruyama *et al.,* 1994). Integrated HBV DNA in the cellular genome (Shafritz *et al.,* 1981) and escape mutation can also lead to persistence of HBV infection. CTLs can suppress antibody production. A study by Barnaba *et al.,* (1990) demonstrated

the generation of HBsAg-specific liver derived T cell clones which could specifically kill anti-HBs producing B cells and thus may contribute to downregulating anti-HBs antibody production. CTLs can also suppress anti-HBs antibody production by releasing a secratible soluble factor (TSF), sometimes found in chronic carriers (Yamauchi *et al.*, 1988).

During chronic infection, the HLA class-II restricted $CD4^+$ T cell response to all viral antigens including HBc/HBeAgs, is much less vigorous than in patients with acute hepatitis (Ferrari *et al.*, 1990; Jung *et al.*, 1991; Penna *et al.*, 1991). Although these T cells can be detected in the liver, they appear to be present at low frequencies (Ferrari, *et. al.*, 1987). Types of $CD4^+$ Th subsets elicited by HBcAg, sometimes may have clinical relevance. Predominance in acute infection of HBcAg-specific Th_1 subsets, which produce IL-2 and IFNs, favours CTL mediated clearance; in contrast, predominance of HBcAg-specific Th_2 cells favours antibody production over cell mediated immunity (Maruyama *et al.*, 1994). Using peptide pulsed or transfected target cells, it was found that, in contrast to the vigorous CTL response to all the viral antigens detectable in the PBMC of the patients who clear the virus, HBV persistence is typically associated with a weak and undetectable CTL response. In one observation, none of the six HLA A2 positive patients with chronic hepatitis produced a detectable CTL response to any of the HLA A2 restricted HBs or HBc specific epitopes (Nayersina *et al.*, 1993). While a vigorous response to CTL epitopes plays an important role in clearing the virus in self-limited acute infection, the weakness or absence of CTL response in chronic infection is probably responsible for indolent necroinflammatory liver disease (Chisari *et al.*, 1995). Recently, Thurz *et al.* (1995) reported a greater frequency of HLA class-II allele DRB1*1302 among children in The Gambia who recovered from HBV infection compared to those who had persistent infection. This suggested that presence of this allele may confer protection against chronic HBV infection. This has been confirmed by others.

Variants in the antigenic determinants may contribute to the chronicity in several ways: they may 'escape' from immune recognition or can show antagonistic effect on T cell receptor (TCR) recognition of standard peptide. In a study by Bertoletti *et al.* (1994), two HLA A2 positive chronic patients were found to show a vigorous CTL response against HBcAg (aa 18-27) despite their inability to clear the virus. Both of them contained variants at aa 21 and 27 which decreased HLA binding to the variant peptide and the TCR recognition. Interestingly, these were not detected in an additional five chronic patients in the same study who did not show any CTL activity against the epitope 18-27. The variant epitope acted as a TCR antagonist by inhibiting the CTL response to wild type epitope.

During chronic infection, there is evidence of failure of interferon production and activation of infected liver cells, so viral protein synthesis is not decreased and there is poor enhancement of HLA display. These may result in failure of presentation of viral peptides to the immune system. Production of suboptimal levels of interferon α and γ by

PBMC in chronic patients was reported in several studies (Abb *et al.,* 1985; Ikeda *et al.,* 1986a). However, a reduced interferon production was also seen in the lymphocytes taken from self limited acute HBV infection (Levin and Hahn, 1982), indicating that reduced interferon production is one of the causes of persistence in some cases. In chronic infection, there is also evidence of abnormal activation of hepatocytes by interferon: levels of hepatic 2-5A synthetase, an enzyme induced by interferon, were minimally elevated (Ikeda *et al.,* 1986b) and the density of HLA class-I protein, which is induced by interferon, was not significantly increased above normal level.

Since lymphocyte production is dependent on specific lymphocyte activation, absence of enriched lymphokine releasing cells may be associated with failure of virus elimination in chronic patients (Jung *et al.,* 1991).

1.3 FULMINANT HEPATITIS

Fulminant hepatitis is a devastating illness in which liver failure occurs within a short period of time. Rapid deterioration of liver function occurs due to extensive hepatocellular destruction.

1.3.1 DEFINITIONS AND AETIOLOGY

i) Definition

There are two definitions commonly used. In 1970, the term fulminant hepatic failure (FHF) was first introduced and defined as i) development of stage II to IV hepatic encephalopathy within 8 weeks of onset of illness, ii) a prothrombin time of less than 40s and no known history of liver disease (Trey and Davidson, 1970). Since then, this definition has been widely used, although over the years several changes in the period between the appearance of symptoms and the onset of encephalopathy have been proposed. A group at King's College Hospital, London have defined an additional late-onset hepatic failure (LOHF) in which encephalopathy appears between 8 to 26 weeks after the onset of symptoms (Gimson *et al.,* 1986). A later definition defined FHF as acute liver failure complicated by hepatic encephalopathy less than 2 weeks after the onset of jaundice (Bernuau *et al.,* 1986a) and is used by a group in Paris. According to this group, acute liver failure complicated by encephalopathy 2 weeks to 3 months after the jaundice, is defined as 'subfulminant liver failure'. 'Subacute liver failure' designates acute liver failure with a protracted course not associated with hepatic encephalopathy (Tandon *et al.,* 1982). Both groups have found that patients with the earliest onset of encephalopathy have the best chance of spontaneous recovery, confirmed in other studies (Takahashi *et al.,* 1991). A new terminology of FHF has been proposed based on studies of more than 600 patients admitted to King's College Hospital, London between 1972 and 1985. This group (O'Grady *et al.,* 1993) suggested 'hyperacute liver failure' for

cases where encephalopathy appears within 7 days of the onset of jaundice. This group of patients have a high rate of cerebral oedema with very good prognosis. The term 'acute liver failure' was suggested for cases with an interval of between 8 and 28 days from jaundice to encephalopathy; these patients also have a high incidence of cerebral oedema but much poorer prognosis without liver transplantation. The term 'sub-acute liver failure' was suggested for cases when encephalopathy occurs within 5 to 12 weeks of symptoms.

In the original definition of acute liver failure, the absence of previous history of liver disease had been a prerequisite. But the new terminology includes cases of acute liver failure due to reactivation resulting from Delta virus superinfection or Wilson's disease.

ii) Aetiology

Fulminant hepatitis can be caused by viruses or agents other than hepatitis viruses. Any virus that causes an acute hepatitis can also cause fulminant hepatic failure. Viruses are the most common cause of acute liver failure world-wide and account for approximately 70% of the cases, although the type of viruses varies geographically.

Hepatitis A virus (HAV) is directly hepatotoxic, so an unusually large inoculum or diminished host defence may contribute to the development of a fulminant course. However, fulminant hepatitis A is rare and mostly prevalent in developing countries (Joshi *et al.,* 1983). In developed countries it is sometimes related to travel to highly endemic areas (Gimson *et al.,* 1983a) or HAV superinfection on pre-existing liver disease (Akriviadis and Redeker, 1989). The survival rate of patients with fulminant A hepatitis is relatively high, 40% or more (Bernuau *et al.,* 1983).

HBV is still the major cause of viral fulminant hepatic failure. Details about fulminant B hepatitis (FHB) are discussed later in this chapter.

Fulminant hepatic failure caused by unknown aetiology is categorised as non A non B (NANB) fulminant hepatitis, often presumed to be caused by one or more viral agents (Wright, 1993). Super-infection or co-infection with HBV has been reported in some cases (Wright *et al.,* 1992). The prevalence of hepatitis C virus (HCV) and hepatitis E virus (HEV) has been reduced in western countries, however, a fulminant course due to HCV has been reported recently from Europe (Liang *et al.,* 1993) and from Japan (Yanagi *et al.,* 1991). Some of the patients in the latter study, though positive for anti-HCV and HCV RNA, were found to be superinfected with HAV or HBV. The survival rate in NANB fulminant hepatitis due to HCV was significantly lower than that caused by HAV or HBV alone (Yanagi *et al.,* 1991). The involvement of HCV in NANB fulminant hepatitis remains a matter of controversy: an investigation in the USA revealed that only a few cases of NANB FHF were positive for HCV markers (Liang *et al.,* 1993). One study by Wright *et al.,* (1992) found that at least 50 percent off the cases diagnosed as NANB were actually caused by HBV as HBV DNA was detectable only using PCR.

There are no risk factors for NANB fulminant hepatitis and no specific diagnostic tests. Clinical features are different from those of type A and B fulminant hepatitis (Gimson *et al.,* 1983b). The disease is recognised by the absence of viral markers and its tendency to run a prolonged, pre-encephalopathic phase; 83 percent of patients in one series of subfulminant liver failure were due to presumed NANB hepatitis (O'Grady *et al.,* 1993). Fulminant NANB hepatitis with late onset was found to be severe with a very low survival rate. Re-infection of the transplanted organ has not been reported.

Hepatitis D virus (HDV) infection occurs either as a coinfection with HBV or as a superinfection of patients with chronic HBV infection. Fulminant D hepatitis does not differ clinically from fulminant HBV with coinfecting HDV (Bernuau *et al.,* 1986a). Such cases of HDV associated infection have been reported from Western Europe and the USA (Smedile *et al.,* 1982; Govindrarajan *et al.,* 1984) and it is evident from these reports that the risk of fulminant hepatic failure is higher in patients with coinfection than with HBV alone.

Hepatitis E virus (HEV) genome has been detected in the serum of patients with fulminant NANB hepatitis (Sallie *et al.,* 1991) but this is rare in Western countries. Pregnant women are at high risk of developing fulminant hepatitis due to HEV (Nanda *et al.,* 1994).

Hepatitis G virus (HGV) or GBV-C, a flavivirus agent, has recently been identified as one of the cause of non-A to E hepatitis and often found to be associated with HBV and HCV infections (reviewed in Karayiannis and Thomas, 1996), was also implicated as a causal agent for fulminant non-A-E hepatitis. This was reported in FHF cases from Japan (Yoshiba *et al,.* 1995) and Germany (Heringlake *et al.,* 1996). However, this was not confirmed by another group in Japan (Kuroki *et al.,* 1996) and found to be transmitted in FHF cases during blood transfusion after the onset of disease (Haydon *et al.,* 1997).

FHF due to non-viral agents has often been reported (Bernuau *et al.,* 1986a), due to drugs or toxins or (such as acetaminophen and mushroom toxins). The toxic effect of *Amanita* mushroom poisoning is mediated by α-amanitin in Western countries. Drugs like halothane, isoniazid, valporic acid and tetracycline have also been reported to cause FHF. Certain conditions such as cardiovascular disease, where disruption of blood flow to the liver can result in acute necrosis. Some metabolic disease such as Reye's syndrome or Wilson's disease may also present as FHF.

1.3.2 CLINICAL FEATURES

i) Encephalopathy

Hepatic encephalopathy is the most important criterion for the diagnosis of fulminant or acute hepatic failure (Trey and Davidson, 1970). Encephalopathy is a reversible clinical state that indicates the residual liver function has fallen below a critical

threshold. It is caused, at least in part, by the accumulation of substances that are normally cleared by the liver. In FHF, encephalopathy is clinically graded from grade 0 to IV on the basis of severity of the disease (Table 1.1). The grades of encephalopathy are used to assess the severity of hepatic failure. Patients who do not develop encephalopathy beyond grade II have a good prognosis. In viral FHF, duration of jaundice more than 7 days before the onset of encephalopathy indicates a poor prognosis (O'Grady *et al.,* 1989). A greater rate of survival was seen in patients who developed encephalopathy within 7 days of onset of jaundice, despite the progression to grade IV (O'Grady *et al.,* 1993), while only 7 percent of patients survived who developed encephalopathy between 8 to 28 days after the onset of jaundice.

Table 1.1

Grade	Clinical features
0	Not encephalopathic
I	Mild or episodic drowsiness, impaired intellect and concentration but rousable and coherent.
II	Increased drowsiness with confusion and disorientation but rousable and conversant.
III	Very drowsy, disoriented, responds to simple verbal commands but often agitated and aggressive.
IV	Unresponsiveness to verbal commands but may move on painful stimulus.

Table 1.1 Clinical features of the grades of hepatic encephalopathy (Table taken from Harrison *et al.,* 1993).

The pathogenesis of hepatic encephalopathy is not well understood. A number of substances have been suggested to play a role. Accumulation of toxic substances are thought to impair neuronal function and damage the blood-brain barrier. Increased gamma aminobutyric acid (GABA) neurotransmission has been implicated in the pathogenesis (Jones *et al.,* 1987), although GABA receptor number and affinity were found to be normal. A number of other substances such as ammonia, mercaptans and medium-chain fatty acids have been proposed to have a toxic effect.

ii) Coagulopathy

Since the liver is involved in producing the majority of the clotting factors, it is not surprising that blood coagulation disturbance is one of the major complications of FHF; levels fall rapidly at the onset (Lechner *et al.,* 1977). Interrelated clotting abnormalities may occur reduced synthesis of clotting factors, consumption of clotting factors, activation of fibrinolysis and platelet abnormalities. The commonly used assay to assess liver disease is the prothrombin time (PT), which measures factors I, II, V, VII and X (all medium in the liver). A prothrombin time greater than 50s indicates a poor prognosis (O'Grady *et al.,* 1989).

Abnormalities in platelet counts, morphology and function have all been described in FHF (O'Grady *et al.,* 1986). The platelet count usually declines progressively during the course of illness. Counts less than 100 000x10^9/L were found in about 70 percent of the cases. Mean platelet counts were lower in patients who died than in survivors. Adhesion abnormalities of platelets have also been reported (O'Grady *et al.,* 1986). Abnormal platelet adhesion causes damage of vascular endothelium by interaction with platelet glycoproteins, and conformational changes occur. As a result, more platelets aggregate at the site of injury, activating and binding with coagulation factors.

iii) Cerebral oedema

Fulminant hepatitis patients who progress to grade IV encephalopathy have a 75 to 80 percent chance of developing cerebral oedema and intracranial hypertension; this is still the major cause of death (O'Grady *et al.,* 1989). Increased intracranial pressure could result from expansion of either the cerebrospinal fluid (CSF), blood volume or blood tissue by 25, 5 and 70 percent respectively (Blei, 1991).

Two pathogenetic mechanisms for cerebral oedema have been suggested. In vasogenic oedema, a breakdown of the blood-brain barrier allows leakage of plasma contents through the capillary endothelium; in cytotoxic (cellular) oedema, net cellular water accumulation occurs as a result of impaired osmoregulation (Blei, 1991).

iv) Other features

Severe circulatory disturbance is characteristic of FHF. There is a low systemic vascular resistance and mean arterial pressure is maintained by a compensatory increase in cardiac output (Rueff and Benhamou, 1973). Patients who progress to grade IV encephalopathy sometimes have pulmonary complications. This is not a serious problem in FHF unless accompanied by a high incidence of lung infection. Renal failure requiring dialysis occurs in about 30 percent of the patients with grade IV encephalopathy (O'Grady *et al.,* 1988). Bacterial infection is found in about 80 percent of cases (Rolando *et al.,*

1990); the prevalent organisms are staphylococci, streptococci and coliform bacteria (Rolando *et al.,* 1990).

1.3.3 FULMINANT HEPATITIS B

HBV is one of the major causes of FHF throughout the world, but the incidence varies geographically. In most developed countries, the incidence has decreased in concert with the increasing awareness of the blood borne viruses and vaccination of high risk individuals. Hepatic failure due to HBV accounts for 45 percent of FHF cases in Paris, 42 percent in Spain, and approximately 20 percent of cases in the United States (reviewed in Norman *et al.,* 1997). The incidence of post transfusion fulminant hepatitis B has decreased since screening of HBsAg among the blood donor population (Gimson *et al.,* 1983). Females appear more likely to be affected than heterosexual males, but whether homosexual males are at risk of developing fulminant hepatitis B is uncertain (Bernuau *et al.,* 1986b). Clustering of cases of fulminant hepatitis B has been reported in groups of intravenous drug-abusers and their sexual contacts.

Most of the early studies analysed differences between prognostic factors in survivors and non-survivors. In one study (Bernuau *et al.,* 1986b), 115 FHB patients, all of twelve factors were found to be different in the two groups. Lower mean age, increased coagulation factor V and presence of detectable levels of anti-HBs were found in the patients who survived. In another study (Tygstrup *et al.,* 1986), those between 30 to 50 years had a better chance of survival. Sex of the patients had little influence on survival rates, though, in other studies, females were found to have a higher fatality rate (Bernuau *et al.,* 1986b).

i) Pathogenesis: early studies

FHB used to have shown abnormally rapid clearance of HBsAg and HBeAg and absence of detectable HBV DNA with early appearance of anti-HBs (Woolf *et al.,* 1976; Gimson *et al.,* 1983a; Brechot *et al.,* 1984), suggesting that the disease results from an excess immune response to the virus rather than a direct cytopathic effect. Early appearance of antibodies, indicating the rapid clearance of their respective antigens, was due to enhanced antibody production. In addition, there was a higher concentration of anti-HBc IgM in patients with FHB compared to acute self-limiting infection (Gimson *et al.,* 1983a). This brisk immune response was believed to be responsible for pathogenicity. Aggregation of excessive HBsAg and anti-HBs complexes was revealed using an electron microscope (Woolf *et al.,* 1976). These were believed to be responsible for blockage of hepatic sinusoids with ensuing ischaemic necrosis of liver cells. The high mortality rate in FHB is due to a lack of sufficient and rapid liver cell regeneration.

As most of the FHB cases have a very low level of serum HBsAg and HBeAg and undetectable levels of viral DNA and as HBsAg and HBcAg could not be detected in

the liver tissue, their role in disease pathogenicity remains unclear. In one study, survivals were significantly associated with the absence of serum HBsAg and presence of anti-HBs (Bernuau *et al.,* 1986b); however, this was not confirmed by others (O'Grady *et al.,* 1990). Persistence of HBsAg and pre-S2 antigens and absence of respective antibodies were found to be associated with a fatal outcome in four of 5 cases indicating continuing viral replication and hepatocyte damage (Brahm *et al.,* 1991). In contrast, early appearance of antibodies, including anti-pre-S2, was found to be associated with clinical recovery, therefore indicating that the early appearance of antibodies may result in a balance between massive liver cell necrosis, early cessation of replication and liver cell regeneration. FHB is also found in infections without any known viral markers. Several such cases have been reported where HBV was detected in 50 percent of the cases using polymerase chain reaction in absence of any other serological markers (Wright *et al.,* 1992).

Spontaneous or drug induced reactivation of chronic HBV infection leading to FHF can also occur (Yoshiba *et al.,* 1992; Meyer *et al.,* 1993). Mayer *et al.* (1993) described two cases where chronic infection spontaneously reactivated to FHF, and Yoshiba *et al.* (1992) described three HBeAg-negative chronic carriers who developed a fulminant course after intensive chemotherapy for non-Hodgkin's lymphoma.

The role of viral heterogeneity in the development of FHF has also been highlighted: an outbreak among two groups of intravenous drug-abusers and their sexual contacts implicated the presence of a viral factors. Transmission of HBeAg negative virus to two successive female sex partners of two unrelated, anti-HBe positive males with subsequent development of FHB (Fagan *et al.,* 1986) indicates an increased virulence of HBeAg negative strains. Similarly, common source outbreaks (Oren *et al.,* 1989) support the involvement of a more infective strain (this is discussed later in this section).

Co-infection or super-infection of HBV and HDV produces a more severe disease in experimental animals (Rizzetto *et al.,* 1980). Several groups have reported such co-infection in severe and fulminant cases of HBV (Shattock *et al.,* 1985; Bensabeth *et al.,* 1987). In one study, HDV super-infection was detected in 74 percent of the FHB cases (Bensabath *et al.,* 1987). HDV super-infections tend to occur even more frequently among intravenous drug-abusers, for example, accounting for approximately 91 percent of the cases (Lattau *et al.,* 1987).

ii) Pre-core variants and FHB

Following the development of more sensitive techniques in molecular biology and the subsequent discovery of the pre-core variant (A_{1896}) (Carman *et al.,* 1989; Brunetto *et al.,* 1989), a number of studies were undertaken to delineate the association of A_{1896} with FHB. HBV strains, with such mutations, have been identified in FHB patients who acquired HBV in various ways: through community transmission (Carman *et al.,* 1991;

Omata *et al.,* 1991), intra-spouse transmission (Yotsumoto *et al.,* 1992), outbreaks (Liang *et al.,* 1991), accidental and post-transfusion transmission (Kosaka *et al.,* 1991; Kojima *et al.,* 1991) and perinatal mother to infant transmission (Terazawa *et al.,* 1991). Most of these patients were HBeAg negative and anti-HBe positive and sometimes negative for both the HBe markers. In the study by Omata *et al.* (1991), sequence was available from seven of 9 FHB patients; all 7 were anti-HBe positive and were infected with A_{1896}. In another study (Yotsumoto *et al.,* 1991), three FHB cases resulted from three anti-HBe positive carriers who transmitted A_{1896} to their spouses and caused a fatal outcome in two of the three cases. Interestingly, in all cases of FHB patients and their contacts, 100 percent of clones tested were found to contain the variant strain. A nosocomial outbreak was reported from Haifa, Israel (Oren *et al.,* 1989) where five individuals were infected from a common source: all five died from hepatic failure within a short period. Sequence analysis of pre-core region showed that all patients and the contact contained A_{1896} (Liang *et al.,* 1991). Perinatal transmission of pre-core variant from mother to their infants has an increased likelihood of developing a fulminant course (Delaplane *et al.,* 1983). Perinatal transmission of HBV from HBeAg positive mothers leads to development of chronic HBV carriage in 90 percent of cases (Beasely *et al.,* 1983). However, vertical transmission of HBV from a HBeAg negative, anti-HBe positive mother seldom gives rise to a chronic state (Raimondo *et al.,* 1993), but either a self-limited acute course or FHB develops with a high mortality. This is believed to be due to absence of HBeAg, which would otherwise induce tolerance of fatal Th cells (Milich *et al.,* 1990). Genetic heterogeneity in the mother's serum may also play a role in the course of infection in the infant. In most FHB cases, only A_{1896} was selected (Raimondo *et al.,* 1993) upon transmission from mothers who were infected with a mixed population of A_{1896} and G_{1896} strains (Terazawa *et al.,* 1991). The presence of A_{1896} in the infant was thought to be associated with immune pressure on the virus (Raimondo *et al.,* 1993).

In all of these studies, cases with A_{1896} strain were found to cause more severe disease and were considered to be one of the major factors associated with the development of FHB. However, the mechanisms of pathogenicity by which the variant strain causes a more severe clinical course is not well explained and most importantly, not all the FHB cases were associated with A_{1896}.

In most of the early FHB studies, the massive hepatocellular injury was thought to be due to enhanced antibody responses with early clearance of virus. Excess production of immune complex might result in blockage of hepatic sinusoids and ischaemic injury. However, after the mapping of T cell epitopes on HBV core and surface antigens, it was suggested that T cells play the key role in clearance of virus infected cells and HBcAg and HBeAg have been implicated as the target of CTL (discussed in section 1.2). One explanation for A_{1896} pathogenesis proposes that if HBcAg epitopes, produced by the

variant strain, are expressed on the hepatocyte membrane (Schlicht *et al.,* 1989) in the absence of circulating HBeAg there may be a stronger immunological attack against HBcAg, resulting in severe hepatic injury (Omata *et al.,* 1991; Carman *et al.,* 1993a). But this does not explain how a non-variant strain causes FHB. It is not clear from most of the studies whether the patients were infected initially with A_{1896} or mixed populations of A_{1896} and G_{1896}. If they were not, it may be that an enhanced immune response is directed against an HBeAg producing strain, leading to extensive hepatocyte destruction and selection of the variant strain. However, during FHB, there is probably insufficient time for a variant to evolve. Thus, this could be explained by the fact that, in most of the cases associated with pre-core variants, the contacts were HBeAg negative and anti-HBe positive and the patients had a short duration of jaundice prior to onset of encephalopathy (Kosaka *et al.,* 1991; Liang *et al.,* 1991; Yotsumoto *et al.,* 1991). Other explanations suggested that the truncated peptide, derived from the pre-core region with a stop codon, may act as an immunological target or that the mutant strain is directly cytopathic to the hepatocytes (Omata *et al.,* 1991). There is no direct evidence for this; however, *in vitro,* deleted pre-core sequence resulted in an increased production of HBcAg (Roossink *et al.,* 1986).

Pre-core variant was not found to be prevalent in all cases of FHB (Carman *et al.,* 1991) and studies from France (Feray *et al.,* 1993) and the USA (Laskus *et al.,* 1993) did not find any significant association of A_{1896} with fulminant hepatitis. This indicated that the host's enhanced immune response to viral antigens was mainly responsible for the development of more severe disease. It is noteworthy that, in the early study by Feray *et al.* (1993); one of 10 patients had the variant strain (A_{1896}); however, 5 others had either HCV or HDV co-infection, indicating that other factors may have contributed to the severity of the disease. Although in the first report from the USA, Laskus *et al.* (1993) reported the presence of A_{1896} in 2 of 37 cases, the other study by Liang *et al.* (1994) reported 17 of 28 cases containing A_{1896}, either alone or with G_{1896}. One of the aims of our study was to resolve this issue once and for all.

