IMMUNOLOGICAL ASPECTS OF HEPATITIS B VIRUS CORE ANTIGEN AND ITS DERIVATIVES

Ai-Li Shiau

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Institute of Cell and Molecular Biology

University of Edinburgh

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DECLARATION

The composition of this thesis and the work presented within it are my own, unless otherwise stated. The experiments presented were devised in collaboration with my supervisor, Professor Kenneth Murray.

ABSTRACT

The use of core antigen (HBcAg) of hepatitis B virus (HBV) to present peptide epitopes to the immune system has been shown to enhance immunogenicity of the peptide epitopes. HBcAg fused to the first 8 amino acid residues of β-galactosidase was exploited to serve as a carrier protein to present the epitopes from the S, $preS_1$ and $preS_2$ regions of HBV at its truncated C-terminus. The emergence of an HBV escape mutant carrying an amino acid substitution from glycine to arginine at amino acid residue 145 of the S domain suggests that it may be necessary to modify future HBV vaccines. The immunodominant region of HBsAg carrying mutant sequence at amino acid residue 145 was also fused to HBcAg. These HBcAg fusion proteins were expressed in *E. coli* and produced in high yields, and assembled into core-like particles morphologically indistinguishable from HBcAg itself. The largest multiple fusion protein, containing a dimer of the $HBs_{(111-156)}$ sequence as well as sequences from $preS_1$ and $preS_2$ regions carried a total of 165 amino acid residues attached to the C-terminus of truncated HBcAg, and could still be accommodated in core-like particles.

The HBcAg fusion proteins displayed similar HBc antigenicity and immunogenicity to the full-length HBcAg. Immunisation of rabbits with the HBcAg fusion proteins elicited T-cell-proliferative responses to HBcAg, HBsAg and $preS_1$ peptides. The T-cell responses to HBcAg were much higher and more consistent than those to HBsAg or $preS_1$ peptide. The HBcAg fusion proteins induced antibodies against the corresponding peptides. The fusions carrying the immunodominant region of HBsAg, either wild-type or gly_{145} mutant with arginine, glutamic acid or lysine substitution, showed HBs antigenicity in the immunoblot analysis and the antigen-capture sandwich radioimmunoassay, albeit at a lesser extent, using two antibodies with different specificity. Furthermore, they elicited anti-HBs responses cross-reactive with wild-type HBsAg. The addition of $preS_1$ and $preS_2$ epitopes to the fusions carrying the S epitopes enhanced anti-HBs production. The ability of HBcAg to induce a strong HBcAg-specific T-cell response in the context of multimeric B-cell epitopes may

explain the carrier effects of HBcAg particles. The results indicate the potential value of these HBcAg fusion proteins as vaccines against wild-type HBV and its variants.

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ABBREVIATIONS

A	adenosine
ATP	adenosine-5′-triphosphate
bp	base pair
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	bovine serum albumin
C	cytosine
°C	degrees centigrade
CAH	chronic active hepatitis
CFA	complete Freund's adjuvant
Ci	Curie
cm	centimetre
CPH	chronic persistent hepatitis
cpm	counts per minute
CTL	cytotoxic T lymphocyte
dATP	deoxyadenosine-5′-triphosphate
dCTP	deoxycytidine-5′-triphosphate
dGTP	deoxyguanosine-5′-triphosphate
DARIP	double antibody radioimmunoprecipitation
ddATP	dideoxyadenosine-5′-triphosphate
ddCTP	dideoxycytidine-5′-triphosphate
ddGTP	dideoxyguanosine-5′-triphosphate
ddTTP	dideoxythymidine-5′-triphosphate
DHBV	duck hepatitis B virus
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide-5′-triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine-5′-triphosphate
E. coli	*Escherichia coli*

EDTA	[ethylene diamine] tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FCS	foetal calf serum
FMDV	Foot and mouth disease virus
g	gram
G	guanosine
β-gal	β-galactosidase
GSV	ground squirrel hepatitis virus
H-2	Histocompatibility-2
HEPES	*N*-2-hydroxyethylpiperazine-*N*´-2-ethanesulphonic acid
HCC	hepatocellular carcinoma
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
hr	hour
IFA	incomplete Freund's adjuvant
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-2	interleukin-2
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kD	kilodalton
KLH	keyhole limpet haemocyanin
l	litre
LB	Luria broth
LCMV	lymphocytic choriomeningitis virus
M	molar
MAb	monoclonal antibody
2-ME	β-mercaptoethanol
MHC	major histocompatibility complex
min	minute