AIM OF THIS STUDY

The pathogenicity of FHB is not well understood. The pre-core variant A_{1896} has been clearly shown to have an association with FHB, but this was not reported in all studies. Moreover, the presence of this variant strain in chronic carriers supports the view that an intense immune response of host to the viral antigens plays the key role in the massive destruction of hepatocytes. The main aim of my thesis was to find a viral factor(s) associated with FHB . When the study was initially undertaken, no reports were found on the sequence analyses of full-length FHBV genome. However, in late 1993 there was a report in which an FHBV strain from an infant, (which had caused the death of two paediatricians) was transmitted to chimpanzees (Ogata *et al.,* 1993). The

chimpanzees developed severe hepatitis. The sequence analysis from the source infant and the animals revealed identical sequences. In addition, there was a significant number of variations which appeared throughout the genome, some of which were in the Enh-I/XP and Enh-II/CP regions.

I performed three different analysis with FHBV cases. Firstly, sequence analyses of complete FHBV genomes to identify variants which could be linked with FHB; of particular interest would be the analysis of sequences in the *cis*-acting regulatory regions and the core gene as theses areas were found to be functionally important in a previous study. All but one of our FHBV samples were collected from FHB patients admitted at King's College Hospital London, over 10 years. None of the cases were linked and some of them were infected from different geographical areas.

Secondly, to compare the FHBV sequences with non-FHBV and to determine if there is a specific lineage among the unrelated FHBV cases.

The massive hepatocellular destruction may be due to enhanced viral replication and extensive production of replicative intermediates. The third aim was to perform functional analysis of some selected FHBV cases by transcription assay, transcription factor binding assay and to examine the *in vitro* distribution of expressed HBcAg.

CHAPTER-2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 PATIENTS

A full lists of patients is given in Tables 2.1 and 2.2.

2.1.2 REAGENTS, SOLUTIONS AND CHEMICALS

i) Plasmids

	Source
pT7-blue	Novagen
pBLt55	Dr. Walter
pRK5	Prof. H Will
pBL	Dr. C Trautwein
pSV	Dr. C Trautwein

ii) Tissue culture systems

HepG2 cells: human hepatoblastoma cells, originally established from a 15 years old male Caucasian. The cells produce a wide range of proteins, including factors prothrombin, antithrombin III, α-foetoprotein, complement, C3 activator and fibrinogen. Grown in Dulbecco's modified Eagles medium (DMEM), supplemented with 10% foetal calf serum, 100 IU penicillin/100 IU streptomycin and 2 mM glutamine.

HeLa cells: human cervix carcinoma cells line. Epithelium like cell line derived from human cervical tissue. Grown in the medium as above, supplemented with 5 percent foetal calf serum.

Huh7 cells human hepatoblastoma cells

COS7 cells: monkey kidney cells, Originally derived from CV-1, an African monkey kidney cell line, Transformed by an origin defective mutant of SV-40. Grown in the medium as mentioned above for HepG2 cells.

iii) Reagents and media for tissue culture

DMEM

Foetal Calf serum

Glutamine

Penicillin /Streptomycin

PBS A 170 mM NaOH, 34 mM KCl, 1 mM Na_2HPO4, 2 mM KH_2PO4, pH 7.2.

Trypsin	0.25% trypsin dissolved in Tris-saline
Versene	600 mM EDTA in PBS A, 0.0015% (w/v) phenol red.

iv) Enzymes

Restriction enzymes were purchased from Boehringer Mannheim or New England Biolabs. RNaseA (Ribonuclease A) and lysozyme were obtained from Sigma Chemicals Co Ltd. T4 polynucleotide kinase, T4 DNA ligase, Proteinase K, *Taq* polymerase used for PCR purchased from Boehringer Mannheim.

v) Commonly used chemicals

All the reagents and chemicals were purchased from BDH Chemicals, UK or from Sigma Chemical Co. unless otherwise mentioned in this section or in the methods section.

Manufacturer	Chemicals
Beecham Research	Ampicillin
Bio-Rad	TEMED, Amonium persulphate
Boehringer Mannheim	Protease inhibitor, PCR reagents, *Taq* DNA polymerase
Prolabo	Boric acid, chloroform, ethanol, glacial acetic acid, glycerol, hydrochloric acid, isopropanol, methanol

vi) Reagents and buffers for PCR, DNA sequencing and cloning

10x PCR buffer	200 mM Tris-HCl (pH 8.4), 500 mM KCl
10x agarose gel-loading buffer	1xTBE, 1% SDS, 50% glycerol 1mg/ml bromophenol blue
10x dNTPs	100 mM of each dATP, dGTP, dCTP, dTTP
10xTBE	89 mM Tris-HCl (pH 8.0), 89 mM boric acid, 1 mM EDTA
10xTAE	0.2 M Tris, 50 mM EDTA (pH 8.0), pH adjusted to 8.0 with acetic acid
10xTE	0.1 M Tris-HCl (pH 8.0), 10 mM EDTA
10x T4 kinase buffer	0.5 M Tris-HCl (pH 7.6), 0.1M $MgCL_2$, 50 mM DTT, 1 mM spermidine-HCl, 1 mM EDTA (pH 8.0)
10x ligase buffer	250 mM Tris HCl (pH 7.6), 50 mM $MgCl_2$, 5 mM DTT, 5 mM ATP, 25% PEG 8000

vii) Reagents and solutions for small scale plasmid preparation

Solution I	25 mM Tris HCl pH 8.0, 10 mM EDTA pH 8.0, 50 mM glucose.
Solution II	0.2 mM NaOH, 1% (w/v) SDS
Solution III	3 M K-acetate, 5 M acetic acid
STET buffer	50 mM Tris HCl pH 8.0, 50 mM EDTA pH 8.0, 0.5% triton X-100, 8% sucrose.

viii) Reagents and solutions for large scale plasmid preparation

Buffer P1	50 mM Tris HCl pH 8.0, 10 mM EDTA pH 8.0 100 µg/ml RNaseA.
Buffer P2	200 mM NaOH, 1% (w/v) SDS
Buffer P3	3 M K-acetate, pH 5.5
Buffer QBT	750 mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0, 0.15% triton X-100
Buffer QC	1 M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0
Buffer QF	1.25 M NaCl, 50 mM Tris HCl, 15% ethanol, pH 8.5

ix) Reagents and solutions for transfection

$CaCl_2$	0.25 M $CaCl_2$, made from 1 M stock
2x HEBS buffer	280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO4.2H_2O$, 12 mM Dextrose, 50 mM HEPES, pH 7.05
2x BBS buffer	50 mM BES, 1.5 mM Na_2PO_4 pH 6.5, 280 mM NaCl
PBS/triton X-100	PBS-A with 0.1% (v/v) triton X-100
PBS/Tween-20	PBS-A with Tween 20

x) Reagents and buffers for luciferase and β-gal assay

5x extraction buffer	125 mM Tris Phosphate pH 7.8, 10 mM EDTA, 10 mM DTT, 50% glycerol, 5% triton X-100
Measuring buffer	25 mM glycyl glycerine, 15 mM $MgSO_4$, 5 mM ATP
Luciferin	250 µM in H_2O, made from 25 mM stock

5x cell lysis buffer	125 mM Tris Phosphate pH 7.8, 10mM DTT, 50% (Promega) glycerol, 5% triton X-100
Luciferase assay reagent	20 mM tricine,1.07 mM $(MgCO_3)_4Mg(OH)_2$ $5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A, 470 μM luciferin, 530 μM ATP, pH 7.8

xi) Reagents and solutions for gel shift analysis

NPBT	10 mM Tris-Cl pH 7.4, 2 mM $MgCl_2$, 140 mM NaCl, 0.5 mM DTT (freshly added), 0.5 mM PMSF, (freshly added), and 0.1% (v/v) triton X-100 (freshly added).
NPB/sucrose	As above with 50% (w/v) sucrose but excluding triton X-100.
Dignam C	20 mM HEPES pH 7.9, 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$. 0.2 mM EDTA, 0.5 mM DTT (freshly added), 0.5 mM PMSF (freshly added).
10x annealing buffer	200 mM Tris-Cl pH 7.6, 100 mM $MgCl_2$, 500 mM NaCl
5x binding buffer	125 mM HEPES pH 7.6, 25 mM $MgCl_2$, 170 mM KCl
10x proteinase inhibitor cocktail	20 mM DTT, 2 mM PMSF
Dialysis buffer	25 mM HEPES pH 7.6, 0.1 mM EDTA, 40 mM KCl, 11.4% (v/v) glycerine

Table 2.1 Clinical data of FHBV and chronic control cases.

Name	Gender/age	Country of origin of infection	Days of illness before sample	HBsAg*	HBeAg	Anti-HBe	HBV DNA (dot-blot)	Transmission route	Clinical features
Patients									
FHBV-1	F/25	UK	7	1:64	-ve	-ve	N/D	?	Pregnant. Died
FHBV-2	F/23	UK	5	1:8	-ve	+ve	-ve	?	Survived
FHBV-3	F/17	UK	2	1;16	-ve	+ve	-ve	?	Survived
FHBV-4	F/18	UK	6	1: 64	-ve	+ve	-ve	Sexual	Survived
FHBV-5	M/19	UK	5	1:6,400	+ve	-ve	-ve	IV use	Survived
FHBV-6	F/23	UK	8	1:3,200	-ve	-ve	N/D	?	Survived
FHBV-7	F/21	Italy		N/D	-ve	-ve	N/D	?	Survived
FHBV-8	F/25	UK	7	1:64	-ve	+ve	N/D	Sexual	Died
FHBV-9	M/23	UK	14	1:3,200	+ve	-ve	+ve	Sexual	Died
FHBV-10	F/30	UK	25	1:6,400	+ve	-ve	+	?	Died
FHBV-11	F/45	Nepal	5	1:8	+ve	-ve	N/D	?	Died
FHBV-12	F/20	Spain	14	1;800	+ve	-ve	+ve	IV use	Died
FHBV-13	M/25	Switzerland		N/D	-ve	-ve	N/D		Died
FHBV-14	F/30	Pakistan	4	1:6,400	-ve	+ve	N/D	?	Pregnant. Died
FHBV-15	M/27	USA	9	N/D	-ve	+ve	-ve	Sexual	Transplanted
FHBV-16	M/30	Cameroon	19	1:6,400	+ve	-ve	+ve	Percutaneous	Died
CHBV-1	M/35	UK	N/A	1:800	-ve	+ve	N/D	N/A	Contact died
CHBV-2	M/35	Pakistan	N/A	1:800	-ve	+ve	N/D	N/A	Contact died
AHBV-1	?	UK	N/A	1:3,200	-ve	-ve	N/D		Acute, contact of case FHBV-6
AHBV-2	M/29	UK	28	1:12,800	+ve	-ve	+ve		Acute
AHBV-3	M/29	UK	28	1:6,400	+ve	-ve	+ve		Acute

M=male, F=female, N/A=not applicable, N/D= not done, +ve= positive, -ve= negative, ++= highly positive, IV= intravenous needle
FHBV= fulminant HBV, CHBV= chronic HBV, dot/blot= DNA was detected by dot-blot hybridisation, ?= not known.*= Titre indicates final dilution at which sample remained positive.

Table 2.2 Clinical data of FHBV (Genbank) and chronic control cases.

Patients	Gender/age	Country of origin	Genotype	HBeAg	Anti-HBe	Transmission route	Disease progression	Clinical features
FHB and contact cases obtained from GenBank included in phylogenetic analysis								
HBVP3CSX	F	Greece	D	-ve	N/D	Sexual	?	±Survived
HBVP2CSX	M	Greece	D	-ve	+ve	?	?	N/A
HBVP1PC	F	Greece	D	-ve	N/D	Sexual	Rapid	±Survived
HBVP2PC	M	Greece	D	-ve	+ve	Contact of HBVP1PC	@N/A	NA
HPBMUT	M	Israel	D	-ve	+ve	Heparin flash bottle	Rapid	Died
HPBETNC	Infant	Japan	C	-ve	-ve	Contact of two cases	@N/A	Died
HPBC5HKO2	F/40	Japan	C	-ve	+ve	Contact of one case	@N/A	Died
HBVP4PCXX	M	Greece	D/A	-ve	N/D	sexual	Rapid	Died
HBVP5PCXX	F	Greece	D/A	-ve	+ve	Contact of case HBVP4PCXX	@N/A	NA
HBVP4CSX	M	China	B	-ve	N/D	?	?	Survived
HPBC4HST2	M/57	Japan	C	-ve	+ve	?	Slow	Died
Chronic carrier controls included in functional analysis								
I-40	F/21	Italy	D	+ve	-ve	N/A	N/A	Chronic carrier
I-59	F/58	Italy	D	+ve	-ve	N/A	N/A	Chronic carrier
I-69	M/31	Italy	D	+ve	-ve	N/A	N/A	Chronic carrier
I-89	M/27	Italy	D	-ve	+ve	N/A	N/A	Chronic carrier
I-95	M/49	Italy	D	-ve	+ve	N/A	N/A	Chronic carrier
I-105	M/33	Italy	D	-ve	+ve	N/A	N/A	Chronic carrier
I-177	M/25	Italy	D	-ve	+ve	N/A	N/A	Chronic carrier

M=male, F=female, N/A=not applicable, N/D= not done, +ve= positive, -ve= negative, FHBV= fulminant HBV, CHBV= chronic HBV, dot/blot= DNA was detected by dot-blot hybridisation, ?= not known. @= Contact had rapid disease progression. ±= received liver transplant.

2.2 METHODS

2.2.1 SEQUENCE ANALYSES OF FHBV GENOME

i) Extraction of FHBV DNA from serum

Digestion of serum and extraction of DNA: Sera from FHBV were digested using the proteinase K method. Twenty five µl of serum were mixed with 1x SDE buffer, (25 mM NaOAc, 2.5 mM EDTA pH 8.0), 1 mg/ml proteinase K, 0.65% SDS and 10 ng tRNA (transfer RNA) and incubated at 37°C overnight. A negative serum control was used after every two of the patient's sera. DNA from proteinase K digested serum was extracted using phenol/chloroform method. An equal volume of tris-saturated phenol was added to the digested serum, vortexed, and centrifuged for 5 minutes at 13,000 rpm on a bench top microfuge. The upper aqueous layer was removed and added to equal volume of chloroform, vortexed and spun as above. The DNA was precipitated from the aqueous layer by the addition of two and a half volumes of 100% ethanol (ETOH) and one tenth volume of NaOAc (pH 5.2). DNA was kept at -70° C for a minimum of 30 mins and precipitated by spinning at full speed for 20 minutes (min), the pellet was washed twice with chilled 70% ethanol, dried under vacuum for 20 mins and finally resuspended in 12-15 µl of distilled water (dH_2O). The DNA was stored at -20°C until used.

ii) PCR amplification of extracted DNA

a) Primers: A range of primers were designed and used for PCR amplification and sequencing across the genomes (Table 2.3). Primers were synthesised in-house on an Applied Biosystems machine. Oligonucleotides were removed from the column with 1.5 ml ammonia solution, deprotected in a 55°C water bath for at least 5 hrs and dried under vacuum overnight (O/N). The oligonucleotides were dissolved in 100-200 µl dH_2O. The optical density (OD) was taken at 260 and 280 nm UV wavelength to measure the concentration. A 50 pmol/µl aliquot was made and stored at-20°C.

b) Polymerase chain reaction (PCR): DNA was amplified in two reaction rounds of the PCR in a 50 µl reaction volume. 5 µl of extracted serum DNA was mixed with 1.5 µl of dNTPs (250 µM of each), 1.5 µl of 15 mM of $MgCl_2$, 5 µl of 10X PCR buffer, 25 pmol of each sense and antisense primer and 1 unit of DNA *taq* polymerase. To avoid evaporation, approximately 30 µl of light weight mineral oil (Sigma) was layered on the reaction mix. The PCR reaction was carried out in a 0.5 ml eppendorf tube using a Biometra Trio Thermoblock (Biometra, UK). After initial denaturation at 94°C for 4 min, the first round PCR program used was as follows:

denaturation	94°C for 1 min
annealing	55°C for 1 min
extension	72°C for 1 min 30 secs
	5 cycles
denaturation	90°C for 1 min
annealing	55°C for 1 min
extension	72°C for 1 min 30 secs
	35 cycles

Followed by a further extension at 72°C for 5 min. 1 µl of first round product was nested or hemi-nested using 25 pmol of sense and antisense inner primer. The number of cycles was reduced to 25 instead of 35 in the second stage.

c) Agarose gel electrophoresis: DNA fragments generated by PCR amplification were resolved by nondenaturing agarose gel electrophoresis. Agarose gels were made using either 1xTBE (Tris, boric acid, EDTA) or 1xTAE (Tris, acetic acid, EDTA) buffer. One per cent agarose gel containing ethidium bromide (EtBr) at a final concentration of 0.3 µg/ml of gel was used to analyse PCR amplified DNA. Five µl of amplified DNA was mixed with 2 µl amount of gel loading dye and was run at 80-100 V. Appropriate size markers were always run alongside the samples. Following electrophoresis, DNA was viewed under short-wave UV light and a picture was taken using the Imager (Appligene) or a polaroid camera.

iii) Sequencing of DNA

a) Purification of PCR product:

a.i) DNA purification using Geneclean**:** DNA purification was performed using the Geneclean kit (Bio lab 101 Inc, CA, USA) either directly from the PCR product or from DNA in an agarose gel slice. A 3x volume of NaI and 5 µl of glass milk was added to a 50 µl of the PCR product (or adjusted to 50 µl with TE buffer when a lesser volume was taken) in a 1.5 ml eppendorf tube. This was vortexed and incubated at room temperature R/T for at least 5 mins with occasional vortexing. The mix was spun for 10-15 seconds at 13,000 rpm in a bench-top microfuge, supernatant was discarded and the pellet was washed 3x using New Wash buffer (provided in the kit). The pellet was dried and finally DNA was eluted in 50 µl of dH_2O by heating at 55°C for 3 mins and spinning for 1 min. When necessary, a second step elution was performed. To an agarose gel slice, 3 volume of NaI and a half of volume of TBE modifier (when TBE gel was used) was added and heated up at 55°C until the gel melted. Glass milk was added and the DNA elution procedure was performed as above.

a.ii) Phenol/ chloroform extraction: To purify DNA by phenol/chloroform extraction method, PCR product was run on 2% Nusieve gel in 1x TBE, the band was excised and the gel was melted with 100 µl TE buffer at 65°C for 10 mins. The mix was washed 3x with tris-saturated phenol and twice with chloroform. The DNA was precipitated on dry ice using two and a half volumes of ETOH and one tenth volume of NaOAc, Following a 20 min spin, pellet was washed once with ice cold 70% ETOH, air dried and diluted in appropriate amount of dH_2O.

a.iii) Dynabead separation: Streptavidin coated magnetic Dynabeads were used to purify DNA from PCR reaction when biotinylated primers were used. Commercial kit dynal (dT) (Dynal. Oslo, Norway.) was used and the method was followed according to the instruction of the manufacturer. Twenty µl (10µg/µl) M-280 Streptavidin beads were washed twice with binding and washing buffer (B and W) and reconstituted in 40 µl volume. 40 µl of PCR product was added and bound to the beads during a 15 min incubation. A magnetic separator was used to separate the unbound DNA which was removed with a pipette. The beads were washed twice with B and W buffer and 8 µl of freshly prepared 0.1M NaOH was added for 10 mins to make single stranded DNA (ssDNA). Beads containing ssDNA were washed once with 0.1M NaOH, once with B and W buffer and once with TE buffer. Finally, beads containing ssDNA were adjusted to a volume of 20 µl and stored at 4°C until sequenced.

a.iv) Purification of product using QIAGEN kits: PCR product was purified using QIAquick gel extraction kit (QIAGEN ltd). DNA was run on a 1% agarose gel, the band containing DNA was excised and 3 volumes of buffer QX1 was added. The gel was melted by dissolving at 50°C for 10 mins. The melted gel was loaded onto a QIA spin column and spun for 1 min at maximum speed. The column was washed once with buffer PE and the DNA was eluted from the column with 10 mM Tris-HCl, pH 8.5.

b) DNA Sequencing

b.i) Dideoxy method: DNA was sequenced using Sequenase Version 2.0 kit (Amersham Life Science, Buckinghamshire, England) according to the manufacturer's instruction. Six µl (1-5 µg) purified DNA was annealed with 5-10 pmol (2 µl volume) of sequencing primer and 2 µl of 5x Sequenase reaction buffer. The DNA-primer annealing reaction was performed on a thermoblock using the program 95°C for 1 min, 65°C for 2 min, 95°C for 45 sec and 37°C for 20-30 min. The annealed DNA was added to 3.5 µl labelling mix containing 2 µl of 1:8 (5x stock) dGTP labelling mix, 1µl of 0.1mM DTT and 0.5 µCi $S^{35}\gamma$ ATP. The reaction was started by the addition of 2 µl of 1:8 sequenase T7 polymerase enzyme at 2°C for 2 mins (using a thermoblock). Then 3.5 µl of radiolabelled DNA was added to each of four tubes containing dideoxy termination mixes (ddA, ddT, ddG, or ddC), which was warmed to 37°C. The termination reaction was continued at 37°C for 5-10 mins and was stopped by adding 4 µl of stop solution.

b.ii) Direct PCR product sequencing**:** A direct PCR product sequencing kit from the same manufacturer (Amersham Life Science, Buckinghamshire, England) was purchased and the methods followed according to manufacturer's instruction. Typically, 5 µl of PCR product from a single, bright band was treated with 1 µl of exonuclease and 1 µl of shrimp alkaline phosphatase at 37°C for 15 min and 80°C for 15 min using a thermoblock, to remove short oligonucleotides and primers. 5-10 pmol (in 3 µl volume) of sequencing primer was added, heated to 100°C for 3 min and cooled down rapidly by chilling on ice. The sequencing reaction was carried out as above.

b.iii) Sequencing of M13 single stranded DNA: To make M13 single stranded DNA, PCR product was first cloned in plasmid pT7-blue (Novagen, AMS Biotechnology UK Ltd, Oxon, UK) (the method is described in section 2.2.3 of this chapter) and transformed in *E.coli* NM522 cells. 2 ml of exponential culture was infected with M13 KO7 helper phage and incubated for 1 hour at 37°C. 400 µl of this culture was transferred to 10 ml L-broth containing 70 µg/ml ampicillin and incubated O/N at 37°C with shaking. 1.5 ml of the culture was spun for 5 mins and the supernatant was added to 300 µl of 2.5 M NaCl/20% PEG. The mixture was kept on ice for 20 min to precipitate the phage, centrifuged at high speed for 5 min to remove any trace of PEG and the pellet was resuspended to 50 µl of TE buffer. Phage DNA was extracted once with an equal volume of phenol/chloroform, purified using Geneclean kit and finally eluted in 20 µl of TE buffer. 7 µl phage DNA was used for sequencing. As phage DNA are single stranded, annealing was done at 65°C for two mins then followed by cooling to 37°C for 20-25 mins. Sequencing reaction was performed as described above.

c) Sequencing gel: Two glass plates of uneven size were washed and wiped twice with 100% ETOH. The smaller one was treated with Repelcote (BDH, Poole, UK) which produces a siliconised surface. A 6% polyacrylamide/7M urea gel of 0.4 mm thickness was prepared from Sequagel 6 (National Diagnostics, Atlanta, Georgia, USA) according to manufacturer's instructions. After pre-electrophoresis with 1x TBE for 20-30 min at 70W, the wells were washed and 3-4 µl of the sequencing reaction was loaded in each well in the order TCGA. Prior to loading, the sequencing reaction was denatured at 95°C for 2 min. Electrophoresis was carried out at 70W for 100 min (until the first blue dye reached the bottom of the gel). For analysis of longer sequence, the gel was run up for as long as 300 min. On completion, the gel was removed from the apparatus, the plates were separated and the plate supporting the gel immersed for 10 min in a buffer containing 10% methanol and 10% acetic acid. This fixes the DNA into the gel and prevents diffusion of the bands. The gel was transferred to 3 MM Whatman filter paper, covered with cling film, dried under vacuum and autoradiographed against Kodak XS-1 film.

d) Interpretation of sequencing data: Sequencing data were analysed using GCG package on computer. The sequences from Genbank and from study patients were

aligned separately and a consensus was made for each. Sequence variations were determined from the consensus. Unique variants (for FHBV sequences) in this study were defined as those variants which did not occur in the Genbank data set nor in non-FHB cases, but only in FHB cases. Rare variants were defined as those variants which occurred rarely (once or twice) in the Genbank data set but occurred in larger numbers in FHBV sequences.