MOPS	3-[*N*-Morpholino]propane-sulphonic acid
mRNA	messenger ribonucleic acid
MW	molecular weight
NBT	nitroblue tetrazolium
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocyte
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenyl-methyl-sulphonyl fluoride
RIA	radioimmunoassay
rNTP	ribonucleotide-5´-triphosphate
rpm	revolutions per minute
S	Svedberg unit
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulphate
S.I.	stimulation index
T	thymidine
TEMED	*N,N,N,N´*-tetramethyl-ethylenediamine
Th	T-helper
Tris	tris(hydroxymethyl)-amino-methane
Triton X-100	octylphenoxypolyethoxyethanol
Tween 20	polyoxyethylenesorbitan monolaurate
U	unit
UV	ultraviolet
V	volt
WHV	woodchuck hepatitis virus
X-gal	5-bromo-4-chloro-3-indol-β-D-galactopyranoside

STANDARD AMINO ACID ABBREVIATIONS

Ala	A	alanine
Cys	C	cysteine
Asp	D	aspartic acid
Glu	E	glutamic acid
Phe	F	phenylalanine
Gly	G	glycine
His	H	histidine
Ile	I	isoleucine
Lys	K	lysine
Leu	L	leucine
Met	M	methionine
Asn	N	asparagine
Pro	P	proline
Gln	Q	glutamine
Arg	R	arginine
Ser	S	serine
Thr	T	threonine
Val	V	valine
Trp	W	tryptophan
Tyr	T	tyrosine

CHAPTER 1: INTRODUCTION

1.1 General

The discovery of Australia antigen in human serum by Blumberg *et al.* (1965) and the subsequent demonstration of the association of Australia antigen with hepatitis B led to the identification and characterisation of hepatitis B virus (HBV) (Dane *et al.*, 1970). HBV was thought to be a unique virus different from viruses of other families until 1978, when Summers *et al.* (1978) found a similar virus which caused hepatocellular carcinoma (HCC) accompanied by a particular form of hepatitis with degenerative and regenerative changes in woodchucks. Now Hepadnaviridae include HBV and the hepatitis viruses of the woodchuck (WHV), ground squirrel (GSV) (Marion *et al.*, 1980), tree squirrel (TSHV) (Feitelson *et al.*, 1986), Peking duck (DHBV) (Mason *et al.*, 1980), and heron (HHBV) (Sprengel *et al.*, 1988). Less well documented findings in other rodents, marsupials, and cats suggest that other hepadnaviruses may have been detected. The host range of Hepadnaviridae is narrow, in fact strikingly species-specific. HBV appears to infect only humans in nature. However, productive infections have been established experimentally in higher primates. Hepadnaviridae also exhibit tropism for hepatocytes although viral DNA sequences have been detected in small quantity in cells other than hepatocytes, such as peripheral blood leukocytes (Laure *et al.*, 1985) and bone marrow (Elfassi *et al.*, 1984). They share similar virion size and ultrastructure, antigenic structure, and molecular as well as biological features (reviewed by Ganem and Varmus, 1987). Their unique features include an unusual genome structure and mechanism of viral DNA replication which involves reverse transcription.

1.2 Clinical aspects of HBV infection

Hepatitis B is a common disease representing a major worldwide health problem, which is especially serious in Southeast Asia and Africa. The infection may lead to a chronic carrier state and may progress to chronic liver disease including HCC, a major cause of death from cancer throughout the world. Perinatal spread from an infected mother to her offspring represents the most important mechanism for

maintenance of HBV carriers in endemic areas of the world. Parenteral transmission is one of the principal modes of spread of hepatitis B in non-endemic areas. The sources include blood, blood products and contaminated needles and other instruments. Since HBV surface antigen (HBsAg) can be detected in almost every body fluid, such as saliva, tears, seminal fluid, vaginal secretions and breast milk, in HBV-infected individuals, contact-associated transmission may occur.