2.2.2 PHYLOGENETIC ANALYSIS

i) Phylogenetic trees

Core and X gene sequences were aligned with the ClustalW programme originally devised by Thomson *et. al.* (1994) and phylogenetic trees were constructed using a maximum likelihood method (program fast DNAml). The maximum likelihood transition:transversion ratio (Ts/Tv) and relative rate test of evolution for the three codon position was estimated using a likelihood program (SPOT). For the 191 core gene sequences, the Ts/Tv was determined to be 1.24 with the relative rates of evolution as 0.87:0.62:1.51 at codon positions 1, 2 and 3 respectively. For the 114 X gene sequences the Ts/Tv ratio and the codon-specific relative rate test of evolution were found to be 1.28 and 1.49:1.04:0.50. In all cases, unrooted phylogenetic trees were constructed and then midpoint rooted for clarity.

ii) Relative rate test of evolution

In order to determine whether there is any difference in the rate of evolution between FHBV and non-FHBV sequences, a relative rate test was undertaken. Distance between FHBV sequence (*a*) and their nearest non-FHBV neighbour (*b*)was compared to their nearest non-FHBV outgroup sequences (*c*) using the DNADIST (for nucleotide) and PROTDIST (for amino acid) programs within the PHYLIP package (Felsenstein, 1993). Thirteen and 14 comparisons were made for the X and core gene respectively. Tests were also performed for the antigenic regions in the core genes as defined in Carman *et al* (1995a) and according to the functional subdivisions in X identified in Yuh *et al.* (1992). Analysis at the protein level was limited statistically to the complex X and core genes and the combined antigenic and non-antigenic regions in core. Relative rate tests were also performed on FHBV sequences with and without the A_{1896} pre-core stop codon variant (9 and 7 comparisons, respectively) and on a set of non-fulminant A_{1896} cases (9 comparisons). In the tests, examining the effects of the A_{1896} variants, sequences from G_{1896} chronic hepatitis B virus cases acted as sequence *b* and *c*. In all cases the Wilcoxon Signed Rank Test was used to test the significance of any differences in evolutionary rate.

Finally, variants were divided into those that altered the encoded amino acid (nonsynonymous changes) and those that did not (synonymous changes) using the

program INA (Ina, 1995) and compared between FHBV and non-FHBV cases. The relative rate of synonymous change is a useful indicator of the strength of natural selection.

iii) Analysis of clustering

A cluster is defined here as an uninterrupted phylogenetic lineage of epidemiologically unlinked FHBV sequences. We undertook an analysis to determine whether FHBV sequences form distinct clusters on the tree more than expected by chance. This was done by treating FHBV status as an additional character state, calculate the expected number of evolutionary steps, given the number of FHBV cases under a parsimony based model of evolutionary change (Carter *et al.*, 1990), as implemented in the program FMAB.

2.2.3 MOLECULAR CLONING AND GENETIC MANIPULATION OF DNA

i) Preparation of insert and vector by restriction endonuclease digestion

Prior to digestion, the insert DNA was purified using Geneclean kit or phenol/chloroform extraction method. Restriction enzyme digestion of insert and vector DNA was carried out with the buffer and incubation temperature specified by the manufacturers. The number of units of enzyme added depended on the activity of enzyme and the amount of DNA being digested. Typically, 10 units of enzyme were added per μg of DNA. To dephosphorylate the restriction endonuclease digestion reaction, 1 unit of Calf Intestinal Phosphatase (CIP) per μg of DNA was added with CIP buffer and incubated at 37°C for 30 min. Digested DNA was run on 1% agarose gel with TBE buffer, visualised under UV, and the appropriate band was excised and purified using Geneclean or phenol/chloroform extraction method.

ii) Ligation of insert DNA and vector

The digested and purified vector and insert DNA were mixed in a ratio of 3 parts inserts to 1 part vector in a total volume of 15 μl containing 1x ligation buffer (Life Technologies, Paisley, UK), and 1 unit of T4 DNA ligase and was incubated for 3-4 hr at 16°C. The ligated DNA was either used immediately in a transformation reaction or was stored at -20°C until required.

iii) Transformation of *E. coli*

a) Preparation of competent cells : 10 ml of L-broth was inoculated with 10 μl of glycerol stock of DH5-α bacteria and incubated in an orbital shaker at 37° overnight to produce a starter culture. 1.5 ml of this culture was used to inoculate 100 ml of L-broth which was grown in a shaker for approximately 2-3 hr or until the culture reached a

density giving an OD_{600} 0.2-0.3. The bacterial cells were transferred to two 50 ml Falcon tubes and spun at 3,000 rpm for 10 mins in a Sorvall RT 6000B centrifuge. Each cell pellet was resuspended in 20 ml of sterile, ice cold $CaCl_2$ (0.1M) and incubated on ice for 2 hr. The cells were then pelleted and resuspended in a total of 2 ml of $CaCl_2$ (0.1M) and incubated on ice for at least 2 hr after which time they were ready for transformation Competent cells were also stored as a 15% glycerol stock in 1 ml aliquots at -70°C.

b) Transformation: 7.5 µl or 15 µl of ligation mix was incubated on ice with 100 µl of competent DH5-α cells. After 40 min the cells were subjected to a 2 min heat shock in a 42°C water bath and immediately placed on ice for 2-5 mins. 100 µl of room temperature L-broth was added to the mix and incubated in a shaker at 37°C for 1 hr. The cells were then pelleted, resuspended in 100 µl of fresh L-broth and plated onto LB agar plates containing 100 µg /ml ampicillin. If β galactosidase selection was used, 20 µl of X-gal (20 µg/µl in dimethyl formamide) and 56 µl of IPTG (100 mM in dH_2O) were initially spread onto the agar plate, and allowed to soak in for 30 mins prior to plating out the bacteria. The plates were dried at room temperature before incubation in an inverted position at 37°C overnight.

iv) Miniprep: small scale preparation of plasmid DNA

a) Alkaline lysis method: Minipreparation of plasmid DNA was done by alkaline lysis method as described by Shambrook *et al.* (1982). A single transformed bacterial colony was transferred into 3-5 ml of L-broth containing 100 µg/ml ampicillin and incubated at 37°C shaker for 16-18 hr. 1.5 ml of each culture was transferred into a 1.5 ml eppendorf tube and spun. The cell pellet was resuspended in 100 µl of solution I. After 5 min at room temperature, 200 µl of freshly made solution II was added, gently mixed 5-6 times and incubated on ice. 150 µl of solution III was added after 5 min; the tube was inverted and vortexed for 10 second. To extract the DNA, 150 µl of phenol/chloroform was added, vortexed and spun for 5-7 mins on a bench top microfuge. The upper clear phase was transferred to a fresh 1.5 ml eppendorf and the DNA was precipitated with two volume of 100% ETOH. The pellet was washed with 70% ETOH and air dried at room temperature. Finally, DNA was resuspended in 50 µl TE containing 20 µg/ml RNaseA and incubated at 37°C for 30 mins.

b) STET prep method: 5 ml of saturated overnight bacterial culture was pelleted for 1-2 mins at 13,000 rpm. Supernatant was removed and the pellet was resuspended in residual medium. 300 µl of STET buffer containing 10 µl lysozyme (from 10 mg/ml stock)was added and boiled at 95°C for exactly 1 min. The denatured cell suspension was spun for 20 mins at 15,300 rpm at 4°C, the pellet was removed with a tooth pick and 400 µl isopropanol was added to the supernatant. After 10-15 mins, DNA was precipitated by spinning at 4°C for 15 mins at 15,300rpm. 50 µl TE containing RNaseA (20 µg/ml) was added to the air-dried pellet.

v) Restriction enzyme digestion of miniprep DNA

Digestion was carried out using the buffer and incubation temperature specified by the manufacturer. For miniprep DNA, typically 2 units of each enzyme was used per reaction containing 10 µl of miniprep DNA in a total of 20 µl volume. DNA was digested for 2-3 hr and 5 µl of digested product was run on a 1% agarose gel containing EtBr and the insert was visualised under UV. As a digestion control, plasmid without insert was also digested with each enzyme, and run on the gel along with digested miniprep and undigested plasmid DNA.

vi) Large scale preparation of plasmid DNA

The QIAGEN midiprep kit was used for large scale preparation of plasmid following the manufacturer's instruction. Either a single large colony from L-broth agar plate or 10 µl of glycerol stock was inoculated into 10 ml of L-broth (Luria broth) containing 1 µg/ml ampicillin and grown in a shaker at 37°C for 3-4 hr to produce a starter culture. One ml of this broth was transferred to 100ml of L-broth containing 100µg/ ml ampicillin in a 200 ml flask and incubated at 37°C overnight. Typically, 25-30 ml of culture was spun at 3,000 rpm for 10 mins in a Beckman RC6000 centrifuge. Cell pellet was resuspended in 4 ml of buffer P1, 4 ml of buffer P2 was added, mixed gently and incubated at R/T. After 5 min, 4 ml of buffer P3 was added, mixed gently 12-15 times and incubated on ice for 15 min. The mixture was transferred in a tube in a SS-34 rotor and spun at 15,000 rpm for 30 mins in a Sorval centrifuge (RC 5B, Du Pont). The supernatant was passed through a QIAGEN midi column which had been previously equilibrated with buffer QBT. The column was washed 2x with wash buffer QC and the DNA was eluted with 5 ml of elution buffer QF. DNA was precipitated by the addition of 0.7x volume of isopropanol, incubated at room temperature for 5 min and spun for 35 min at 11,000 rpm in an SM-24 rotor in a Sorval centrifuge. After a 70% ETOH wash, the DNA pellet was resuspended in 100 µl of TE.

Plasmid was purified in large-scale using Hybaid recovery™ plasmid midiprep kit (HYBAID, Middlesex, UK) according to manufacturer's instructions. A saturated overnight culture was spun, pellet was resuspended in 500 µl dH_2O and transferred to a 1.5 ml eppendorf. The cell suspension was centrifuged again and the pellet was resuspended in 200 µl pre-lysis solution. 400 µl of alkaline solution was added, mixed gently and incubated at room temperature for 5 mins. To the mix, 300 µl ice-cold neutralising solution was added and vortexed for 5 seconds at full speed. The suspension was incubated on ice for 5 min, spun for 5 mins and the supernatant was added to 1.2 ml of binding buffer. The DNA/binding buffer complex was settled at room temperature for 2-5 min, supernatant was discarded and the complex was transferred to a spin filter, spun

for 2-5 min and washed once with washing buffer. Finally DNA was eluted in 350 µl dH_2O or TE.

vii) Sequencing of cloned DNA

8 µl of plasmid DNA was mixed with 2 µl of 2M NaOH and incubated at room temperature for 10 min to make single stranded DNA. 7 µl of dH_2O, 3 µl of 3M NaOAc and 50 µl of 100% ethanol was added to the mix which was incubated on dry ice for at least 20 min. DNA was precipitated by spinning at 13,000 rpm for 20 min and the pellet was washed with ice cold 70% ETOH, dried and resuspended in 6-7 µl of dH_2O. The DNA was then annealed with appropriate primers and sequenced as described before (Table 2.3).

2.2.4 TRANSFECTION

i) Tissue culture

Cells were grown in a medium (80 cm^2) or large (175 cm^2) tissue culture flask until confluent. To split cells, the cells were washed with versene and then trypsinised with a solution of trypsin and versene (1:4 ratio) for 5 mins at 37°C. Cells were resuspended in 20 ml of DME (Life Technologies, Paisley, UK.) medium (DMEM) containing 10% FCS (foetal calf serum), 2 mM glutamine, 100 IU/µl penicillin and 100 IU/µl streptomycin, and triturated using a fine tip plastic pasteur pastette and seeded to new plates and flasks containing DMEM and grown in a 5% CO_2 incubator at 37°C. Flasks were seeded at 1:10 dilution and 35 mm plates were seeded at $3x10^5$ cells per plate.

ii) Transfection

Cells were transfected using calcium phosphate method when they were 50-60% confluent. Typically, for a 60 mm plate, 150 µl of 0.25M $CaCl_2$ containing 5 µg plasmid DNA was prepared and 150 µl of 2x HEBS (HEPES buffered saline) buffer was added slowly, dropwise to the DNA/ $CaCl_2$ solution to allow a $CaPO_4$ precipitate to form a $CaPO_4$ /DNA complex. The mixture was incubated at room temperature for 30 mins to 2 hr and then added dropwise across the plate. After 8-16 hr, the medium was changed and incubated at 37°C in 5% CO_2 for further 24-48 hr until the cells were harvested.

As an alternative method, 2x BBS (BES buffer saline) buffer was used instead of HEBS. After transfection, the plates were incubated in 3% CO_2 until the medium was changed at 8-16 hr when the plates were transferred to the 5% CO_2 incubator.

2.2.5 FUNCTIONAL ANALYSIS

i) Expression of core protein by immunofluorescence

a) Construction of plasmids: Core gene was expressed both with its homologous promoter and also using a heterologous promoter. To construct plasmid with its homologous promoter, an area spanning nt1549-2458 containing the negative regulatory elements (NRE), the core upstream regulatory sequence (CURS), the basal core promoter (BCP), enhancer II (EnhII) and the core gene was generated by PCR amplificaton. The primers used were C9y and C2y with *Xho 1* and *Pst 1* restriction site respectively as outer sense and antisense primers and nested with C8y and C4y as inner sense and antisense primers with the same restriction sites (Table 2.3). The DNA fragment was cloned into vector pT7-blue (Novagen, AMS biotechnology, Oxon, UK Ltd.). The *Xho-1* and *Pst-1* digested product was excised from the gel, purified and recloned into pKLt-55 vector whose own promoter had been removed.

To study the effect of core protein sequence variability on intracellular distribution without any effect from its homologous promoter, a DNA fragment of nt1818-2458, containing core gene only was generated by PCR from the clone with homologous promoter using the primers C5e and C4b with *Eco R1* and *Hind III* restriction site and cloned into vector pRK-5. This vector contains the SV40 origin of replication and CMV early promoter.

b) Immunofluorescence: Intracellular distribution of core protein was performed by standard immunofluorescence technique. 5 μg plasmid DNA was transfected onto cells on a coverslip in a 35 mm dish.. 48-72 hr after transfection, the cells were harvested. The cell monolayer was washed 3x with PBS-A and fixed with paraformaldehyde (PFA) for 10-15 min at R/T. Cells were washed again 3x with PBS-A and permeabilised for 10 min with 0.1% triton X-100 in PBS. Cells were washed again twice with PBS-A, twice with 0.05% Tween 20 in PBS-A and labelled with 30 μl of anti-core polyclonal rabbit antibody (Zymed, CA, USA) on each coverslip. The dish containing the coverslip was incubated in a moist box for 1 hr at R/T in the dark, washed twice with PBS-A, twice with PBS-tween 20 and incubated with 30 μl of FITC-labelled mouse anti-rabbit IgG for 30 min at R/T in dark. After 3 washes with PBS-A, the coverslip was dried and mounted on glass slides with 5 μl Citifluor, a glycerol/PBS solution (University of Canterbury, Kent, UK). Cells were viewed using a Nikon Microphot-SA fluorescence microscope with appropriate filters; pictures were taken using Kodak Film.

ii) Transcription analysis by luciferase assay

a) Construction of plasmid: Plasmid constructs were based on pBL vector which includes the luciferase gene. The plasmid was derived form P-bluescript vector whose own promoter site was removed (Figure 2.3). A DNA fragment of 400 nt (nt1549-1974) which includes NRE, CURS, BCP, enhancer II and approximately 100 bases from the beginning of the core gene was generated by PCR. The primers C8m and BC-3 with *BamH I* and *Pst I* resriction sites respectively were used as sense and antisense and cloned into the pBL vector upstream of the luciferase gene.

b) Luciferase assay: Luciferase assay was performed according to the method described by Wood, (1991). Typically, 10 μg of plasmid DNA was transfected into two 60 mm plates containing 50-60% confluent Huh7 or HepG2 cells by standard transfection method. To overcome the effect of variable transfection efficiency, each plate was cotransfected with 0.2 μg pSV vector containing β-galactosidase gene. 72 hr after transfection, cells were harvested for the assay. Cell lysates were made according to the method described by Wood, (1991). Cells were washed 2 times with PBS; 350 μl cell extraction buffer was added to each plate, incubated at room temperature for 10-15 min and the cell lysates were transferred to an eppendorf. Cell lysate were spun at 13,000 rpm for 2 min to precipitate the cell debris; supernatants were removed to a fresh eppendorf and used immediately for the assay or stored at-20^{o}C.

The assay was done by adding 50 μl cell lysate to 350 μl measuring buffer containing 530 μM freshly added ATP in a Starstedt tube; (two tubes were taken for each plate) shaken quickly and measured in the luminometer (Lumac, model LB-95001, Beathold, Hannover, Germany) in which 100 μl luciferin (25 μM) was injected automatically.

Alternatively, the assay was done by using the Promega luciferase kit (Promega, Madison, USA) according to manufacturer's instructions. Typically, 250 μl of lysis buffer was used to make a cell lysate and 50 μl cell lysate was mixed with 100 μl assay buffer containing 470 μM luciferin and measured in a luminometer.

β-galactosidase assay was performed using the same cell extracts. 50 μl cell extract was added to 350 μl of β-gal assay buffer containing 1mg/ml ONPG, and incubated at 37°C until pale yellow colour developed. The OD at 405 nm was measured. The luciferase value of each experiment was calculated by dividing the mean unadjusted luciferase value by the mean β-gal value and the luciferase value for each construct was the mean value of all experiments.

For each construct, both luciferase and β-galactosidase assays were performed at least 3 times; for each assay, duplicate plates were used and, for each plate, duplicate measurements were taken.

iii) Mobility shift assay

a) Preparing nuclear extracts: Nuclear extracts were made from Huh7 and HeLa cells. Cells were grown for five days in 5% CO_2 incubator at 37°C. The method followed was originally described by Dignam *et al.* (1983). Cells were washed twice with ice cold PBS, scraped with a cell scraper and taken into a 1.5 ml eppendorf tube. Cells were spun at 4,000 rpm for 5 mins on a bench top microfuge at 4° C. Two hundred µl of NEBT buffer was added to approximately 10^6 cells, mixed quickly and incubated on ice for 3-5 min to lyse the cells. The cell lysates were then layered on equal volume of NPB buffer containing 50% sucrose to separate nuclei from the cytoplasmic fraction. Nuclei were then pelleted by spinning at 13,000 rpm for 12 mins at 4°C and resuspended in 50 µl of buffer Dignam C and rotated gently for 30 mins to 1 hr at 4°C to lyse the nuclei. The lysate was then spun at 13,000 rpm for 12 min at 4°C and the supernatant containing nuclear extract was snap-frozen in liquid nitrogen and stored at -70°C until required.

b) Estimation of protein: The amount of protein in nuclear extract was estimated by Bradford assay. The Bradford (Bio Rad, Hemel Hempstead, UK) solution was diluted at a 1:5 ratio in dH_2O and into 500 µl of this solution, 2,4 and 10 µg BSA (New England Biolab, Hertfordshire, UK) and 10 µl of nuclear extract were added. OD was taken at 595 nm and the amount of protein in the nuclear extract was calculated.

c) Annealing of oligonucleotides: Complementary oligonucleotide pairs corresponding to the variant HBV sequences were made either using in-house oligo synthesiser or purchased from Oligold (Table 2.4) (Eurogentec, Seraing, Belgium). The oligonucleotides were removed from the column, deprotected and dried as described in section 2.2.1. 50 µg of each sense and antisense oligonucleotide were annealed with annealing buffer (20 mM tris pH 7.6, 10 mM $MgCl_2$, 50 mM NaCl) in 260 µl volume at 70°C for 5 mins and cooled down very slowly. Annealed oligos were radiolabelled or stored at 4°C until used.

d) Labelling of oligonucleotides: 250 ng of annealed oligonucleotide was labelled with 10 units of T4-polynucleotide kinase (New England Biolab, Hertfordshire, UK) and 40µCi $^{32}P\gamma$ ATP (in 20 µl volume) at 37°C for 30-45 mins. Radiolabelled oligonucleotides were purified from unincorporated ^{32}P using a Sephadex G-50 spin column (Pharmacia, Uppsala, Sweden). Before use, the column was washed twice with 1x TE buffer by spinning at 500g for 4 min. Labelled oligonucleotides were diluted to a volume of 70 µl, placed onto the Sephadex bed and eluted by spinning at 500g for 4 min. The radiolabelled oligonucleotides were stored at -20°C.

e) Gel-shift assay: 30,000-50,000 cpm of radiolabelled oligonucleotides were mixed with 4 µl of 5x binding buffer, 2 µl of proteinase inhibitor cocktail, 1 µg poly dIdC, and 2 µg BSA in a total volume of 10 µl and incubated on ice. The labelled oligonucleotide was then added to 10 µl dialysis buffer containing 0, 1 and 3 µg of

nuclear extract. The reaction mix was incubated at R/T for 15-20 min and shift was analysed by electrophoresis on a 6% polyacrylamide nondenaturing gel. Two µl of 20% Ficoll was added prior to loading. The gel was fixed in gel fixing buffer containing 10% methanol and 10% acetic acid and dried under vacuum. The dried gel was exposed overnight using a Kodak XS-1 film.

Table 2.3 List of primers for PCR and sequencing

Fragment	Primer	Sequence	Restriction site	sense/antisense inner/outer	Nucleotide position
Primers for PCR					
1	G1	CCGGATCCCCACCGTGAACGCCCA	*Bam* H1	sense, outer	1615-1630
	G2	CGGGATCCTGCCCAAGGTCTTACATAAG	*Bam* H1	sense, inner	1639-1658
	C2N	CCCC/AGTAAAGTTC/TCCG/CACCTT	-	antisense, outer	2475-2495
	C4	GAGAATTCTACTAACATTGAGATTCCCG	*Eco* R1	antisense, inner	2441-2460
2	19A	TATCAACACTTCCGGAA/GACT	-	sense	2319-2338
	21A	GAGAATTCCTGAGCCTGAGGGCTCCAC	*Eco* R1	antisense, outer	3082-3099
	22A	CTCCAACTGA/GTGA/GTCGGG	-	antisense, inner	2972-2990
3	23A	CGGATCCGCCTCATTTGC/TGGGTCAC	*Bam* H1	sense	2809-2826
	26	GAGAATTCGGTATTGAGAGGATTCTTG	*Eco* R1	antisense	222-240
4	S1	CCTGCTGGTGGCTCCAGTTC	-	sense, outer	56-75
	S6	CGGGATCCGAGGACTGGGGACCCTG	*Bam* H1	sense, inner	130-146
	S2	TGACATACTTTCCAATCAATA	-	antisense, outer	972-992
	S7	GAGAATTCTTAGGGTTTAAATGTATACC	*Eco* R1	antisense, inner	823-842
5	S4	GTATGTTGCCCGTTTGTCCTC	-	sense, outer	459-479
	12A	GGGCCAAGTCTGTACAA/GCATC	-	sense, inner	758-778
	14	GAGAATTCTAG/AACAAAGGACGTCCCGCG	*Eco* R1	antisense	1408-1427
6	O1	GCCTGTTTTGCTCGCAGC	-	sense	1288-1305
	O2	CACCACCCCCAACACCTC	-	antisense	1734-1752

Table 2.3 List of primers (continued)

Primers used for making plasmid constructs				
C9y	CGAGCTCGAGACCACGGGGCGCAC CTCTCTTTAC	*Xho* 1	sense, outer	1516-1539
C8y	CGAGCTCGAGGTCTGTGCCTTCTCA TCTGCC	*Xho* 1	sense, inner	1549-1571
C2y	GACCTGCAGCCCA/CGTAAAGTTTC CC/GACCTT	*Pst* 1	antisense, outer	2476-2495
C4y	GACCTGCAGCCTTATGAGTCCAAGG G/AATA	*Pst* 1	antisense, inner	2459-2479
C8m	TCGACGGATCCGTCTGTGCCTTCTC ATCTGC	*Bam* H1	sense	1549-1571
BC3	GCATTCTGCAGGAAAGAAGTCAGAA GGCAA	*Pst* 1	antisense	1956-1974
C5e	AGTCGAATTCCA/CCCTCTGCCTAA CATCTC	*EcoR* 1	sense	1828-1845
C4h	GGACAGAAGCTTCCTTATGAGTCCA AGGG/ATA	*Hind* III	antisense	2459-2479
Primers for sequencing				
BC1	GAAAGAAGTCAGAAGGCAA	-	antisense	1956-1974
Cα	CATACA/TGCACTCAGGCAAGC	-	sense	2055-2073
KC1	GCGAATCCACACTCCA/GAAAGA	-	antisense	2261-2271
S8	GAAGATGAGGCATAGCAGC	-	antisense	415-434
S3	AATGGCTCTAGTAAACTGAGCC	-	antisense	669-690
16r	CTGCGAGAGCAAAACAAGC	-	antisense	1288-1304
14r	GCGGGACGTCCTTTGTC/TTA	-	sense	1408-1427
C7	CAC/AACAGTCTTTGAAGTAT/GG	-	antisense	1702-1721
S1r	GAACTGGAGCCACCAGCAGG	-	antisense	56-75
21r	TGGAGCCCTCAGGCTCAG	-	sense	3082-3099

Table 2.3 shows the primers used for PCR, sequencing and cloning. 6 overlapping fragments of PCR products were generated to sequence full-length genome. The underlined nts denote restriction sites. sense= sense strand, antisense= antisense strand, inner= primers used in nested PCR, outer= primers used in 1st round.

Table 2.4 Sense strand oligonucleotides used for gel shift

OL-wt	1742	GGGGGAGGAGATTAGGTTAAAGGTCTTTGTATTAGGAGGCT 1783
OL-CHBV-2		----------C---A----T-A-T-A--C----------
OL-FHBV-4		----------C---A----T-A-T-A----------
OL-FHBV-14		----------A------T-G----------
OL-FHBV-5		----------C---A----T-A-T-A----------
OL-FHBV-1		----------T----------C---------
OL-FHBV-15		----------C----A----------C---------
OL-ew.1	1031	CACAATGTGGTTATCCTGCGTTAATGCCCTTGTATG 1066
OL-em.1		----------T--------T-------
OL-ew 2	1231	CGCATGCGTGGAACCTTTGTGGCTCCTCTGCCGATC 1266
OL-em.2		----------TC----------
OL-lt.wt	1783	GTAGGCATAAATTGGTCTGCGCACCAGCACCATGC
OL-lt.in		----------A--------
		AACTTTTTCACCTCTGCCTAA-TCAC 1842
		----------A----

Table 2.4 shows the oligonucleotide sequences used for gel shift analysis. Only sense strands are shown here. Nucleotide positions of 5' and 3' ends, numbered from *Eco R1* site.

CHAPTER-3

RESULTS

The aim of this study was to determine whether viral factors are associated with the development of FHB. The study was divided into three parts. The first part was to sequence FHBV DNA extracted from patients' sera. This was followed by phylogenetic analyses of FHBV sequences to determine whether they are within particular lineages. The third part involved functional analyses of some selected FHBV cases. The first section of this chapter consists of sequence analyses of FHBV genomes. In the second section, the sequence data for core and X genes alone of FHBV cases were analysed compared to 161 and 86 core and X genes respectively from non-FHB cases. These analyses were carried out in collaboration with the Wellcome Centre for the Epidemiology of Infectious Diseases at Oxford University. The results in the third section include transcription analyses, nuclear factor binding assays and core protein expression and distribution in selected cases.

3.1 SEQUENCE ANALYSES OF FHBV GENOMES

Twenty one patients were included. Sixteen were FHBV, 3 were contacts of FHBV cases and 2 had self-limited acute infection. Of these 21 patients, sequencing was performed across the full length genome for 8. These 8 comprised 5 FHBVs, 2 symptomless carrier contacts, and 1 contact with acute hepatitis who was the source of infection to her sibling (FHBV-6). For 10 patients, sequences were generated from the begining of Enh-I to the end of the core gene. For the remaining 3 patients, the genome was sequenced from the begining of Enh-II to the end of the core gene. In summary, the following sequences were generated from a total of 21 patients:

Full genome	8 patients
Enh-I/X promoter, X gene, Enh-II/BCP, core gene	10 patients
Enh-II/BCP, core gene	3 patients

This section discusses the detailed sequence analysis of the above mentioned regions for the 21 study patients and compares these with 11 published sequences from three recent studies on FHB cases (Ogata *et al.*, 1993; Hasegawa *et al.*, 1994, Sterneck *et al.*, 1996). The phylogenetic section includes analysis of variants in the X and core gene, so there is some repetition. Other studies (Yotsumoto *et al.*, 1991; Karayiannis *et al.*, 1995) have shown that FHB patients and their contacts have identical or nearly identical sequences; therefore, they are considered here as FHB cases.

i) Nucleotide variants clustered in four positions of Enh-I region

Sequencing data were available in the Enh-I and X promoter regions from 18 cases. Fourteen of them were FHB, 3 were contacts and 1 was an acute hepatitis case. Table 3.1 shows the distribution of nucleotide variation in the Enh-I and X promoter region in 17 FHB cases in this study in comparison with 11 other FHB cases from three other groups and also non-FHB cases in this study (1 case) and from Genbank (22 cases). Most of the variants in this region were found to be unique to one case and absent in the others and were distributed throughout the entire Enh-1 and X promoter region. However, a significant number of patients had variants in four nucleotide positions. $T/C_{1050}G$ was found in 10 of our 17 FHB cases, but not in the acute case studied at the same time. This variant was also seen in 2 of 11 other FHB cases but occurs only once in 22 non-FHB sequences in Genbank. $T_{1059}C$ was seen in 10 of our 17 FHB cases, 7 of 11 FHB cases reported previously and was seen in only 1 of 22 Genbank non-FHBV sequences. Clustering of variants was also seen at positions 1249 and 1250. At nt 1249, common HBV sequences contain either a G or a C. Ten of our 17 FHB cases showed T_{1249}. In addition, $T_{1250}C$ was observed in 12 of our 17 FHB cases. These substitutions were also observed in other reported FHB cases (5 and 6 of 11 cases respectively). T_{1249} was also found in 1 of 22 non-FHB cases. It is to be noted that 10 out of 12 cases C_{1250} appeared with T_{1249}; whereas in two other cases, it appeared with G_{1249} or C_{1249}. Interestingly, these substitutions are found in the basal X promoter or accessory module of Enh-I (Trujillo *et al.*, 1991) but not in the liver specific basal module where most of the nuclear factors bind. Occurrence of variation in the basal X promoter region may be significant. The second section of this chapter provides further detail.

ii) Higher number of variants in Enh-II/CP are found in the FHB cases

Sequence analysis of the Enh-II/CP complex was available for all 21 of my patients. Table 3.2 shows the distribution of variants compared to the other 11 reported FHB cases and the 22 non-FHB sequences. A specific variant that was common to all fulminant cases could not be identified. However, some of the variants occurred more commonly in FHB cases than in non-FHB cases. The most common variants found were T_{1676}, G_{1754}, A_{1757}, T_{1762}, A_{1764} and T_{1766}. Most of these variants are also observed in 22 non-FHB sequences obtained from Genbank data, but the number of occurrences are lower than in fulminant cases. T_{1676} occurs in 12 of 19 FHB cases analysed here, but is not seen in either of the two acute cases and is seen in only 1 of 22 non-fulminant Genbank sequences. Eight of 11 FHB cases from other studies also contained this variant. $T_{1754}G$ was found in 6 cases in this study and also in one case in a previously reported study, but is absent in the Genbank data.

Table 3.1.1 Variants associated with fulminant cases in Enh-I/X promoter

Nucleotide Position	T_{996}	G_{1010}	G_{1019}	G_{1023}	A_{1040}	G_{1050}	A_{1055}	C_{1059}	G_{1061}	C_{1064}	A_{1069}	T_{1093}	C_{1126}	T_{1148}	C_{1151}	A_{1157}	C_{1184}	T_{1186}	G_{1187}	G_{1213}	A_{1215}	A_{1233}	A_{1237}	T_{1249}	C_{1250}	T_{1262}	T_{1268}	C_{1290}	T_{1350}
Fulminant or contact cases (17)	3	1	1	1	2	9	2	10	2	2	1	1	1	1	1	1	2	2	3	1	3	1	1	10	12	1	1	1	1
Acute cases (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Other FHBV cases (11)	-	-	-	-	-	2	-	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5	6	-	-	-	-
Genbank non-FHBV (22)	1	-	-	-	-	1	-	1	-	-	-	-	-	-	-	-	-	-	1	-	1	-	-	1	-	-	-	-	-

Table 3.1.1 Nucleotide changes in the Enh-I/ X promoter regions. The number in the ( ) denotes the number of cases in each group. - = no variation. Enh-I= enhancer I. FHBV= fulminant HBV. CHBV= chronic HBV. AHBV= acute HBV. Variants in nt 1050 and 1059 appeared together in 8 cases; in one case (FHBV-12), G_{1050} appeared alone, whereas in cases FHBV-14 and CHBV-2, C_{1059} appeared alone. Similarly, T_{1249} and C_{1250} were seen together in 10 cases; they appeared alone in cases FHBV-3 and CHBV-2. When compared 17 fulminant cases with 22 non-fulminant Genbank cases, the P value for G_{1050}, C_{1059}, T_{1250} and C_{1250} are 0.0009, 0.0002, 0.0003 and 0.00001 respectively (deretmined by Fishcher's exact test)

Table 3.2 Variants in Core Promoter/Enhancer-II and in pre-core regions of HBV genome

	CURS	BCP																		
		Enh-II														Pre-core				
Nucleotide in consensus	C_{1653}	A_{1676}	T_{1703}	A_{1752}	T_{1753}	T_{1754}	G_{1757}	A_{1762}	G_{1764}	C_{1766}	T_{1768}	T_{1773}	T_{1790}	G_{1809}	C_{1812}	A_{1819}	T_{1821}	T_{1826}	G_{1896}	G_{1899}
FHBV1	-	T	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	**A**	A	-
FHBV2	-	T	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	A	-
FHBV3	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**C**	-	A/G	A
FHBV4	-	T	-	-	T(C)	**G**(T)	A	A(T)	G(A)	C(T)	(T)**A**	-	-	-	-	-	-	-	A	G(A)
FHBV5	-	T	-	-	T(C)	**G**(T)	A	A(T)	G(A)	C(T)	(T)**A**	-	-	-	-	-	-	-	A	G(A)
FHBV6	-	T	-	-	-	**G**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FHBV7	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FHBV8	**T**	-	-	-	T(C)	-	A	T	T(A)	C(G)	-	-	-	-	-	-	-	-	A	G(A)
FHBV9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FHBV10	-	-	-	-	-	-	-	-	-	-	-	**C**[$]	-	-	-	-	-	-	-	-
FHBV11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FHBV12	**A**	T	**C**	-	-	**G**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FHBV13	-	-	-	-	-	-	-	-	-	-	-	**C**[$]	-	-	-	**C**	-	-	-	-
FHBV14	-	T	-	-	-	-	A	-	T	G	-	-	-	-	-	**C**	-	-	A	A
FHBV15	-	T	-	**C**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-
FHBV16	-	-	**C**	-	-	-	-	-	-	-	-	-	-	**T**	**T**	-	-	-	-	-
CHBV1 (C)	-	-	-	-	-	-	-	-	A	-	-	-	**A**(T)	-	-	-	**C**	-	-	-
CHBV2 (C)	-	T	-	-	C	-	A	T	A	T	**A**	-	-	-	-	-	-	-	A	A
AHBV1 (C)	-	T	-	-	-	**G**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AHBV2	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AHBV3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Other FHBV (11)*	-	8	1	3	2[A/C]	1	7	4	5[A]	2	1	-	-	-	-	-	-	-	6	4
Genbank data (22)	-	1	1	-	1	-	2	4	4	1	-	-	-	-	-	-	-	-	1	-

Table 3.2 shows the distribution of nucleotide changes in the Enh-II/CP complex. - denote where the same sequence observed as consensus. Nucleotide in ( ) denote when changes are observed in the clone used for transcription assay but not on direct sequencing. Enh-II= enhancer II. BCP=basal core promoter. CURS= core upstream regulatory sequence. FHBV= fulminant HBV, CHBV= chronic HBV, AHBV= acute HBV. (C)= contact to fulminant cases. *= variations away from consensus in this many cases out of 11 total. $= C_{1773} in these cases are outside of genotype context. Bold nucleotides are unique to particular case or reported in FHB cases only. Underlined nucleotides were reported from both FHB and non-FHB (Genbank) cases; here they are significant because they appeared as 'motifs' or combinations.

A G to A conversion at nt 1757 (A_{1757}) was also observed in 6 of 19 FHB cases in this study and 7 of 11 cases in other studies, but seen in 2 of 22 non-FHB cases and was not in the acute cases. The association of T_{1676} and A_{1757} with FHB is a matter of little debate. In one study they are mostly found to be associated with genotype D and therefore not believed to have a major contribution in the development of FHB (Bollyky *et al.,* 1997, submitted). However another recent study by Sterneck *et al.* (1996) it was reported that T_{1676} and A_{1757} are not genotype D specific and they occurred rather infrequently in 35 non-fulminant cases in Genbank (17 and 14 percent cases respectively).

Two other frequently described variants in the BCP are T_{1762} and A_{1764}. These variants were found in 5 of 19 cases at each position in our study and also occurred in 4 of 11 and 5 of 11 fulminant cases respectively in other studies. However, they are also seen in non-fulminant cases (4/22 for each variant) showing that these are not unique to FHBV sequences. Later analyses (section 2 and 3 of this chapter) suggest that presence of these two variants is significant when they appear in combination with one or more other variants in the same region. In two of the cases in our study, these variants were observed in the form of mixed populations (FHBV-4 and 5); in two other cases $G_{1764}T$ or $G_{1764}T/A$ were observed (FHBV-8 and FHBV-14). There are two other variants, T_{1766} and A_{1768}, which were reported to be mostly associated with FHB cases, and these were also seen in my study. A variant at nt 1766 was found in 5 of 19 FHB cases. In two cases it was seen as a mixture of C/T, in one, a mixture of C/G, and in another case as a G. Similarly, T_{1768} is also found in mixed or non-mixed form in a total of 3 cases. These two variants were also seen in the other 11 reported FHB cases (2 and 1 cases respectively). A recent study (Baumert *et al.,* 1996) showed that these two variants are associated with high replication efficiency which is due to higher rate of encapsidation of pregenomic RNA, even though they are approximately 75 nt upstream of ε. In the present study, it was shown that these two variants are associated with a higher rate of transcription (section 3.3 of this chapter).

Other variants unique to a particular case were distributed throughout the entire enhancer-II and core promoter region. A T to C substitution at nt 1703, at nt 1773 and at nt 1821 were seen in 2, 3 and 2 cases respectively. A C to T or C at nt 1653, A to C at nt 1752, T to A or T at nt 1809, A to C at nt 1819 were also seen in one or two cases. Although they do not occur in high numbers, these are unique to FHB cases presented here or elsewhere.

The pre-core variant, A_{1896}, was also observed in 9 of 19 cases. This is the most common variant previously found to be associated with both severe anti-HBe positive chronic disease and fulminant hepatitis. A_{1896} was also seen in 6 of 11 other previously reported fulminant cases. Here, in 5 of 9 A_{1896}-FHB cases, the variant was the dominant population; in 4 cases it was mixed. Nonetheless, appearance of A_{1896} is strongly correlated with HBeAg negative status of the patients, all but one being HBeAg negative

on admission (Table 3.8). Another variant, A_{1899}, was also seen in 5 of 19 cases but always with A_{1896}.

Two of our cases had an insertion at nt 1838, just after the X ORF. Case CHBV-1 was a HBeAg negative, anti-HBe positive carrier, pre-core sequence contained G_{1896} and an A insertion at nt 1838 which prevented production of HBeAg. FHB-15 had an AC insertion at the same position despite the presence of A_{1896}.

Most of the variants clustered in the BCP are in the binding sites of most of the nuclear factors (Figure 1.4).

iii) X protein has aberrant occurrence of cysteine, serine and methionine residues

Table 3.3 shows the substitution of amino acids in the X protein. As X protein overlaps with the Enh-II/CP complex, some of the nucleotide changes in this region led to aa changes in the X protein. The rate of aa substitution in the X protein compared to non-FHB is described in the second section. Amino acid substitutions were observed in a total of 21 positions of the X gene in 18 of the fulminant cases in this study. Many of these are unique variants, seen in only one or two cases and not seen in any of the non-FHBV cases. However, in the X protein there is no particular variant which was common to all fulminant cases, but a significant number of cases showed aa substituted to cysteine, serine and to methionine. A substitution of Arg26Cys was observed in 9 of 18 FHB cases in this study and 5 of 11 FHB cases in other studies. A similar change was observed in 1 of 22 non-FHB Genbank cases, but not in our acute case. The presence of cysteine was also seen at aa 6 in one patient and at aa 72 in another. Cysteines are highly conserved in X protein and the presence of additional cysteine residues may play an important role in determining its tertiary structure. Similarly, Ile88Met and Ile127Met substitution occurred in 5 of 17 and at 6 of 17 FHB cases respectively in this study. Leu130Met substitution was also seen in 6 of 17 FHB cases, confirmed in other studies where this appeared in 2 of 11, 3 of 11 and 4 of 11 FHB cases respectively. In Genbank non-FHB sequences, Leu130Met was seen in only 4 of 22 cases. The impact of the presence of aberrant methionine residues in the X protein is not known but their appearance in greater numbers in FHB cases may be significant. Substitution of Pro/Leu33Ser was found in 7 of 17 FHB cases in this study and 9 of 11 FHB cases in other studies. Serine substitution was also found at aa 44 (1/17 cases), aa 136 (1/17 cases) and at aa 147 (2/17 cases) (overall in 3 FHB cases). Again the presence of higher number of serine residues may contribute to the transactivation function of the X protein or it may be genotype D specific, as most of the cases in our study belong to genotype D. Other aa substitutions were also found in the X protein, some of them unique to a particular case (Table 3.3).

Table 3.3 Amino acid variants in X- protein

aa position	F_6	C_{26}	S_{33}	S_{44}	T_{68}	C_{72}	F_{75}	M_{88}	Y_{95}	D_{126}	M_{127}	M_{130}	I_{131}	Y_{132}	S_{136}	Q_{139}	M_{141}	A_{146}	S_{147}	L_{150}	L_{151}
FHBV-1	-	+	+	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	+
FHBV-2	-	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
FHBV-3	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
FHBV-4	+	+	-	+	-	-	-	+	-	-	+	±	±	±	-	-	-	-	-	-	-
FHBV-5	-	+	+	-	+	-	-	+	-	-	+	±	±	±	-	-	-	-	-	-	-
FHBV-6	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
FHBV-7	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
FHBV-8	-	-	-	-	-	-	-	-	+	+	±	±	+	-	-	-	-	-	-	-	-
FHBV-9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FHBV-10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FHBV-12	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
FHBV-13	-	+	+	-	-	-	-	+(N)	-	-	-	-	-	-	-	-	-	-	-	-	-
FHBV-14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
FHBV-16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
CHBV-1 (C)	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-	+	-
CHBV-2 (C)	-	-	-	-	+	-	-	-	-	-	+(T)	+	+	+	-	-	-	-	-	-	-
AHBV-1 (C)	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
AHBV-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AHBV-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Other FHBV (11)	-	5	9	-	-	-	-	2	1	1	3	4	5	1	-	-	-	-	-	-	-
Genbank non-FHBV (22)	-	1	2	-	-	-	-	-	-	-	-	4	3	-	-	-	-	-	-	-	-

Table 3.3 shows the amino acid substitutions in the X gene. - not observed, + = substitution observed, ±= found in mixed form. FHBV= fulminant HBV, CHBV= chronic HBV, AHBV= acute HBV. Number in the ( ) denotes number of cases considered, (C)= contact of the FHB cases. Substitutions observed in higher numbers in FHB cases than in non-FHB cases are shaded. Aberrant methionines at aa 88 and 127 were found only in FHB cases and are shown in bold. Other aa substitutions which are unique to one or two cases in this study are not highlighted as they may be coincidental. The P values for C_{26}, S_{33}, M_{88} and M_{127} in 17 fulminant (FHBV+CHBV) cases are 0.0005, 0.01, 0.0009 and 0.003 respectivly when compared with 22 non-FHBV Genbank cases.

iv) Rate of nucleotide and amino acid substitution in pre-S/S regions is low

The S and polymerase ORFs were sequenced for eight patients in this study. Variations in nucleotide and the predicted amino acid changes in these 8 cases was compared with the data available for another 11 FHB and 22 non-FHB cases found in the GenBank. As can be seen from table 3.4, only a few nucleotide changes occurred in the pre-S regions in each case. Four cases in this study did not show any variation. Two of the 8 cases had 2 variations in each, and one had just one. Surprisingly, one contact case (CHBV-1), showed 11 variants in pre-S, all clustered between nt 92-111. As this area is not in any of the surface promoter regions, their functional impact is not known. These variations were not detected in any of the 11 FHB cases or in 22 non-FHB sequences. A similar low rate of amino acid substitution was found. Five cases did not show any substitution, one case had one substitution, and another had two. Case CHBV-1, who had 11 nucleotide changes, showed 7 amino acid substitution.

When variability in the HBsAg was compared with the other 11 FHBV and 22 non-FHB (Table 3. 5), no significant clustering pattern was seen. Most of the nt variants were unique to a particular case and some occurred twice. But two variants (T_{190} and T_{526}) occurred in three of the FHB cases and in one non-FHB case. None of the variants except T_{346} identified in this region were reported before in FHB cases. T_{346} was found in one case in this study and in 3 other FHB cases (Sterneck *et al.,* 1996). In HBsAg, the contact case CHBV-2 showed the most variation; 7 of the 19 unique variants were found in this one case. Most of these nucleotide changes did not lead to predicted amino acid changes. Amino acid variability was low with a range of 1 to 3 substitution for each case; FHBV-16 was invariant. This is consistent with other studies; the surface was less variable than any other region and no significant clustering was observed. Met125 (in the MHR) was seen in 3 of 8 FHBV cases in this study and 2 of 11 FHBV cases in the other three studies, but was also found in 1 of 22 non-FHBV cases.

Table 3.4 Nucleotide variants in the pre S regions

Nucleotide position	FHBV-3	FHBV-6	FHBV-9	FHBV-14	FHBV-16	AHBV-1	CHBV-1	CHBV-2	Other fulminants (11)	Genbank non-fulminant cases (22)
G_{2940}	+	-	-	-	-	-	-	-	-	-
A_{2969}	-	-	-	-	-	-	-	+	-	-
G_{41}	-	-	-	-	-	-	-	+	-	-
G_{70}	+	-	-	-	-	-	-	-	-	-
C_{92}	-	-	-	-	-	-	+	-	-	-
G_{94}	-	-	-	-	-	-	+	-	-	-
A_{95}	-	-	-	-	-	-	+	-	-	-
A_{96}	-	-	-	-	-	-	+	-	-	-
T_{97}	-	-	-	-	-	-	+	-	-	-
T_{99}	-	-	-	-	-	-	+	-	-	-
G_{100}	-	-	-	-	-	-	+	-	-	-
C_{101}	-	-	-	-	-	-	+	-	-	-
C_{104}	-	-	-	-	-	-	+	-	-	-
A_{107}	-	-	-	-	-	-	+	-	-	-
A_{111}	-	-	-	-	-	-	+	-	-	-
T_{129}	-	-	-	-	+	-	-	-	-	-

Table-3.4 shows the distribution of nucleotide variations in the pre-S1 and preS2 regions combined. Nucleotide changes in this region from 8 cases in this study were compared with the 11 fulminant cases from the other 3 studies (ref as mentioned in the text) as well as 22 non-fulminant cases from Genbank. FHBV= fulminant HBV, CHBV= chronic HBV, AHBV= acute HBV. - = not observed, += observed.

Table 3.5 Nucleotide variation in S gene

Nucleotide position	FHBV-3	FHBV-6	FHBV-9	FHBV-14	FHBV-16	AHBV-1	CHBV-1	CHBV-2	Other FHBV (11)	Genbank non-FHBV (22)
G_{161}	+	-	-	-	-	-	-	-	-	-
T_{190}	+	+	-	-	-	+	-	-	-	1
T_{202}	+	-	-	-	-	-	-	-	-	-
C_{235}	-	-	-	-	-	+	+	-	-	-
T_{290}	-	-	-	-	-	-	-	+	-	-
C_{298}	-	-	-	-	+	-	-	-	-	-
T_{325}	+	-	-	-	-	-	-	-	-	-
T_{346}	-	-	-	-	-	-	-	+	3	-
G_{370}	-	-	+	-	-	-	-	-	-	-
T_{390}	-	-	-	-	-	-	-	+	-	-
G_{391}	-	-	-	-	-	-	-	+	-	-
C_{461}	-	-	-	+	-	-	-	-	-	-
T_{527}	+	+	-	-	-	+	-	-	-	1
A_{663}	-	-	-	-	-	-	-	+	-	-
C_{709}	-	-	-	-	+	-	-	-	-	-
C_{742}	-	-	+	-	-	-	-	-	-	-
A_{767}	-	-	-	-	-	-	-	+	-	-
C_{770}	-	-	-	-	-	-	-	+	-	-
C_{800}	-	-	-	-	-	-	-	-	-	-

Table 3.5 shows nucleotide variation in the HBV S gene of 8 fulminant cases in this study, compared with 11 FHB cases from other 3 studies and 22 non-FHB cases from Genbank. Variants were distributed throughout the S gene. At 2 nt positions (190 and 527), variants were seen in 3 out of 8 FHB cases and only 1 case from 22 non-FHB (Genbank).

Table 3.6 Amino acid changes in preS/S regions

Amino acid position	FHBV-3	FHBV-6	FHBV-9	FHBV-14	FHBV-16	AHBV-1	CHBV-1	CHBV-2	Other fulminant cases (11)	Genbank non-fulminant cases
Pre-S regions										
I_{20}	-	-	-	-	-	-	+	-	-	-
Q_{28}	+	-	-	-	-	-	-	-	-	-
E_{121}	-	-	-	-	-	-	+	-	-	-
A_{150}	+	-	-	-	-	-	+	-	-	-
P_{154}	-	-	-	-	-	-	+	-	-	-
N_{155}	-	-	-	-	-	-	+	-	-	-
M_{156}	-	-	-	-	-	-	+	-	-	-
L_{157}	-	-	-	-	-	-	+	-	-	-
L_{158}	-	-	-	-	-	-	+	-	-	-
L_{166}	-	-	-	-	+	-	-	-	-	-
S region										
D_3	+	-	-	-	-	-	-	-	-	-
H_{16}	+	-	-	-	-	-	-	-	-	-
N_{27}	-	-	-	-	-	-	+	-	-	-
L_{41}	-	-	-	-	-	+	-	-	-	-
S_{46}	-	-	-	-	-	-	-	+	-	-
L_{79}	-	-	-	-	-	-	-	+	-	-
L_{103}	-	-	-	+	-	-	-	-	-	-
A_{113}	-	-	+	-	-	-	-	-	-	-
R_{117}	-	+	-	-	-	-	-	-	-	-
M_{125}	+	+	-	-	-	+	-	-	2	-
C_{196}	-	-	-	-	-	-	+	-	-	-
M_{205}	-	-	-	-	-	-	-	+	-	-

Table 3.6 shows the amino acid substitutions in the pre-S1, preS2 and S regions

v) Variants in polymerase protein are discrete

In the polymerase protein, variation was found to be more discrete than any other region and distributed throughout the entire region. As the P ORF overlaps the surface, Enh-I and X-protein region, some of the changes in these regions also appear in the polymerase protein. As most of the X variants described above were at the C-terminal half, they did not lead to change in the polymerase protein. Table 3.7 shows the distribution of variants in the polymerase protein in 8 of the FHBV cases.