1.2.1 Acute and chronic hepatitis

The course of HBV infection varies widely in different individuals, which depends on the host immune response, rather than on the virus itself, since HBV is not cytopathic to the hepatocytes. In addition to the inapparent or subclinical infections, patients may develop anicteric or icteric hepatitis. Patients may recover completely, progress to chronic hepatitis, or, in very rare cases, develop fulminant hepatitis, in which massive sections of the liver are destroyed and is often fatal. Acute infection in adults is usually self-limited, but 5-10% of infected individuals become carriers (i.e. chronically infected). The carriers are identified by the persistence of HBsAg and anti-HBc for at least 6 months. The carrier state is more likely to be established following childhood infection. The HBsAg carriers may be divided into two groups. Those that are HBeAg and virion positive have been shown to be highly contagious. Those that are HBeAg and virion negative and anti-HBe positive have low infectivity. Chronic infection can be associated with nearly normal liver function, or may lead to the development of chronic persistent hepatitis (CPH), chronic active hepatitis (CAH), cirrhosis and HCC.

1.2.2 Hepatocellular carcinoma

Several lines of evidence support the connection between chronic HBV infection and HCC. 1) The epidemiologic evidence is the geographical coincidence between a high prevalence of HBV infection and a high incidence of HCC. Geographic areas with the highest incidence of HCC are also areas where HBV infection is common and

where persistent HBV infections occur at the highest frequency. 2) The incidence of HCC is much higher in HBV-infected humans, woodchucks, ground squirrels and Chinese ducks than in uninfected members of the species. A large, prospective study in Taiwan has shown the incidence of HCC to be more than 100- to 200-fold higher in HBsAg-positive than in HBsAg-negative individuals (Beasley *et al.*, 1981), and a prospective study in Japan yielded a similar result. Further evidence of a link comes from animal studies. More than 90% of woodchucks infected with WHV at birth died of HCC within 2-3 years (Popper *et al.*, 1987). 3) Almost all HBV-related HCCs harbour integrated viral DNA, often in multiple copies. In some cases, tumours are clonal with respect to these insertions, indicating that integration of viral DNA precedes or accompanies the transforming event (Tanaka *et al.*, 1988; Hino *et al.*, 1989).

Although the molecular mechanisms leading to oncogenesis are still not understood, evidence supporting the direct or indirect contribution of HBV DNA to HCC has accumulated. Integrated viral DNA contains extensive deletions and rearrangements, which are different for each integrated viral sequence, indicating that integration is a random event. There is no evidence that the HBV genome contains viral oncogenes. HBV does not appear to activate known proto-oncogenes by the integration of viral sequences in the flanking host DNA although there are certain exceptions. However, in animal models, it has been demonstrated that WHV contributed to neoplastic transformation through insertional activation of cellular proto-oncogenes, eg. *c-myc* and N-*myc* (Hsu *et al.*, 1988; Fourel *et al.*, 1990). Besides the activation of cellular proto-oncogenes, integration of viral DNA can lead to deletions and rearrangements of cellular DNA at sites of integration. These events could also contribute directly to growth deregulation. Transactivation of cellular genes by the truncated products of integrated HBV $PreS_2$/S genes (Kekule *et al.*, 1990) and by HBxAg (see Section 1.5.4), and long-term storage of the three HBV envelope glycoproteins (Chisari *et al.*, 1989) have been proposed as causative or contributory factors in HBV-associated oncogenesis.