Table 3.7 Distribution of variants in polymerase protein

	Distribution of changes
FHBV-3	S_{42} D_{175} C_{373} F_{414} S_{670}
FHBV-6	P_{176} A_{689}
FHBV-9	A_{429} Y_{553}
FHBV-14	G_{38} P_{405} P_{542}
FHBV-14	S_{459}
AHBV-1	S_{95} S_{397} A_{689}
CHBV-1	T_{49} S_{68} L_{82} Y_{117} C_{157} R_{303} S_{336} S_{340} H_{341} A_{383} H_{384} D_{466} L_{579}
CHBV-2	S_{68} L_{81} L_{526} N_{561} A_{562} K_{657} L_{668}

vi) HBe antigen status is correlated with disease progression

Twelve of our 19 FHB cases had rapid disease progression; 10 were HBeAg negative (the 2 exceptions being FHBV-6 and FHBV-11). All 10 had a higher number of variants compared to HBeAg positive cases, particularly in the Enh-II/BCP complex, X and core gene (Table 3.8). FHBV-6, negative for both HBeAg and anti-HBe, was infected from her sister (AHBV-1) by sharing a needle, AHBV-1 was HBeAg positive and anti-HBe negative. Both had G_{1896}. FHBV-11 was HBeAg positive and anti-HBe negative, had G_{1896} and is the only example of rapid disease progression despite the presence of an HBeAg-producing strain. 4 of 6 other HBeAg positive FHB cases (excluding case AHBV-1, as it was an acute contact case) had slow disease progression, were less variable in the Enh-II/CP complex, X and C gene and had a higher fatality rate (5 of 6 cases including FHBV-11). The only HBeAg positive survival was case FHBV-5 who, although HBeAg positive on admission, later seroconverted to anti-HBe, had both A_{1896} and G_{1896} and rapid disease progression. Except for FHBV-6, 3 other FHB patients were both HBeAg and anti-HBe negative; two of them died and one received a liver transplant (case-FHBV-7). This last patient had very slow disease progression and grade II-III coma when transplanted, so had no chance of natural survival. Other factors also seemed to contribute to disease severity. Three of the patients were pregnant (two died and one had a liver transplant) and two cases were coinfected with HDV, both of whom died.

Table 3.8 Distribution of variants in different regions of the genome

Patients	Distribution of variants						Clinical features				
	Enh-1/XP	*X- gene$^{\alpha}$	Enh-II/CP	Core gene$^{\alpha}$	PreS/S gene$^{\alpha}$	A_{1896}	HBeAg	Anti-HBe	Disease progression	Outcome	Comments
FHBV-1	7	2	3	2	n/d	+	-	-	R	Died	Pregnant
FHBV-2	4	5	2	3	n/d	+	-	+	R	Survived	
FHBV-3	4	3	2	4	5	±	-	+	R	Survived	
FHBV-4	5	4	8(5)	-	n/d	±	-	+	R	Survived	
FHBV-5	4	4	8(5)	1	n/d	±	+	-	R	Survived	Seroconverted
FHBV-6	5	2	2	1	2	-	-	-	R	Survived	
FHBV-7	6	1	1	-	n/d	-	-	-	S	Transplanted	Pregnant
FHBV-8	2	-	6(2)	7	n/d	±	-	+	R	Died	
FHBV-9	1	1	-	-	1	-	+	-	S	Died	
FHBV-10	1	-	1	-	n/d	-	+	-	S	Died	
FHBV-11	n/d	n/d	-	3	n/d	-	+	-	R	Died	
FHBV-12	8	2	4	1	n/d	-	+	-	S	Died	HDV coinfection
FHBV-13	4	3	-	3	n/d	-	-	-	S	Died	HDV coinfection
FHBV14	3	-	6	5	1	+	-	+	R	Died	Pregnant
FHBV-15	n/d	n/d	3	5	n/d	+	-	+	R	Transplanted	
FHBV-16	2		4	2	1	-	+	-	S	Died	
CHBV-1(C)	2	3	4	6	10	-	-	+	R**	Died	
CHBV-2(C)	7	8	7	9	3	+	-	+	R**	Died	
AHBV-1(C)	4	2	2	1	2	-	+	-	n/a	n/a	
AHBV-2	-	-	-	-	n/d	-	+	-	n/a	n/a	
AHBV3	n/d	n/d	-	4	n/d	-	+	-	n/a	n/a	

Table 3.8 shows the number and distribution of variants in the different regions of the genome. α= only non-synonymous changes were considered. *= only variants which did not overlap the Enh-II/CP complex were considered. **= their wives had rapid disease progression. AHBV= acute HBV. FHBV= fulminant HBV. CHBV= chronic HBV. (C)= contact of FHB cases. Enh= enhancer. XP= X promoter. CP= core promoter. n/d= not done. n/a= not applicable. R= rapid. S= slow. Numbers in ( ) denote number of variants which occurred in mixed form. As can be seen from the Table, anti-HBe posititve cases had a higher number of variants and rapid disease progression. 4 out of 5 HBeAg positive cases had lower number of variants with fatal outcome; 2 of which had HDV coinfection and the other was pregnant. 2 of 3 pregnant cases died, 1 received liver transplant. A_{1896} occurred (either alone or in mixed form) in HBeAg negative cases, except cases FHBV-5 which was HBeAg positive but later seroconverted to anti-HBe. Similarly, case CHBV-1 was HBeAg negative due to a nucleotide insertion, with a resulting frame shift of the pre-core region.

vii) FHB patient has a heterogeneous population compared to her acute contact

Whole genome sequences were available from the acute /fulminant family pair. There was a considerable heterogeneity between the two cases. The sequence from AHBV-1 showed a single population of subtype *ayw* whereas, although FHBV-6 contained same subtype ayw as the dominant population, she had another strain as a minority (Table 3.9). We are not certain about the subtype of this strain. This contradicts with the previous reports where source and contacts had identical sequences. This may be because, unlike other studies, the source had an acute self-limited disease.

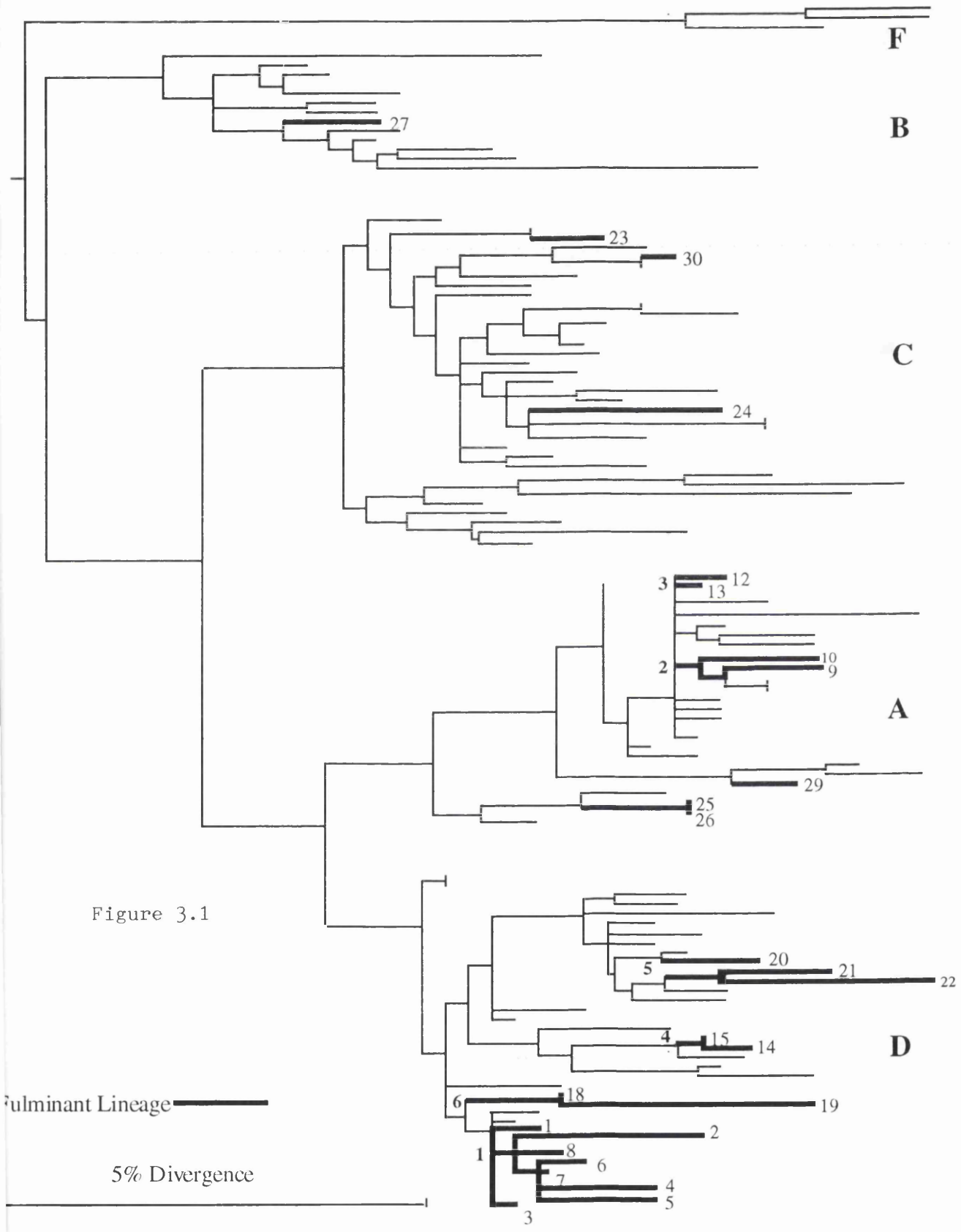
Table 3.9 Genetic heterogeneity of a pair of fulminant (FHBV-6) and acute contact (AHBV-1) cases.

nt position	FHBV-6	AHBV-1	nt position	FHBV-6	AHBV-1
S gene					
10	C	T	2200	C(A)	C
275	T	C	2212	C(T)	C
505	A	C	2215	T(A)	T
Enh-I/XP			2221	T(C)	T
926	A	C	2224	C(T)	C
1127	A	C	2239	A(T)	T
Enh-II/BCP			2242	C(T)	C
1637	C	G	2245	A	T
1768	T(C)	T	2246	T	A
Core gene			2248	A(C)	A
1956	T	A	2251	A	G
1978	A	G	2260	G(C)	G
1979	G	A	Polymerase gene		
1997	C	G	2554	T	C
2160	G(A)	G	2581	G	C
2170	C(T)	C	2826	C	T
2176	T(C)	T			
2189	T(A)	T			

Table 3.9 shows the nucleotide variability of case FHBV-6 and its acute contact. Nucleotides in the ( ) denote the minority population observed on direct sequencing.

3.2 PHYLOGENETIC ANALYSES

For the phylogenetic study, 30 FHBV core gene sequences from 27 unlinked episode were analysed, of which 11 were obtained from GenBank sequence databases. For X gene, 26 FHBV sequences representing 25 unlinked episodes were analysed, of which 9 were obtained from the sequence databases. With respect to the non-FHBV sequences, 1 X and 2 core gene sequences were from this study and 159 core gene and 86 X gene sequences were obtained from the sequence databases. This produces a total of 191 core gene and 114 X gene sequences.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26
D
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20
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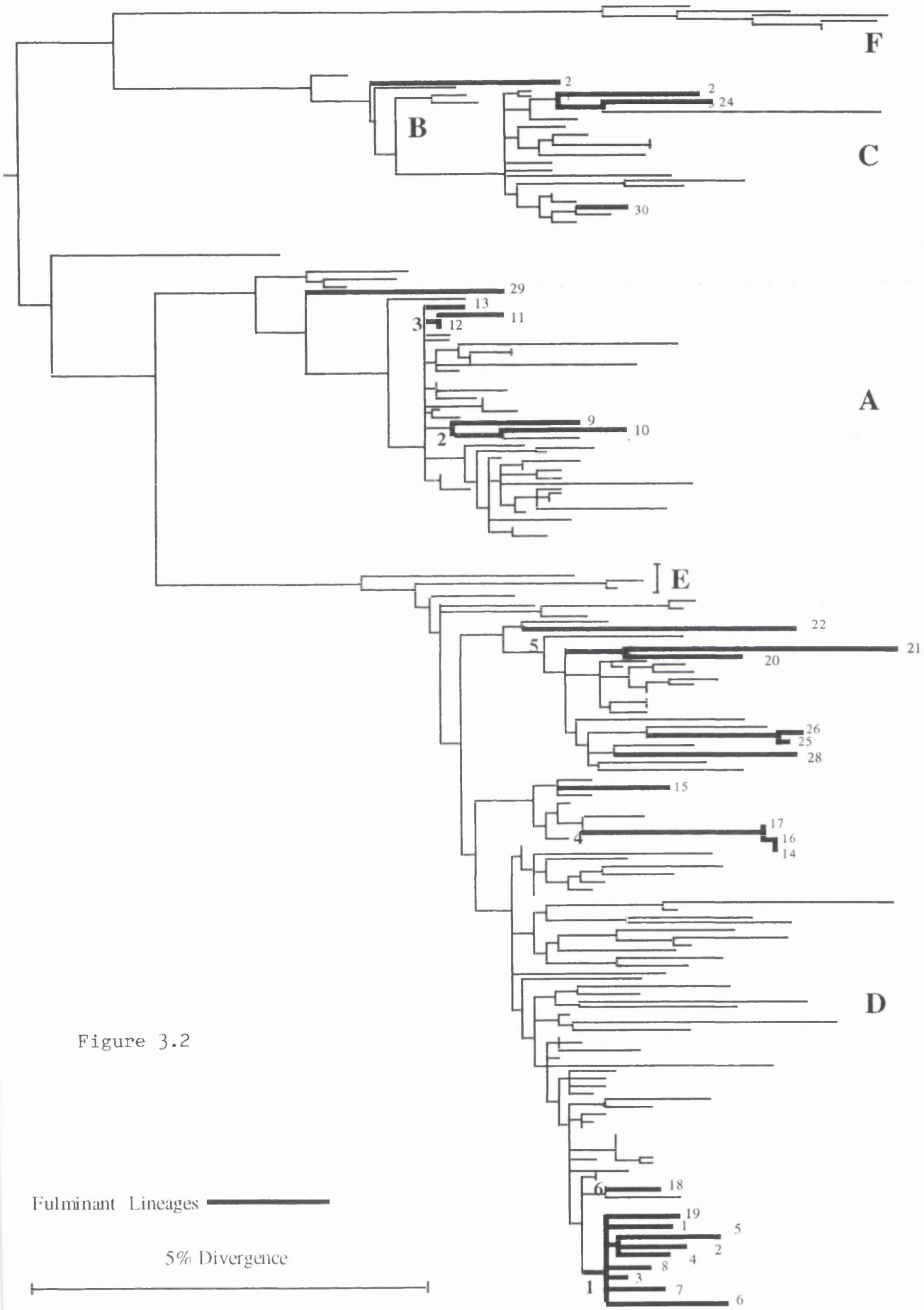
22
4
15
14
6
18
19
1
2
1
8
6
7
4
5
3
Fulminant Lineage
5% Divergence

Figure 3.1

Figure 3.1 Clustering of FHBV sequences on the maximum likelihood tree of the X gene. For the 114 X gene sequences the Ts/Tv and the codon-specific relative rates of evolution were found to be 1.28 and 1.49:1.04:0.50 at codon positions 1, 2 and 3, respectively. Lineages which possess FHBV status are indicated. Horizontal branch lengths are drawn to scale. Clusters of sequences, as defined as FHBV lineages which have no intervening non-FHBV sequences, are numbered in bold. Genotype of each major branch is given.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16
14
6
18
19
1
5
2
4
8
3
7
1
6
Fulminant Lineages
5% Divergence

Figure 3.2

Figure 3.2 Clustering of FHBV sequences on the maximum likelihood tree of the core gene. For the 191 core gene sequences the Ts/Tv was determined to be 1.24 with the relative rates of evolution as 0.87:0.62:1.51 at codon positions 1, 2 and 3, respectively. Lineages which possess FHBV status are indicated. Horizontal branch lengths are drawn to scale. Clusters of sequences, as defined as FHBV lineages which have no intervening non-FHBV sequences, are numbered in bold. Genotype of each branch is given. As most of the patients in the cluster-1 belonged to UK, to overcome the epidemiological bias, removal of all UK cases from cluster-1 altered the P value but still significant ($P= 10^{-2}$)

i) Epidemiologically unlinked FHBV cases have genetic similarity

Figures 3.1 and 3.2 which show the phylogenetic trees of X and core genes respectively; and shows that FHBV sequences are clustered. For X gene, the distribution of the 25 independent FHBV sequences on the phylogenetic tree could be accounted for most parsimoniously by 14 unambiguous evolutionary steps, fewer than the minimum of 21 expected under a random model of sequence evolution ($P= 0.1x10^{-5}$). In the core gene, the 17 observed unambiguous evolutionary steps for the 27 independent FHBV sequences was again less than the 25 expected if these sequences were not clustered ($P= 0.4 \times 10^{-7}$). This clustering reflects a high degree of genetic relatedness amongst FHBV sequences. Generally the same clusters were found in both genes indicating that the viruses themselves are related; however, sequences FHBV-19, FHBV-21 and FHBV-23 occupied different positions on the X and core gene trees. Whether these discrepancies reflect recombination events, as was clear in the case of non-clustered FHBV sequences FHBV-25 and FHBV-26, or the effect of rapid evolution associated here with FHBV sequences was unclear.

ii) Clusters were linked to clinical outcome

Clustering FHBV sequences was strongly linked to mortality. Every cluster was uniform with respect to outcome (i.e. survival or death) with two exceptions, one being a liver transplant recipient while the other was pregnant and died. All FHB cases in individuals who were pregnant, HDV coinfected, or over 30 years of age were fatal, with the exception of those patients who received liver transplants. Clustering was also uniformly linked to the speed of onset of hepatic encephalopathy and clearance of HBsAg and HBV DNA, with two exceptions. The two patients who were coinfected with HDV were found in the same cluster.

iii) FHBV sequences were characterised by a different rate of nucleotide evolution

Relative to their non-FHBV phylogenetic neighbour, the FHBV sequences had a significantly higher rate of nucleotide substitution in both the X and core genes (Tables 3.10 and 3.11 respectively). In the X gene, this increase was also significant at the amino acid level and localised to the BCP at both the nucleotide and amino acid levels, FHBV sequences also had a higher rate of synonymous changes in the X gene, indicating an elevated rate of background mutation. This relationship is also reflected in the codon position specific weighting ratio determined for the X gene phylogenetic tree, where first and second codon position changes occur at an elevated rate relative to third position changes. In the core gene, the elevated rate of evolution in FHBV sequences was significant at the nucleotide level for the whole gene and for the non-antigenic regions.

Table 3.10 Relative rate tests for the X gene comparing rates of evolution between FHBV and non-FHBV sequences.

Nucleotide Sequence*		Amino acid sequences±	
Region		Region	
Full sequence	**0.024**	Full sequence	**0.050**
1373-1631	0.906	1-86	0.255
(CURS) 1631-1742	0.678	(CURS) 87-12	0.636
(BCP) 1742-1838	**0.009**	(BCP) 124-154	**0.042**

Table 3.10 All results are expressed as P = the probability that FHBV sequences are evolving faster than non-FHBV sequences. Significant results are in bold type. *Nucleotides are numbered from the unique *EcoR1* site. ± Amino acids are numbered from the start of the X gene. CURS = Core Upstream Regulatory Sequence, BCP = Basal Core Promoter. Relative rate test results for synonymous and nonsynonymous changes were significantly different only for the number of synonymous changes in the X gene between FHBV and non-FHBV sequences ($P = 0.014$).

Table 3.11 Relative rates test for core gene

Nucleotide Sequence*

Region	FHBV	G_{1896} FHBV	A_{1896} FHBV	A_{1896} non-FHBV
Full sequence (1901-2458)	**0.045**	**0.022**	**0.009**	**0.018**
Region 1 (1963-2020)	0.154	0.787	0.076	0.715
CD4 epitope (2050-2107)	0.610	0.361	0.944	**0.036**
Anti-HBc / e1 (2122-2161)	0.919	0.281	0.141	**0.036**
Anti-HBc / e2 (2200-2251)	0.724	0.281	**0.014**	0.281
Anti-HBc / e3 (2290-2305)	0.787	1.000	**0.022**	0.181
Antigenic regions	0108	0.052	**0.009**	**0.014**
Non-antigenic regions	**0.039**	**0.043**	**0.013**	0.051

Amino Acid Sequence

Region	FHBV	G_{1896} FHBV	A_{1896} FHBV	A_{1896} non-FHBV
Full sequence	0.108	0.151	**0.013**	**0.013**
Antigenic regions	0.162	0.295	**0.009**	**0.014**
Non-antigenic regions	0.235	0.052	**0.024**	0.183

Table 3.11 shows relative rates tests for the core gene comparing rates of evolution in the complete FHBV dataset, A_{1896} FHBV variants, G_{1896} FHBV variants, and non-FHBV A_{1896} variants, all against G_{1896} non-FHBV sequences. All results are expressed as P = the probability that FHBV sequences are evolving faster than non-FHBV sequences. Significant results are in bold type. *Nucleotides are numbered from the unique *EcoR1* site. Relative rate tests for synonymous and nonsynonymous changes were not significantly different between FHBV and non-FHBV sequences in any areas tested.

iv) No particular variant was associated with clusters of sequences

There was no single unique variant linked to all individual FHBV sequences or group of sequences. However, particular variants characterised FHBV sequences and clusters of sequences (Table 3.12). On the nucleotide level, these included variants in enhancer I, (which regulate transcriptional function of X promoter and also has a stimulatory affect on BCP), the NRE and CP/Enh-II complex. At the amino acid level, these included uncharacteristic patterns of otherwise highly conserved cysteine residues, which would be expected to play a central role in determining the tertiary structure of protein products of X, and aberrant methionine residues, which might be expected to alter levels of the three protein products thought to be coded by the X gene and thus affect on transcriptional transactivation of HBV.

v) Particular 'motifs' or combinations of variants were associated with FHBV cases

The FHBV sequences possess particular motifs or combination of variants which distinguish them from non-FHBV sequences. The combination of either aberrant methionine residues, aberrant cysteine residues, or one of three identified nucleotide variants in Enh-I with any of a group of notable variants in the NRE, the BCP or the A_{1896} pre-core variant was nearly exclusive to FHBV sequences (Table 3.13). T_{1762} and A_{1764} characterised some clusters of FHBV sequences but were not by themselves disproportionately represented in FHB cases. However, where both these variants are present with either T_{1766} or A_{1768}, 3 of 4 examples were FHBV. With a single exception, whenever the A_{1896} variant was found with the aberrant cysteine and methionine residues in X protein or Enh-I variants described here, it was in a sequence associated with FHBV.

vi) A_{1896} is linked to different patterns of evolution in FHBV and chronic sequences

In G_{1896} FHBV sequences and in the complete FHBV dataset (A_{1896} plus G_{1896} FHBV sequences), changes in the complete core gene and in non-antigenic regions accumulated faster than in non-FHBV sequences. A_{1896} FHBV sequences had significantly higher rates of nucleotide evolution than in G_{1896} non-FHBV sequences in the complete core gene and in both non-antigenic and antigenic regions, particularly within the anti-HBc/e2 and anti-HBc/e3 epitopes, as well as significantly higher rates of amino acid evolution in all areas tested. These results for A_{1896} FHBV also differed from the non-FHB A_{1896} controls which did not accumulate changes at a significantly increased rate in non-antigenic regions and differed in the specific antigenic regions affected

Table 3.12 Variants suggested to play a role in FHB and their distribution in the dataset.

	Enhancer-I			NRE		$^{\$}$/Enhancer-II/BCP					Pre-core	Aberrant cysteine				Aberrant Methionine			Genotype,
Nucleotide variant	G_{1050}	T_{1249}	C_{1250}	*1633	*1634	T_{1762}	A_{1764}	T_{1766}	A_{1766}	C_{1773}	A_{1896}	*1390	T_{1449}	T_{1587}	*1605	*A_{1386}	G_{1637}	G_{1754}	Died/
Translation in X						M_{130}	I_{131}	Y_{132}				C_6	C_{26}	C_{72}	C_{78}	M_5	M_{88}	M_{127}	Survived
FHBV prevalence	10/26	10/26	14/26	10/26	13/26	6/26	7/26	4/26	2/26	4/26	16/30	2/26	11/26	1/26	1/26	3/26	9/26	5/26	
Non-FHBV prevalence	5/52	3/52	3/52	3/88	6/88	16/88	15/88	3/88	3/88	0/88	43/161	2/88	9/88	1/88	0/88	1/88	2/88	0/88	
Cluster-1																			
1. FHBV-1	+	+	+	+	-	+	-	-	-	-	+	-	+	-	-	-	+	-	D, died
2. FHBV-2	+	+	+	+	+	-	-	-	-	-	+	-	+	+	+	-	+	-	D, survived
3. FHBV-3	+	+	+	+	-	-	-	-	-	-	±	-	+	-	-	-	+	-	D, survived
4. FHBV-4	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	+	+	D, survived
5. FHBV-5	+	+	+	+	+	-	-	-	-	-	+	-	+	-	-	-	+	+	D, survived
6. FHBV-6	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	+	D, survived
7. AHBV-1	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+	+	D, survived
8. FHBV-7	+	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	+	-	D, survived
Cluster-2																			
9. FHBV-8	-	-	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	A, died
10. CHBV-1	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	A, died
Cluster-3																			
11. FHBV-9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		A, died
12. FHBV-10	-	-	-	-	-	-	-	-	-	+@	-	-	-	-	-	-	-	-	A, died
13. FHBV-11	n/a	n/a	n/a	-	-	-	-	-	-	-	-	n/a	n/a	-	-	-	-	-	A, died
Cluster-4																			
14. HBVP3CSX	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	D, survived
15. HBVP3CSX	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D,died
16. HBVP1PC	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	+	n/a	n/a	n/a	n/a	n/a	n/a	n/a	D, survived
17. HBVP2PC	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	+	n/a	n/a	n/a	n/a	n/a	n/a	n/a	D, survived

Table 3.12 (continued)

Cluster-5																			
18. FHBV-12	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	+	D, died
19. FHBV-13	+	+	+	+	+	-	-	-	-	+@	-	-	+	-	-	-	-	-	D, died
Cluster-6																			
20. FHBV-14	-	+	+	-	-	-	+(T)	+(G)	-	+	+	-	-	-	-	-	-	-	D, died
21. CHBV-2	-	-	+	-	-	+	+	+	+	+	+	-	-	-	-	+	-	-	D, died
22. HPBMUT	-	-	-	-	-	+	+	+	+	+	+	-	+	-	-	-	-	-	D, died
23. HPBC5KO2	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	+	-	-	C, died
24. HPBTNC	-	-	-	-	+	+	+	-	-	-	+		-	-	-	+	-	-	C, died
25. HBVP4PCXX	-	-	+	-	+	-	-	-	-	+@	+	-	-	-	-	-	-	-	D/A, died
26. HBVP5PCXX	-	-	+	-	+	-	-	-	-	+@	+	-	-	-	-	-	-	-	D/A, died
Individual cases																			
27. HBVP4CSX	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	B, survived
28. FHBV-15	n/a	n/a	n/a	n/a	-	-	-	-	-	-	+	-	n/a	n/a	n/a	-	n/a	n/a	D, survived
29. FHBV-16	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-		A, died
30. HPBC4HST2	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-	-		C, died

Table 3.12 Distribution of variants in Enh-I, NRE, Enh-II/BCP complex, pre-core and aberrant cysteine and methionine in X gene. += that variant observed otherwise mentioned in a parenthesis ( ). - = variant not observed. * = Variation related to FHBV at these sites involves deviations from otherwise highly conserved genotype nucleotide identity. More than one nucleotide variant therefore describes the relevant variation at this position. @ = C_{1773} in these cases are outside of genotype context. n/a = not available. BCP= basal core promoter. Enh= enhancer. NRE= negative regulatory element. $= there were a number of other variants in the Enh-II/BCP complex whose incidence was unique to a single sequence or a pair of sequences. These were A_{1779}, A_{1790}, A_{1794} (CHBV-1); T_{1810}, T_{1811} (HPBC4HKO2, HPBC4HST2); A_{1826} (FHBV-1).

Note: Some of the variants which have been shown in the table 3.2 are not included here. One reason is some of them more prevalent in a particular genotype (e.g. T_{1676} and A_{1757} are mostly seen in genotype D). The other reason is that variants which led to change in aa in X protein are not mentioned separately in this table (e.g. G_{1754} lead to change in M_{127}).

Table 3.13 Motifs or combinations of variants, suggested to play a role in FHBV and their occurrence in dataset.

	A_{1896} only				A_{1896} plus G_{1896}					
*Motif	1	2	3	total (45)	4	5	6	7	8	Total (114)
FHBV prevalence	4/13	8/13	8/13	11/13	4/26	8/26	13/26	11/26	14/26	21/26
Non-FHBV prevalence	0/32	0/32	1/32	1/32	1/88	0/88	4/88	2/88	1/88	6/88
Cluster-1	3/3	3/3	3/3		2/8	2/8	8/8	8/8	8/8	
Cluster-2	-	-	-		1/2	1/2	-	-	-	
Cluster-3	-	-	-		0/2	0/2	0/2	0/2	0/2	
Cluster-4	0/2	0/2	1/2		0/2	0/2	0/2	0/2	2/2	
Cluster-5	-	-	-		0/2	0/2	2/2	1/2	2/2	
Cluster-6	1/3	1/3	2/3		1/3	1/3	1/3	0/3	0/3	
	0/2	2/2	0/2		0/2	1/2	0/2	0/2	0/2	
	0/2	2/2	2/2		0/2	2/2	2/2	2/2	2/2	
Individual cases	0/2	0/2	0/2		0/3	0/3	0/3	0/3	0/3	

Table 3.13 shows the motifs of each clusters and the number of cases showing each motif. Only sequences for which complete information was available for the areas in question are listed. From a total of 114 sequences, 26 were FHBV (14 A_{1896} and 12 G_{1896}) and 88 were non-FHBV (31 A_{1896} and 57 G_{1896}). Cluster 3 and 3 sequences from individual cases did not show any motifs. Motifs in here defined as:

Motif 1= A_{1896} + aberrant cysteine residue in X protein.

Motif 2= A_{1896} + aberrant methionine residue in X protein.

Motif 3= A_{1896} + enhancer-I nucleotide variant.

Motif 4= BCP nucleotide variant + aberrant cysteine residue in X protein.

Motif 5= BCP nucleotide variant + aberrant methionine.

Motif 6= NRE nucleotide variant + aberrant cysteine residue in X protein.

Motif 7= NRE nucleotide variant + aberrant methionine residue in X protein.

Motif 8= NRE nucleotide variant + enhancer-I nucleotide variant.

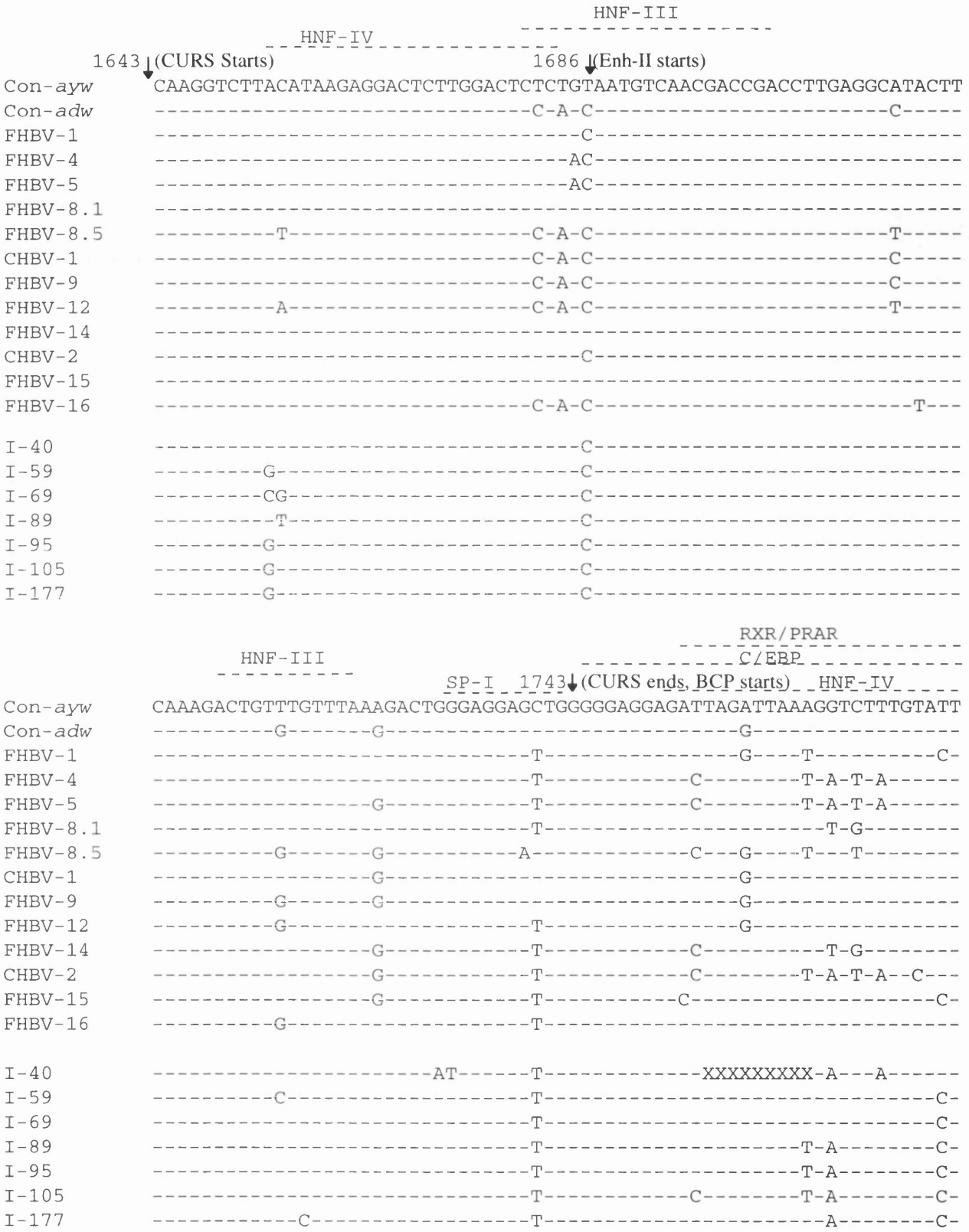
3.3 FUNCTIONAL ANALYSES

i) Fulminant hepatitis B virus constructs have a higher transcription level

A sequence greater than 400 nt, containing the NRE, CURS, BCP, Enh II and the 5' end of the core gene, from a total of 20 patients was cloned into a luciferase-expressing vector. Eleven of 20 were FHB; 4 of them were HBeAg negative in the first available samples. Two were laboratory standard *adw* (HBV *adw*2 in Genbank; genotype A) and *ayw* (Xxhepav in Genbank; genotype D) subtype controls (both HBeAg producing) and the remaining 7 were CHB controls with well known clinical characteristics. Table 3.14 shows the luciferase level correlated with variation in the *cis* acting and pre-core regions. Seven of 11 FHB cases, all with variation in the first two AT rich regions of the BCP (Figure 3.3) showed substantially higher luciferase activity compared to control *adw* which we considered as background or normal. Six of these 7 cases also had A_{1896} and one with G_{1896} sequence had an A insertion at 1838 and was therefore an HBeAg negative strain. Two of 11 cases (FHBV-8 and FHBV-9) had an intermediate level of luciferase level compared to control *adw*. For case FHBV-8, 2 clones were tested, one with A_{1896} and the other with G_{1896}, but both had variant BCP regions. Both clones showed the same level of luciferase activity, but this was not consistent (higher standard deviation). Case FHBV-9 was G_{1896} and invariant in the BCP, but had a single variant in the CURS. The last 2 of the 11 FHB cases (FHBV-12 and FHBV-16) had normal luciferase activity and contained G_{1896} with almost identical variants in the CURS and BCP. Only one of these variants was in the third AT rich region and both sequences were invariant in the first two AT rich regions. Six of 7 control CHB cases showed normal luciferase production comparable to *adw* control. Three of 7 CHB controls had HBeAg-producing sequences (G_{1896}); the remainder were A_{1896}.

Turning to specific variants, of those in the BCP, T_{1762} (in three) and A_{1764} (in five) were the most common in CHB (5 of 7 cases). These variants were also common in FHB cases that were associated with high luciferase expression, but were always accompanied by T_{1766} and/or A_{1768}. This indicates that T_{1762} and A_{1764} alone are not sufficient to affect transcription in our system. However, T_{1762} (without A_{1764}) with one other unique variant (A_{1826}) appeared to be crucial in case FHBV-1. The only CHB control case which had intermediate luciferase activity (I-40) was HBeAg positive and contained a deletion in the BCP from nt1754-1762 (Figure 3.3). The functional effect of combinations of non-unique variants was further seen in the linkage of variants at nts 1727 and 1740. G_{1727} and T_{1740} together were observed 4 times, but only in the group of 7 high luciferase-producing FHB sequences. The control *ayw* subtype (con-*ayw*) had higher luciferase activity compared to *adw* and had considerable differences in the BCP/enhancer II complex, particularly at positions 1678, 1727, 1740 and 1773 (Figure 3.3).

Figure 3.3


HNF-III
HNF-IV
1643 (CURS Starts)
1686 (Enh-II starts)
Con-ayw CAAGGTCTTACATAAGAGGACTCTTGGACTCTCTGTAATGTCAACGACCGACCTTGAGGCATACTT
Con-adw
FHBV-1
FHBV-4
FHBV-5
FHBV-8.1
FHBV-8.5
CHBV-1
FHBV-9
FHBV-12
FHBV-14
CHBV-2
FHBV-15
FHBV-16
I-40
I-59
I-69
I-89
I-95
I-105
I-177
RXR/PRAR
HNF-III
C/EBP
SP-I
1743 (CURS ends, BCP starts)
HNF-IV
Con-ayw CAAAGACTGTTTGTTTAAAGACTGGGAGGAGCTGGGGGAGGAGATTAGATTAAAGGTCTTTGTATT
Con-adw
FHBV-1
FHBV-4
FHBV-5
FHBV-8.1
FHBV-8.5
CHBV-1
FHBV-9
FHBV-12
FHBV-14
CHBV-2
FHBV-15
FHBV-16
I-40
I-59
I-69
I-89
I-95
I-105
I-177

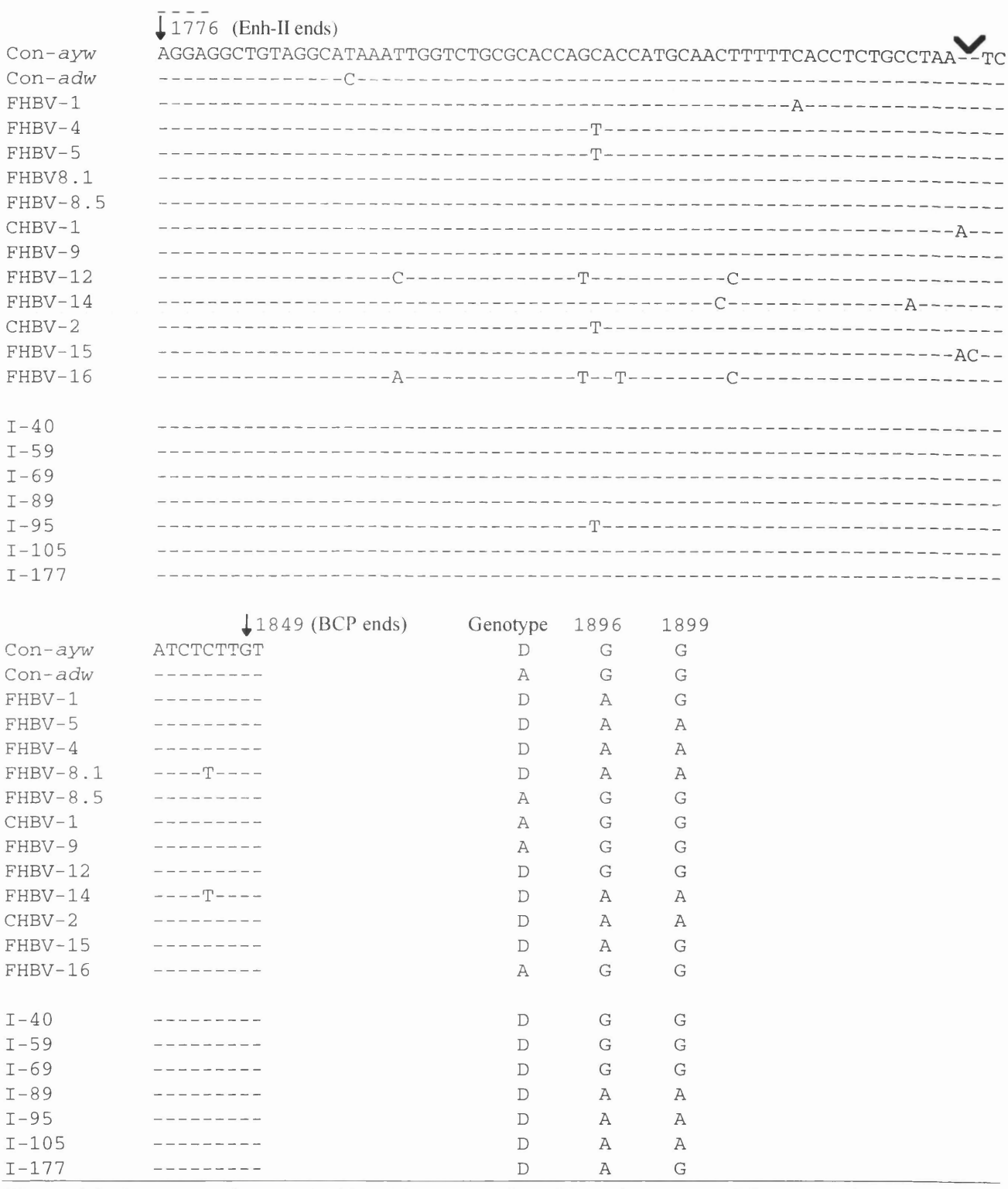


Figure 3.3: Sequences from nt 1549 to 1974 were cloned into the vector pBL. Here, the sequence of CURS, BCP and Enh-II only is shown along with the major nuclear factors binding sites. CHBV-1 and -2 are contacts of FHB cases. All I- numbers denote chronic carrier controls. BCP=basal core promoter. CURS=core upstream regulatory sequence. = insertion. X= deletion

Table 3.14 Luciferase value correlated with variation in the CURS, BCP and pre-core region.

Phylogenetic Cluster	Patients and controls	Luciferase (x 10^6)	Standard deviation ±	Precore	Variants in the CURS/ BCP	Disease progression
1	FHBV-1	11.51 (H)	3.57	A_{1896}	T_{1762} A_{1826}	Rapid
	FHBV-4	8.81 (H)	1.77	A_{1896} A_{1899}	C_{1753} T_{1762} A_{1764} T_{1766} A_{1768} T_{1810}	Rapid
	FHBV-5	10.25 (H)	2.29	A_{1896} A_{1899}	C_{1753} T_{1762} A_{1764} T_{1766} A_{1768} T_{1810}	Rapid
2	FHBV-8.1	2.76 (I)	1.64	A_{1896} A_{1899}	T_{1678} T_{1764} G_{1766} T_{1845}	Slow
	FHBV-8.5	3.65 (I)	1.16	G_{1896}	C_{1740} C_{1753} T_{1762}	Slow
	CHBV-1	10.70 (H)	0.82	G_{1896}	C_{1740} $_{1838}$ A_{1839}	Rapid
3	FHBV-9	4.06 (I)	0.58	G_{1896}	C_{1740}	Slow
5	FHBV-12	0.60 (N)	0.30	G_{1896}	T_{1703} C_{1794} T_{1809} T_{1812} C_{1821}	Slow
6	FHBV-14	11.39 (H)	4.71	A_{1896} A_{1899}	T_{1678} T_{1764} G_{1766} A_{1834} T_{1845}	Rapid
	CHBV-2	9.68 (H)	2.46	A_{1896} A_{1899}	C_{1753} T_{1762} A_{1764} T_{1766} A_{1768} C_{1771} T_{1810}	Rapid
Individual cases	FHBV-15	7.98 (H)	1.12	A_{1896}	T_{1678} C_{1752} $_{1838}AC_{1839}$	Rapid
	FHBV-16	1.13 (N)	0.22	G_{1896}	T_{1703} A_{1794} T_{1809} T_{1812} C_{1821}	Slow
Controls	I-40	4.18 (I)	1.61	G_{1896}	Δ1754-1762 A_{1732} T_{1733} A_{1764} A_{1768}	N/A
	I-59	0.77 (N)	0.36	G_{1896}	C_{1719}	N/A
	I-69	0.55 (N)	0.29	G_{1896}	-	N/A
	I-89	0.78 (N)	0.30	A_{1896} A_{1899}	T_{1762} A_{1764}	N/A
	I-95	0.56 (N)	0.28	A_{1896} A_{1899}	C_{1719} T_{1762} A_{1764} T_{1811}	N/A
	I-105	1.25 (N)	0.79	A_{1896} A_{1899}	C_{1753} T_{1762} A_{1764}	N/A
	I-177	0.62 (N)	0.39	A_{1896}	C_{1721} A_{1764}	N/A
	adw	1.5 (N)	0.84	G_{1896}	C_{1740}	N/A
	ayw	4.21 (I)	1.74	G_{1896}	T_{1678} C_{1740}	N/A

Table 3.14 Variants found in the region between 1549-1974, which contains the CURS and the Enh-II/CP complex, are considered. The clustering pattern and patient number follows according to table 3.2.1. CURS=core upstream regulatory sequence, BCP= basal core promoter. Enh-II= Enhancer II. CHBV-1 and -2 are infective contacts of fulminant cases. H= High (luciferase value>7), I= Intermediate (luciferase value 2.0-7.0), N/A= Not applicable, N= Normal (luciferase value <2), Δ= deletion. $_{1838}A_{1839}$ and $_{1838}AC_{1839}$ denote an insertion of A or AC between the noted nucleotide positions. The standard deviation is derived from at least 6 replicates for each construct. Rapid/ slow disease progression was a clinical judgement; allocations to categories were made before these studies were undertaken. (There are some discrepancies about the variants shown in this table and table 3.2, the reason for this is that some of the variants which are rare but not unique [e.g. C_{1740}, T_{1678} etc.] are included here; as in combination with unique variants, they had functional effect).

Con-*ayw* had A_{1727} and C_{1740}; a number of FHB and control CHB cases also contained A_{1727}, but always with T_{1740}. This is a remarkable parallel with our finding that 4 FHB cases had G_{1727} and T_{1740} . T_{1773} was found in 8/11 of the FHB cases with high or intermediate luciferase activity. Three FHB cases had T_{1678}; 2 of them had high and 1 had intermediate luciferase activity. Con-*ayw*, the control with intermediate activity was the only control sequence to have T_{1678}. In summary, although the BCP is generally variable, some unique variants, but more importantly, combinations of variants ('motifs'), were associated with high or intermediate transcriptional activity, nearly always in FHB cases.

ii) Luciferase activity correlates with disease progression

There was a correlation between high luciferase expression, rapidity of disease progression, and seroconversion to anti-HBe. All 7 FHB cases with high luciferase expressing sequences had rapid disease progression regardless of their eventual clinical outcome. In contrast, FHB cases with intermediate or normal luciferase expression had slow disease progression. There was a correlation between high luciferase expression and HBeAg status in FHB cases. Six of 7 FHB cases with high luciferase expression were HBeAg negative on admission and seroconverted to anti-HBe; the other 4 FHB cases (with intermediate or normal luciferase expression) were HBeAg positive on admission and 1 of them seroconverted to anti-HBe. In contrast, 4 of 7 HBeAg negative CHB cases had normal luciferase expression (and 3 had an intermediate level).

iii) BCP and enhancer I sequences from FHB have different nuclear factor binding patterns to non-FHB controls

To investigate the effect of BCP variation on binding of transcription factors derived from nuclear extracts, pairs of complementary oligonucleotides spanning nts 1742-1783 from 6 FHB cases with variability in the BCP and from *adw* subtype (as a non-FHB control) were synthesised. As can be seen from Figure 3.3, the chosen oligonucleotides included most of the variants previously identified as linked to FHB. Figures 3.4s give the results of nuclear factor binding and figure 3.6 compares the sequence to cartoons of the banding patterns. The non-FHB sequence (OL-*adw*) shows 3 clear complexes (Figure 3.4a), whereas all FHB sequences except one (FHBV-1), which had only one mutation (T_{1762}), bound poorly, or not at all, with complexes II and III. Case FHBV-1 (Figure 3.4c) which showed a similar binding pattern compared to non-FHB control (OL-*adw*), had two bands in complex-II. In order to identify liver specific complexes, nuclear extracts from HeLa cells were made, and the binding assay repeated using oligonucleotides from 3 FHB and 1 control non-FHB cases. From figure 3.5b, it is clear that complexes II and III are not hepatocyte specific.

Figure 3.4

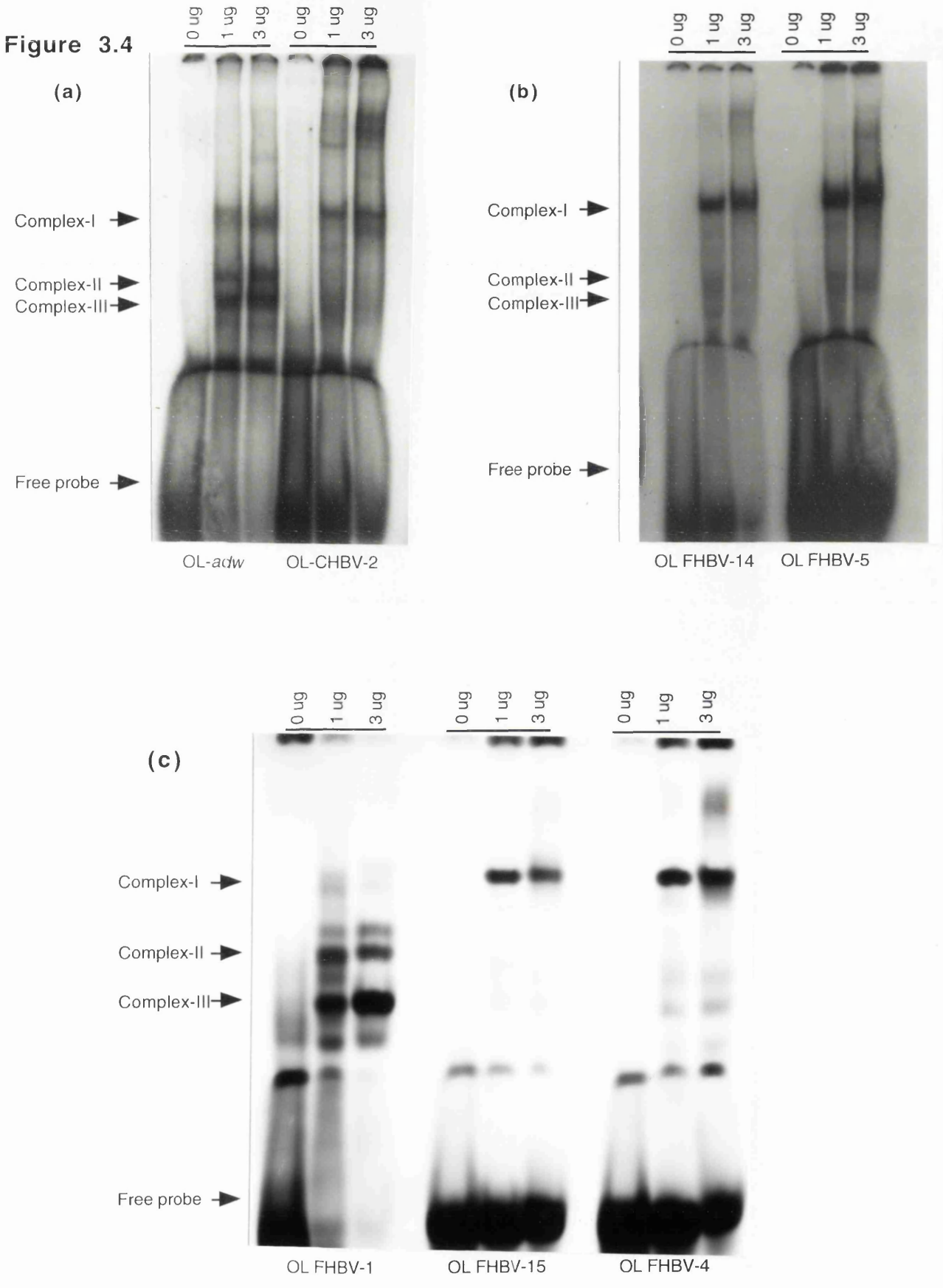

(a)
0 ug
1 ug
3 ug
0 ug
1 ug
3 ug
Complex-I
Complex-II
Complex-III
Free probe
OL-adw
OL-CHBV-2
(b)
0 ug
1 ug
3 ug
0 ug
1 ug
3 ug
Complex-I
Complex-II
Complex-III
Free probe
OL FHBV-14
OL FHBV-5
(c)
0 ug
1 ug
3 ug
0 ug
1 ug
3 ug
0 ug
1 ug
3 ug
Complex-I
Complex-II
Complex-III
Free probe
OL FHBV-1
OL FHBV-15
OL FHBV-4

Figure 3
(continued)

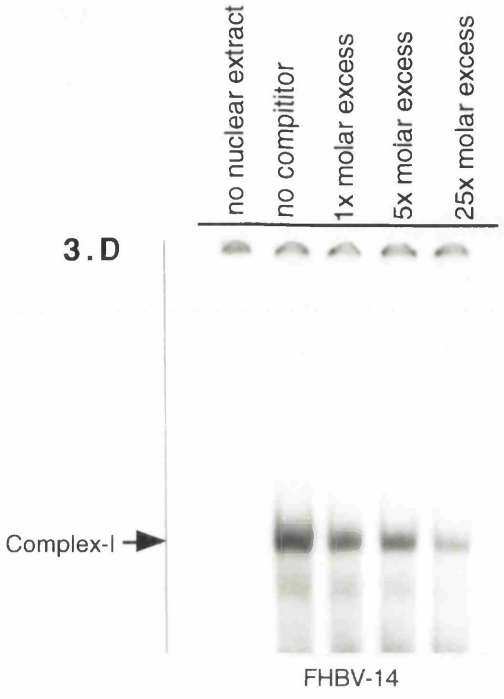


Figure 3 :Nuclear factor binding pattern of 'normal' and variant oligonucleotides. Nuclear extracts were prepared from HuH7 cells. 0, 1 and 3 ug of nuclear extracts were mixed with 30,000 cpm ^{32}p labelled oligonucleotides (oligos) and run on 6% polyacrylamide non-denaturing gel. Figure 3. A shows nuclear factors binding pattern of oligos from *adw* and CHBV-2. Figure 3.B and 3.C show nuclear factor binding pattern of 5 more variant oligos. In figure 3. D , 30,000 cpm ^{32}p labelled oligonucleotide from FHBV-14 was mixed with 1x, 5x and 25x molar excess of unlabelled oligonucleotide as cold competitor (lanes 3,4 and 5), added to 3 ug nuclear extract and resolved on 6% polyacrylamide gel. lane 1=no nuclear extract, lane 2= no cold competitor.

Figure 3.5

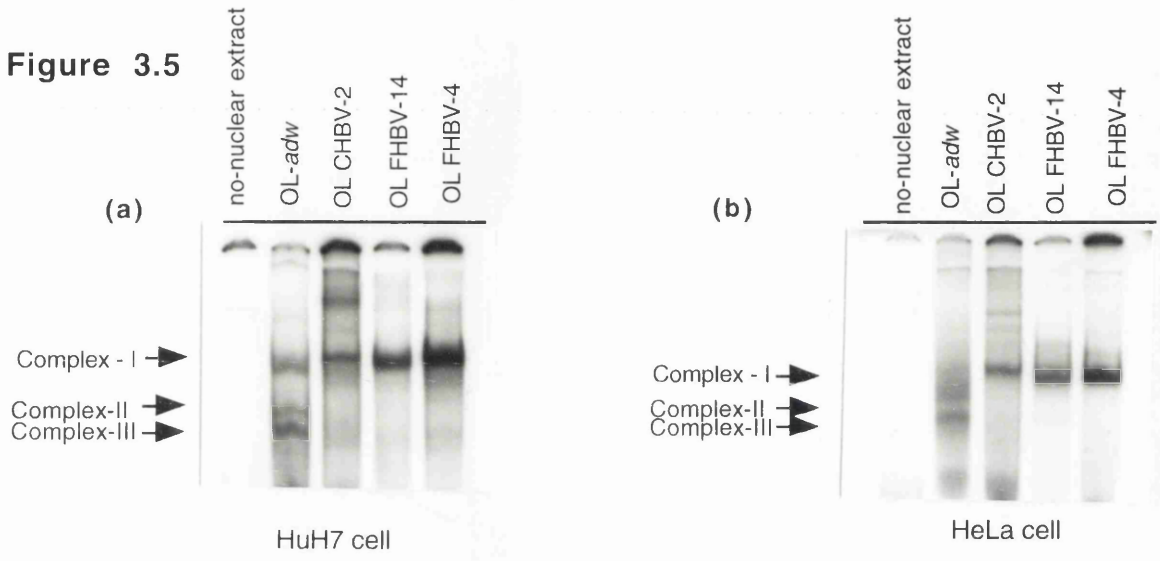


Figure 3.5: Shows the nuclear factor binding pattern of HuH7 and HeLa cells. Nuclear extracts were made from HuH7 cells and HeLa cells. 0, 1 and 3 ug of nuclear extracts were mixed with 30,000 cpm ^{32}P labelled oligonucleotide and resolved on 6% polyacrylamide non denaturing gel. Figure 3.5(a) shows binding of oligonucleotide derived from con-*adw* (OL-*adw*), CHBV-2,FHBV-14 and FHBV-4 with nuclear extracts from HuH7 . Figure 3.5 (b) shows same oligonucleotides binding with nuclear extract made from HeLa cells.

Cases	Luciferase value x10^6	Variations in the BCP
Con-*adw*	1.50	
FHBV-1	11.51	T_{1762} A_{1826}
FHBV-4	8.81	C_{1753} A_{1757} T_{1762} A_{1764}, T_{1766} A_{1768} T_{1810}
FHBV-5	10.25	C_{1753} A_{1757} T_{1762} A_{1764}, T_{1766} A_{1768} T_{1810}
FHBV-14	11.39	A_{1757} T_{1764} G_{1766} A_{1834} T_{1845}
CHBV-2	9.68	C_{1753} A_{1757} T_{1762} A_{1764} A_{1768} C_{1771} T_{1810}
FHBV-15	7.98	T_{1678} C_{1752} A_{1757}

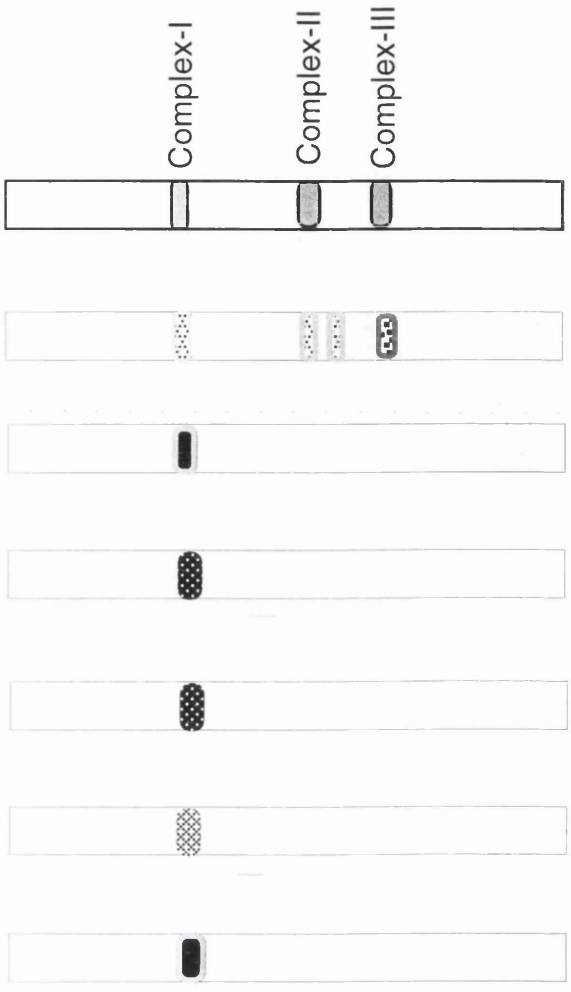


Figure 3.6 Cartoon representation of nuclear factor binding assay showing luciferase values and variants in the BCP

Figure 3.7

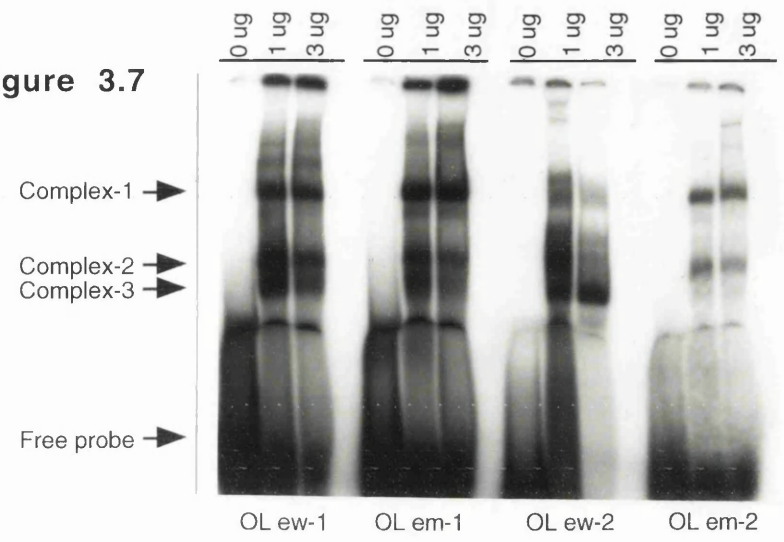


Figure 3.7: Oligonucleotides were made from normal C_{1050} (OL ew-1), variant G_{1050} (OL em-1), normal C_{1249}, T_{1250} (OL ew-2) and variant T_{1249}, C_{1250} (OL em-2). Oligonucleotides were mixed with 0,1 and 3 ug of nuclear extracts from HuH7 cells and run on 6% polyacrylamide non-denaturing gel.

The BCP variant oligonucleotides showed a similar pattern of binding to both nuclear extracts (Figure 3.5b, lane 3, 4 and 5). A competitive experiment using unlabelled oligonucleotides as a cold competitor showed the specificity of the interactions (Figure 3.4d).

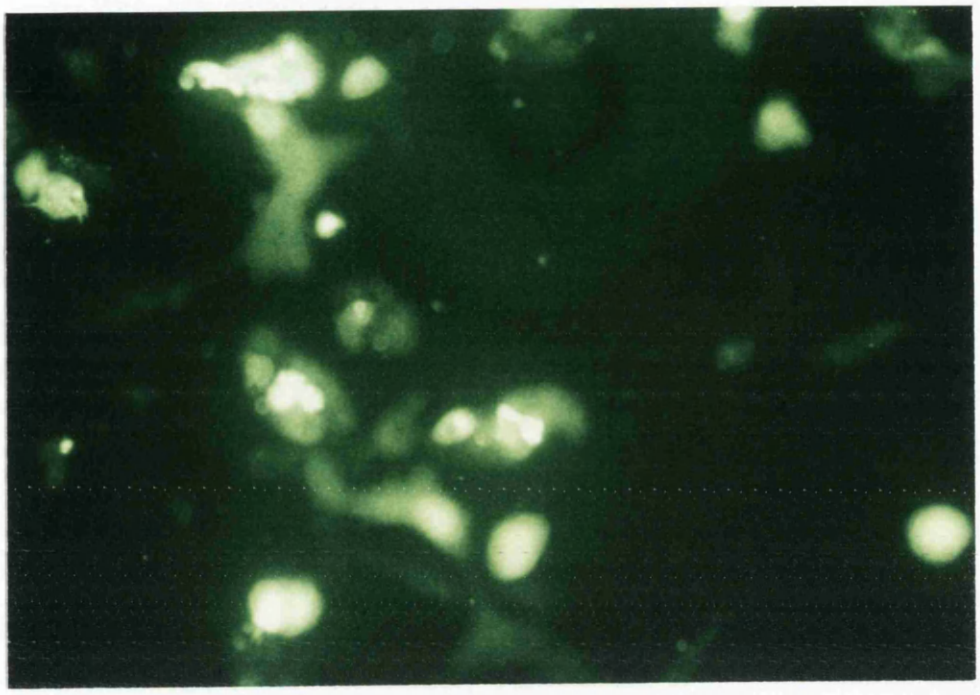
A number of FHB sequences had variants in Enh-I, particularly $T_{1050}C$, $G_{1249}C$ and T_{1250}. Four pairs of oligonucleotides with or without Enh-I variants were synthesised (Table 2.4) and bound to nuclear extracts of HuH7 cells. Figure 3.7 shows that variant G_{1050} (oligonucleotide em-1) does not have any effect on the nuclear factor binding pattern, but variants T_{1249} and C_{1250} (oligonucleotide em-2), in contrast to G_{1249} and T_{1250} (oligonucleotide ew-2), do not give rise to complex 3.

Finally, a nuclear factor binding assay was performed using an oligonucleotide with an insertion of A at 1838 as detected in two patients (CHBV-1 and FHBV-15). No difference was observed in the binding pattern (data not shown).

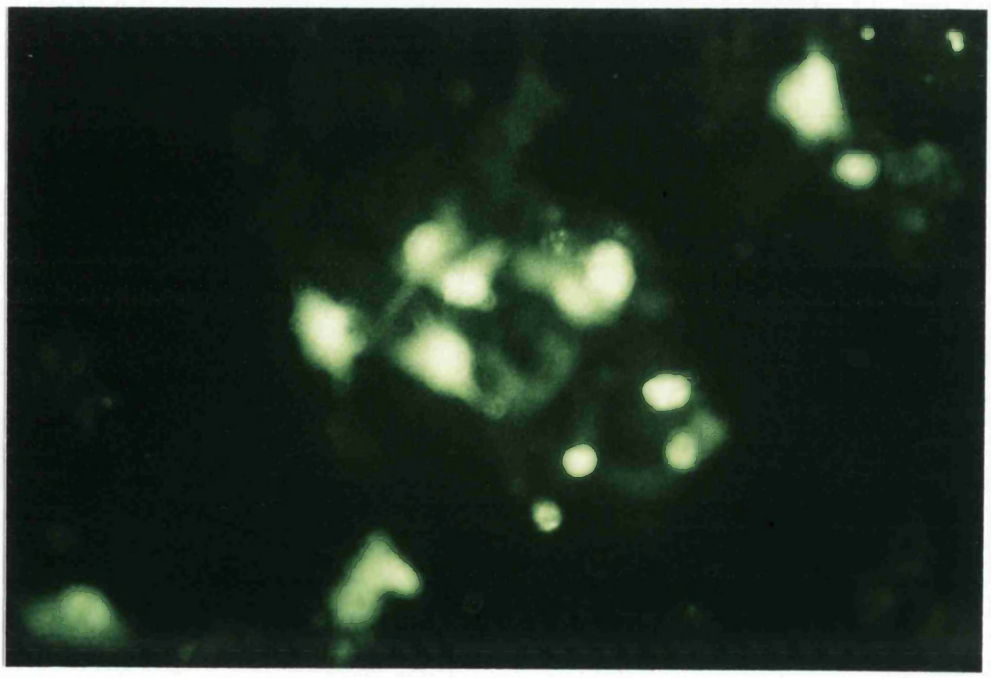
iv) Expression and distribution of core protein was not different in FHBV.

To investigate the pattern of core protein and its intracellular distribution under the control of the homologous promoter, an area spanning nt 1549-2458 from 13 patients (8 FHB or contacts of FHB, 4 CHB and 1 AHB) was cloned into vector pKLt55. Core proteins were expressed in HepG2 cells and labelled with polyclonal anti-core rabbit IgG. The influence of core protein sequence on intracellular distribution was addressed using core nt 1818-2458 from 11 FHB, 4 CHB and 1 AHB cases in vector pRK5 under control of the CMV early promoter. The core proteins were expressed in both COS7 and HepG2 cells. HBcAg distribution was observed in both cytoplasm and nucleus and there was no obvious difference in the level of HBcAg expressed in FHB and control cases. A similar level of expression was observed using either homologous or heterologous promoters (Figure 3.8)

Figure 3.8

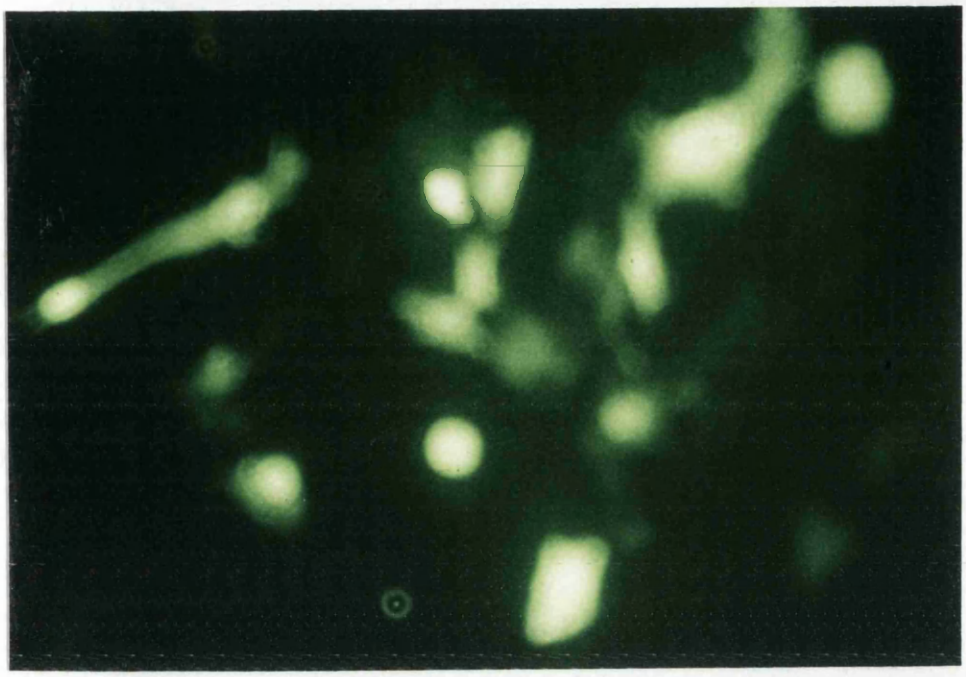


3.8a HBcAg expression in HepG2 cells from an FHB case

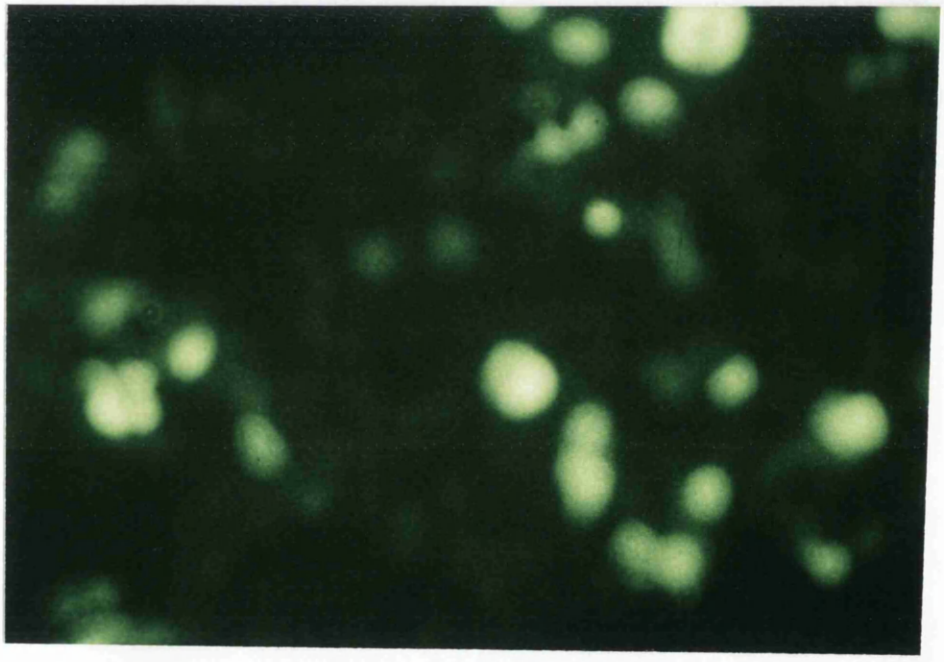


3.8b HBcAg expression in HepG2 cells from a contact case

Figure 3.8 (continued)



3.8c HBcAg expression in HepG2 cell from a control carrier case



3.8d HBcAg expression in HepG2 cells from positive control case.

Figure 3.8 shows HBcAg expression from various plasmid constructs with homologous promoter. The positive control contains heterologous promoter.

CHAPTER-4

DISCUSSION

HBV infection is associated with a wide spectrum of liver injury, ranging from acute self-limited infection to an asymptomatic chronic carrier state, chronic hepatitis with or without progression to cirrhosis and fulminant hepatitis (FHB). FHB is a severe manifestation of acute hepatitis with a mortality rate of 50 percent. In early studies, a high mortality rate was reported with rapid clearance of viral antigens and appearance of respective antibodies (Brechot *et al.,* 1984). Pathogenesis was believed to be due to over production of antigen-antibody complexes which eventually caused the blockage of liver sinusoids and resulted in ischaemic liver cell necrosis (Woolf *et al.,* 1976).

Are FHBV strains genetically related?

In the phylogenetic study, we showed that FHBV sequences have substantial genetic similarity; sequences from epidemiologically unrelated FHBV cases were statistically clustered within particular viral lineages. For X gene, 26 cases from 25 independent episodes had 14 evolutionary steps, which is fewer than the minimum of 21 expected by a random model ($P= 0.1x10^{-5}$). In the core gene, 17 evolutionary steps were observed in 30 cases from 27 unlinked episodes which would be 21 if the sequences were not clustered ($P= 0.4x10^{-7}$). This is conclusive evidence of a viral genetic basis to FHBV. Each cluster is associated with a particular variant 'motif' or combinations of variants. We have also shown that variants out of their genotype context occur in HBV. In this study, we have shown that the Enh-II/CP complex of A_{1896} associated FHBV sequences has significantly increased transcriptional efficiency (see later).

The phylogenetic clustering of unlinked FHB cases, when FHB is itself a viral transmission dead end, would suggest that certain viral genetic contexts, such as those defined by strain and genotype identity, might either be particularly susceptible to acquiring specific FHB associated variants or already possess some elements of the FHB motif combinations. We propose that the symptomless contact who transmits HBV resulting in fulminant hepatitis has a heterogeneous population of HBV strains, or a quasispecies characteristic of quickly evolving viruses (Duarte *et al.,* 1994), some of which all posses the necessary elements required to cause FHB in a *de novo* infection by triggering a massive immune response in the presence of elevated rates of viral replication.

Are there specific unique variants associated with all FHBV?

In our study, no specific variants were identified which were common to all FHB cases. Recently, a number of studies on sequencing entire genomes from FHB cases have been reported. In two of these, (Ogata *et al.,* 1993; Hasegawa *et al.,* 1994), FHBV

sequences showed a significantly higher number of nucleotide substitutions in all four open reading frames and also in the *cis*-acting regions. In our study we have shown that there are a number of unique variants found in some FHB cases, but not in others, and some variants were identified which occurred in significantly higher numbers in FHB cases when compared with non-FHB cases. The *cis*-acting regions were areas of especial interest as they regulate viral transcription. Variants in the Enh-II/CP have been reported in FHB (Ogata *et al.,* 1993) and chronic cases (Okamoto *et al.,* 1994). Sixteen of the 19 FHB cases in our study possessed notable variants in the Enh-II/CP complex. The two most common variants in the BCP were T_{1762} and A_{1764}; both have been reported in FHB and chronic cases (Sato *et al.,* 1995; Kurosaki *et al.,* 1996) and can also appear occasionally after seroconversion to anti-HBe. However, in FHB cases these are linked to fatality (Laskus *et al.,* 1995; Kaneka *et al.,* 1995). Previous studies (Okamoto *et al.,* 1994; Sato *et al.,* 1995) found that variation in the Enh-II/CP complex was associated with seroconversion to anti-HBe, so it was believed to contribute to downregulation of pre-core mRNA transcription. However, this has not been found in all anti-HBe positive cases (Kurosaki *et al.,* 1996) and has been reported from HBeAg positive chronic HBV cases (Okamoto *et al,*. 1994; Laskus *et al.,* 1995); there was an association with lower levels of HBeAg production. In one study, it was found that these two variants in chronic patients has no effect on transcription (Nishizono *et al.,* 1995). In our study, 6 of 7 cases with T_{1762} and/ or A_{1764} had A_{1896} containing sequences, excepting case CHBV-1 who possessed G_{1896} despite having one of these variants (Table 3.8). CHBV-1 had an A insertion at nt 1838 (within pre-core) resulting in a frame shift and was therefore HBeAg negative. Thus, for our patients, it seems that there is a correlation with core promoter variants and HBeAg negative phenotype.

Why are the motifs important?

The first novel concept to come from our study is that, individually, many of these variants are not unique to FHBV sequences but that their pathogenic significance lies in their interaction. The second novel concept is that there can be an interaction between protein and nucleotide variants. Twenty-one of 26 FHBV X gene sequences had a combination of: i) variants in the *cis*-acting region which regulates pregenomic and pre-core mRNA synthesis; ii) the pre-core variant $A_{1896;}$ and iii) one of the three alterations which might be expected to affect the synthesis and function of X gene products (aberrant methionine or cysteine residues or enhancer I variants). These combinations or motifs were nearly exclusive to FHBV sequences. The interactions between the nucleotide changes in *cis*-acting regulatory elements and aberrant cysteine and/or methionine residues (which may have altered the structure) of X protein can have functional effect (cluster 1 and 5, chapter 3.2). Similar motifs were also found in A_{1896} and Enh-II/CP variants (cluster 6, chapter 3.2). We have shown that a specific combination of variants in Enh-

II/CP resulted in an increase rate of transcription which was probably due to a protein-DNA interaction resulting in high transcription efficiency. In this study we have conclusively shown that unique variants in combination with 'normal' variants can cause abnormality; as we have shown in case FHBV-15 (discussed below).

We have also shown that the significantly higher rates of nucleotide evolution in the core and X genes and in the HBV genome as a whole, suggest that an increased rate of evolution is a hallmark of FHBV. The elevated rate of synonymous changes in X and in non-antigenic regions in the core gene are consistent with this interpretation.

What is the importance of A_{1896}?

There is no simple answer to this question. A_{1896} has been implicated in the development of FHB in some studies (Liang *et al.,* 1991, Omata *et al.,* 1991). However, the HBeAg producing strain, G_{1896}, is also found in FHB cases (Carman *et al.,* 1991a, Laskus *et al.*, 1993; Feray *et al.*, 1993) and A_{1896} has also been found as the dominant strain in patients with acute (Carman *et al.,* 1991b; Uchida *et al.,* 1993; Mphahlele *et al.*, 1997) or chronic hepatitis (Carman *et al.*, 1989; Brunetto *et al.*, 1989; Okamoto *et al.,* 1990; Tong *et al.,* 1990). Nine of 19 FHBV cases in our study harboured A_{1896}, the others being G_{1896}. Our data confirm that A_{1896} is not invariably found in FHB patients. Presence of A_{1896} was strongly correlated with the HBeAg status of our patients. Eight of 9 patients containing A_{1896} were HBeAg negative and had seroconverted to anti-HBe. The only exception was case FHBV-5, who was HBeAg producing despite containing A_{1896}.

Yet, there is an epidemiological association between HBeAg negative infection and FHF. HBeAg negative infection of all clinical types is strongly associated with the A_{1896} variant. Transmission of A_{1896} from an anti-HBe carrier resulting in FHB has been documented after sexual exposure (Fagan *et al.,* 1986), outbreaks from a common source (Liang *et al.,* 1991), intrafamilial contact (Kosaka *et al.,* 1991) and mother to infant transmission (Terazawa *et al.,* 1991). Most of these studies reported rapid and severe liver failure with a fatal outcome. In our study, 12 of the 19 FHB cases were HBeAg negative on admission and 9 had rapid disease progression accompanied by the appearance of anti-HBe in most cases, regardless of clinical outcome. The mechanism of pathogenesis by an HBeAg negative variant is poorly understood. However, HBeAg is probably an immune modulator (Milich *et al.*, 1990) and its absence in a naive host may be responsible for rapid hepatocyte destruction by CTL directed to HBcAg epitopes (Mondelli *et al.,* 1987). Infants born to an anti-HBe positive mother seldom develop the chronic state, but either a self limited acute hepatitis or FHB (Beasley *et al.*, 1983; Delaplane *et al.*, 1983). Recently, transmission of a pure strain of A_{1896} to an adult resulting in acute infection and development of anti-HBe has been reported (Mphahlele *et al.*, 1997). However, it is not clear from most FHB studies whether patients were

exposed to a variant strain as opposed to a mixture of variant and normal strains. If they were not, then it may be that there was an initial massive response to the HBeAg producing strain (Carman *et al.,* 1993a) leading to selection of the variant strain; or if they were initially exposed to the variant strain, than the absence of immune modulation may lead to immune elimination of hepatocytes expressing HBcAg epitopes on their membrane.

$CD4^+$ Th cells play an important role in the clearance of virus during acute infection but are less active in chronic patients except during acute exacerbations (Ferrari *et al.,* 1991; Penna *et al.,* 1992). $CD4^+$ cells recognise peptides presented by HLA class-II molecules and HBe and HBc Ags are cross-reactive at the T cell level (Milich *et al.,* 1988). A recent study found that, in transgenic mice, HBe and HBc Ags elicited different subsets of Th cells (Milich *et al.,* 1997); HBcAg was preferentially recognised by Th_1 cells, leading to enhanced cytokine activity with liver cell injury, whereas Th_2 cells elicited by HBeAg were involved in secretion of anti-inflammatory cytokines and antibodies. Presence of both antigens thereby maintains a balance between antigen production and massive CTL response. On the other hand, the absence of an anti-inflammatory HBeAg-specific Th_2 like response may increase the risk of an unchecked and aggressive HBeAg/HBcAg-specific Th_1 cell dominated response.

Although there is substantial evidence for the occurrence of A_{1896} in chronic carriers, FHB is rare. This is probably because, in the course of chronic infection, a prolonged and imbalanced immune response leads to evolution of this variant, which upon transmission to a naive host may lead to FHB in a proportion of cases. Another explanation is that A_{1896} with other variants in the genome may be responsible for FHB. Transmission of A_{1896} can also result in acute infection but it is rare (Mphahlele *et al.,* 1997). We show here that the diversity of A_{1896} associated illness can be explained by the presence of variants elsewhere in the genome.

So, what is the role of A_{1896}?

Different cases of FHB are associated with distinct selective forces: FHBV sequences with A_{1896} were shown to have different patterns of evolution in the core gene compared to FHBV sequences without this variant. However, we have recently shown that transmission of the A_{1896} variant and, presumably HBeAg negative status, occurs on a significant scale, yet clearly does not often lead to FHB (Bollyky *et al.,* submitted). In this study we have conclusively shown that the combination of A_{1896} with other FHBV-associated variants distinguishes A_{1896} CHB from A_{1896} FHB cases. Lending further support to this hypothesis of non-equivalent effects of A_{1896} in FHB and CHB cases, we have also shown here that A_{1896} FHBV sequences have different patterns of evolutionary change from A_{1896} CHBV sequences. This may be because a prolonged selective force in CHB is associated with evolution of A_{1896}; there is also evidence that a proportion of A_{1896}

in CHB is due to superinfection by A_{1896} containing virus rather than emerging *de novo* from G_{1896} status during seroconversion (Bollyky *et al.*, submitted).

Do the motifs have any functional effect?

Another novel concept of this study is that the multiple sequence motifs are associated with a common functional effect. One hypothesis to explain the massive liver cell injury in FHB is that altered binding of transcription factors leads to increased virus replication which, in predisposed individuals, induces an exaggerated immune response. We have shown clearly that *cis*-acting regions from four phylogenetically linked clusters of FHB viral sequences (which are epidemiologically unrelated), have enhanced transcriptional activity *in vitro*. Further, this effect was confined to those FHB associated sequences containing variants in the BCP as well as A_{1896}. The only exception to this rule was CHBV-1, a symptomless male contact implicated in causing FHB in successive wives (Fagan *et al.*, 1986). His sequence also showed high luciferase expression despite having G_{1896} but this was accompanied by an A insertion at nt1838 and C_{1862} which lies within the encapsidation signal and may have altered the RNA secondary structure. FHB cases with G_{1896} sequences (clusters 3 and 7) had normal or intermediate luciferase levels. Two of these cases with normal transcriptional activity had variants in the BCP, one of which was in the third AT rich region (at nt1794); its effect on transcription is thus unknown, but is unlikely to be important. It is illuminating that A_{1896} sequences from chronic carriers not associated with FHB cases had normal transcriptional activity, whilst A_{1896} containing contacts of FHB cases had levels similar to A_{1896} FHB cases. A_{1896} containing chronic carrier controls also had variants in the BCP, commonly T_{1762} and A_{1764}. However, in FHB cases these two variants were accompanied by T_{1766}, A_{1768} or both. In a recent study, based on sequence generated from a single patient, these latter two variants were found to be associated with an elevated rate of replication (Baumert *et al.*, 1996). It is clear that the two most common variants in the BCP (T_{1762} and A_{1764}) do not contribute to the level of transcription unless they occur in combination with other variants. This substantiates the conclusions from the phylogenetic analysis that there are multiple sequence motifs in FHB associated cases which have a common functional outcome and that A_{1896} itself is not, *per se*, the major factor but is a marker for other variation within the *cis* -acting region of the genomes associated with it. As FHB as a clinical entity is rarely, if ever, transmitted, it must also be true that this increased transcriptional activity must be suppressed or otherwise offset in chronic carriers who have it.

Is sequence related to clinical outcome in FHB?

Motifs which characterise individual clusters of FHBV sequences correlate with distinct clinical features. The sequence cluster relationships were linked to death or

survival in the absence of confounding prognostic factors such as pregnancy, coinfection with HDV or older age. A fatal outcome was associated with every FHBV sequence with either the T_{1762} or A_{1764} variants. In two other studies, 3 of 4 (Laskus *et al.,* 1995) and 4 of 5 (Kaneko *et al.,* 1995) such cases were fatal. In our study, variants in these two positions occurred in 5 cases as the dominant population: the outcome in all cases was fatal. In a recent computer modelling study, these variants have been suggested to lead to alteration in RNA superstructure encompassing the ε loop from which RNA is encapsidated (Kidd *et al.,* 1996). They are also the part of the TATA-like regions involved in initiation of pre-core and pregenomic transcripts (Okamoto *et al.,* 1994). A further novel, though related, concept to arise from this study is that specific variants can be linked to different clinical outcomes if they occur outside the usual genotype context. Every FHBV case associated with an aberrant C_{1773} was fatal. Although this study was not designed to address this issue, particular genotypes may predispose patients to FHBV: 15 of 26 FHBV X gene sequences were genotype D.

Interestingly, there was a correlation between clinical parameters and transcriptional activity. High transcriptional activities were found in "rapid FHB" and their symptomless contacts. Rapid FHB is characterised by undetectable viral antigens with rapid seroconversion to anti-HBe and decline into coma within two weeks of the first symptoms and a similarly rapid spontaneous recovery. Both symptomless contacts studied here were implicated in rapidly progressive FHB in two successive wives each (Fagan *et al.,* 1986). Liver cell necrosis and ensuing rapid clinical deterioration is explained by the triggering of a massive immune response which, ultimately, favours clearance of virus. Similarly, early and complete cessation of virus replication should favour liver regeneration and explain the rapid clinical recovery and, ultimately, good prognosis associated with this subgroup of patients with FHB. Clearly, high levels of viraemia are not always linked to high luciferase activities *in vitro* because serum HBV DNA levels typically were low in such contacts and undetectable in these FHB cases except within the first week and only by nested PCR. Also, massive liver injury is not inevitable with high transcriptional activity as both contacts had symptomless mild chronic hepatitis. In contrast, those patients with 'slow FHB' typically show a more protracted clinical and serological course, over several weeks, and have lower transcriptional activities than 'rapid FHB'. Early complete cessation of virus replication is lacking; seroconversion may be delayed as HBV DNA levels remain detectable for several weeks from the first symptoms.

Is a specific variant associated with impaired nuclear factor binding?

We have found that binding of one or more nuclear factors is impaired in most cases with increased transcription. Oligonucleotides were synthesised which encompass the regions in the BCP which bind several transcriptional regulatory proteins including a

liver specific factor C/EBP (Lopez-Cabrara *et al.,* 1990). A recent study found that inhibition of binding of nuclear factors by T_{1762} and A_{1764} (Buckwold *et al.,* 1996) is liver specific, as nuclear extracts from HeLa cells led to fewer bands. In our study, the missing bands were not liver specific, as nuclear extract from HeLa cells showed the same pattern of binding. Figure 3.6 which schematically correlates the variation in the BCP with nuclear factor binding patterns, indicates that particular nuclear protein binding patterns are associated with BCP variants. The oligonucleotide containing only one variant (FHBV-1) showed a similar pattern of binding to the non-FHB control, indicating that T_{1762} alone is unlikely to inhibit binding of nuclear factors. However, the oligonucleotide derived from case FHBV-15, which contained one unique variant (C_{1752}) and one variant out of its usual genotype context (A_{1757}) showed binding inhibition. Oligonucleotides from other FHBV cases with variants in the BCP showed a similar pattern of inhibition. This indicates that a number of combinations can lead to lack of binding to transcription factors and thus to a similar functional outcome. All of these variant cases were associated with high or intermediate luciferase activity; we therefore conclude that these two complexes have an inhibitory effect on transcription.

Which factor(s) is responsible for G_{1896} associated FHB?

A_{1896} has not been reported in association with FHB in the regions where genotype A is more prevalent (Feray *et al.,* 1993; Laskus *et al.,* 1993). In the whole genome sequencing studies, greater numbers of variants were observed when A_{1896} was also present, particularly in the X, core and the Enh-II/CP complex. This raised the question of the factor(s) responsible for G_{1896} associated FHB. There is no direct answer but it could be explained in different ways: firstly, most FHB cases with G_{1896} showed slow disease progression (our study; Brahm *et al.,* 1991) with a high mortality rate. Continuing viral replication and immune elimination of infected liver cells for a longer period without producing neutralising antibodies may contribute to this high mortality. Secondly, there may be confounding factors. In the study by Laskus *et al.* (1993), 10 of 37 cases were coinfected with HDV, always found with G_{1896}. HDV coinfection was also found in the French study by Feray *et al.* (1993); in France, the prevalence of A_{1896} is low overall. In our study, HDV coinfection was found in 2 cases with G_{1896} and both had a fatal outcome. Thirdly, we do not exclude a role for a genetically heightened immune response. Recently, it was found that two patients with FHB, but not their contacts, had one or more alleles for DR 13 at the HLA class-II locus, which was previously shown to be associated with clearance of virus in acute infections (Karayiannis *et al.,* 1995).

Do the variants in our study correlate with the findings of others?

There were a number of variants observed in the Enh-II/CP complex. The two other common variants were T_{1676} and A_{1757} which occurred in 12 and 6 (out of 19) cases

respectively in this study and in significant numbers in other studies (Hasegawa *et al.*, 1994; Sterneck *et al.*, 1996). These variants were also reported from non-FHB cases and they were probably linked to a particular genotype. Along with the 3 other variants in the Enh-I/XP complex (G_{1050}, T_{1249}, C_{1250}), C_{1059} was at higher frequency in our study as well as other FHB studies. Sterneck *et al.* (1996) found that the frequencies of occurrence of C_{1059}, T_{1676} and A_{1757} were 78, 89 and 78 percent respectively in FHB cases in comparison to 17, 14 and 26 percent in non-FHB cases and suggested that these variants were not genotype D specific. Their presence in higher frequencies in the FHBV cases may be important as they occurred in functionally significant areas. They may play a role in pathogenesis although they are not absolutely specific for the FHB group. We believe that they are genotype D specific and that this genotype may be more likely to cause severe disease then other genotypes. In fact, 13 of the 19 FHB cases sequenced in this study (and 18 out of 30 FHBV core gene sequences in the phylogenetic analysis) belong to genotype D.

A number of variants in the Enh-II/CP complex, some of which are unique to FHBV cases. They may not have any obvious effect on pathogenesis when they appear singly, as they did not occur in high numbers, but as showed, they may have a functional effect when they appear in combination. As the X-ORF overlaps with the Enh-II/CP complex at its carboxy-terminal end, some of the variants in this region also lead to amino acid substitutions in the X-protein. The X protein functions as a transcriptional transactivator (Miller and Robinson, 1987) and the carboxy terminal end (particularly a domain between aa 110-143), interacts with TBP (Qadri *et al.*, 1995). The X protein also interacts with other cellular proteins to exert its transactivating function (Maguire *et al.*, 1991). X-gene sequence was available for 17 of our patients, 15 of which showed amino acid substitutions in X-protein. A number of cases showed substitution to cysteine, serine or methionine at different positions (Table 3.3). The presence of cysteines may contribute to an alteration in the secondary structure of the protein and thereby influence the binding to other trans-activating proteins. The significance of these additional serine residues is unknown and requires further analysis. An additional methionine upstream of the X-ORF may cause early initiation of translation and generate an abnormally sized protein (Takahashi *et al.*, 1995). Although the significance of additional methionine residues within the reading frame is not known, one can imagine that translation from these methionines is possible and can generate X-proteins of smaller size. These may still retain transactivating activity.

In the core region, variants were discretely distributed and no clusters of amino acid substitution were seen. This is not consistent with the findings of Ehata *et al.* (1993), where two subtype specific clusters of amino acid substitution were seen in the patients with both FHB and severe exacerbations of chronic hepatitis. Variation in the specific amino acid positions (aa 77 and 113) was also noted in one case (Nakayama *et al.*, 1995).

Here we have shown that a higher number of core variants was associated with A_{1896} but mostly confined to non-antigenic regions. A study by Aye *et al.* (1994) also reported a higher level of core variation in A_{1896} FHB cases, but without any definite clustering.

The occurrence of variants in the pre-S1, pre-S2, S and P-ORFs was also examined. Unique or rare amino acid substitutions occurred less frequently in these ORFs than in the core or X ORFs. The cysteine residues at positions 48, 65 and 69 in SHBs, which are essential for secretion of viral particles (Mangold *et al.,* 1993), were conserved in all FHBV sequences. The SP-I and SP-II promoters remained invariable in all cases. No clusters of amino acid variability were observed in the surface and polymerase proteins. This is consistent with the recent studies on whole genome sequencing from FHB cases (Sterneck *et al.,* 1996; Karayiannis *et al.,* 1995; Alexopoulou *et al.,* 1996). None of these studies could find any specific variants associated with FHB.

Previous studies showed that FHB and their contact cases had identical sequences (Aye *et al.*, 1994; Karayiannis *et al.*, 1995). In our study we had a pair of patients, where the contact had a self limited acute hepatitis but her sibling who shared the same needle developed FHB. The sequence analysis showed considerable disimilarity, contradicting previous findings. This may be because, unlike other studies, the contact is a an acute case.

Does subtype or genotype have any role in FHB?

Transmission of quasispecies from anti-HBe positive mothers with selection of a specific subtype or variant has been reported in a number of studies, indicating that certain subtypes or genotypes may be more likely to cause severe disease. In our study, in 9 out of 12 genotype D FHBV cases, the infection source was either from the UK (8 cases) or the USA (1 case), where genotype A is prevalent. The FHBV sequence associated with the outbreak in Israel also belonged to genotype D. In Mediterranean countries, where chronic HBV with cirrhosis is prevalent, the dominant genotype is D. Similarly, contribution of a particular subtype to disease severity is possible. Transmission of subtype *ayw* from a mixed population in the mother to infants was found to be associated with fatal outcome; survival was linked to transmission of both *adw*2 and *ayw* (Bahn *et al.,* 1995; von Weizsacker *et al.,* 1995). In one study, 2 of 3 newborns had *ayw* as a dominant population during the phase of severe disease with a fatal outcome. However, the infant who survived had a mixed population of *adw*2 and *ayw* in the anti-HBe positive phase but only *adw*2 after recovery (Bahn *et al.,* 1995). This indicates that the transmitted subtype which is the cause of massive hepatitis is the primary target of an immense immune (probably CTL) response.

In the end, what causes FHB?

From the above discussion it reveals that there is no single 'factor' that can be implicated as the cause of FHB. However, in this study we have conclusively shown that:

i) FHBV sequences from unrelated cases have considerable sequence similarity indicating a specific viral genetic factor is associated with disease severity.

ii) No single variant can cause FHB (not even A_{1896}, although it plays a significant role). Specific combinations of variants, affecting functional properties (e.g. increase in rate of transcription and/or replication) can cause FHB.

iii) Anti-HBe positive cases have higher number of variants especially in the *cis*-acting regions, with elevated rate of transcription; indicating that a highly replicative strain, in absence of immune modulation via HBeAg, is responsible for massive hepatocyte destruction.

iv) Non-synonymous amino acid substitutions in the functionally significant areas of X protein and in the non-antigenic areas of core protein reflected the fact that FHBV strains are highly evolved and highly replicative.

v) HBeAg positive cases had less number of variants and 2 of our FHBV cases did not show any variants in the *cis*-acting regions. In such cases, several other factors can play role: (a) a slow disease progression without producing any neutralising antibodies; (b) co-factors such as HDV coinfection or pregnancy and (c) specific HLA type which we could not assayed in our study.

Future work

It is clear from this and other studies that looking for a single variant for FHB is far too simplistic. In our study, we have shown that combinations of variants which form a 'motif' is responsible for severe disease via a common functional effect. Higher transcription rates were found to be associated with combinations of variants in the *cis*-acting regulatory elements. In our study we could assay only a selected number of cases. A study with a greater number of cases including various combinations of variants could give a more conclusive result, and different parallel experiments for the same assay could strengthen the result.

A high rate of transcription must be associated with high replication rate which is responsible for high infectivity of FHBV. Thus, it is conceivable that a study on the replication efficiency of variant strains will differentiate FHB from non-FHBV cases. In fact one study has already shown that two variants in *cis*-acting region are associated with high rate of replication (Baumert *et al.*, 1996). A replication study using annexin-V cells (which are susceptible to HBV infection *in vitro*) transfected with various combinations of variants and assaying their replication rate from one passage to another could help to dissect out the replication competent strains. Only one study has been reported on

transmission of FHBV-infected serum to chimpanzees with subsequent development of severe hepatitis (Ogata *et al.,* 1993). Although laboratory use of chimpanzee is not ethical, at present this is the only animal susceptible for HBV. An animal study on chimpanzees infected with cloned variant and non-variant Enh-II/CP will give an idea about *in vivo* pathogenic effect of a particular variant(s).

In a number of FHB cases, mixtures of variant and normal strains are seen. If it is possible to obtain and assay samples in different disease phases, it would give us a better idea about the strain/genotype/subtype which is the primary target of immune elimination; one strain may be more virulent than another.

Most of the FHB cases with G_{1896} have shown the least number of variants with low luciferase values. A host factor is probably playing the major role in pathogenesis in these cases. Recently, Karayiannis *et al.* (1995) have shown that patients with FHB, but not their chronic contacts, contained one or more HLA class-II alleles which were found to be associated with the clearance of virus in acute infection. Although, in that study patients were infected with HBeAg negative strain and virus could not be cleared before substantial liver damage occurred (as one patient died and one had a liver transplant) indicating the involvement of a highly replicating virus; it is clear that HLA typing and Th cell proliferation assay should be included. Another reason is that Th cells play an important role in producing neutralising antibodies.

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