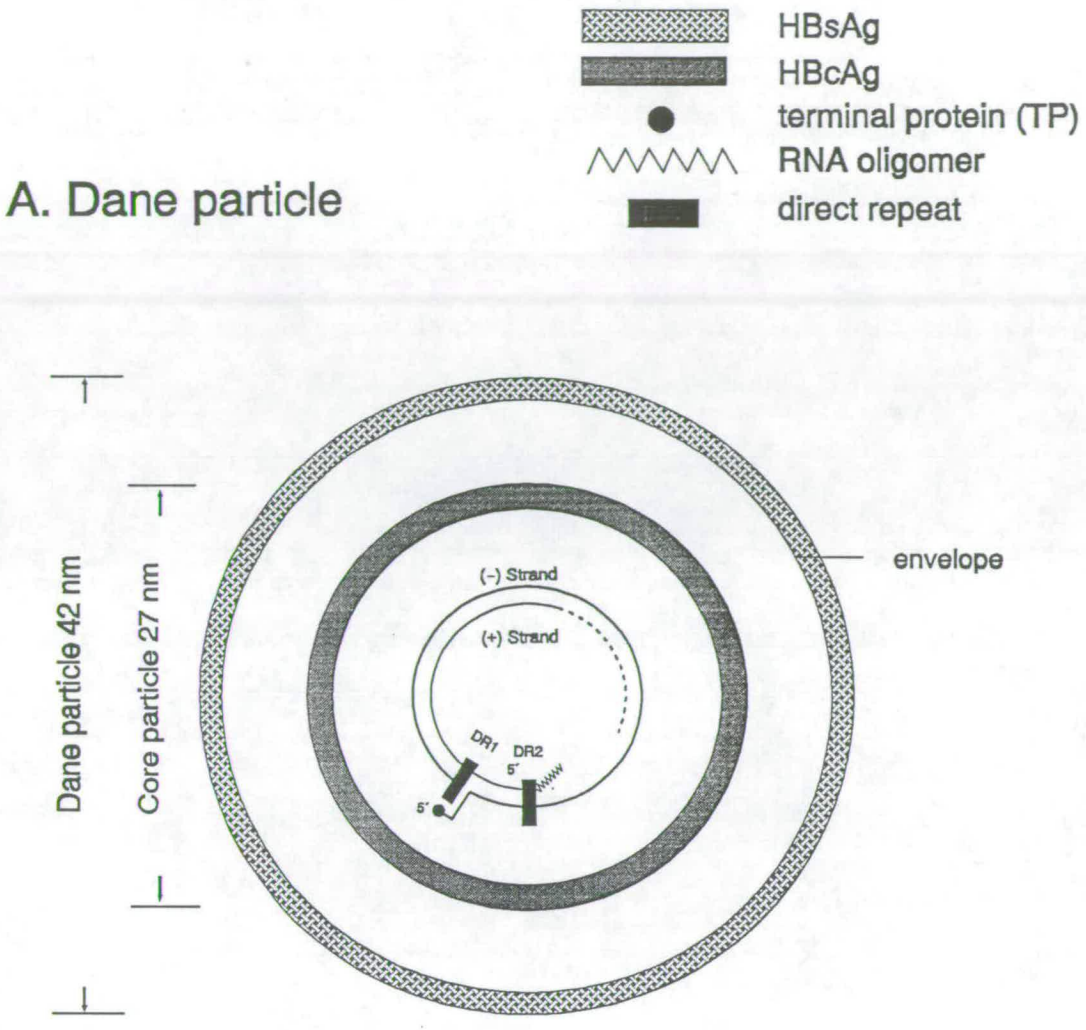
Finally, HBV may also contribute indirectly to oncogenesis, for infection generally results in hepatocyte necrosis, which may trigger a host response marked by chronic inflammation, fibrosis and hepatocyte regeneration. This regenerative hyperplasia expands the population of cells at risk of subsequent genetic alterations that further deregulate cell growth. Therefore, the host immune responses to HBV infection which lead to the destruction of infected hepatocytes and trigger these changes may contribute to oncogenesis. It is tempting to speculate that HBV DNA may contribute both directly or indirectly to HCC *in vivo*.

1.3 Virion structure

1.3.1 Ultrastructure

The structure of the hepatitis B virion in presented in Figure 1.1. The virion was first observed under the electron microscope in 1970 as a spherical particle approximately 42 nm in diameter (Dane *et al.*, 1970), with an electron-dense, spherical inner core with a diameter of approximately 27 nm surrounded by an envelope approximately 7 nm in thickness. In addition to the virion, or Dane particle, which is found in the liver and blood of acutely infected individuals, there are more numerous particulate forms that bear viral surface antigen (HBsAg) in the serum of infected hosts. These are small spherical particles with a diameter of 22 nm and filamentous forms 22 nm wide and varying in length. They are considered to be incomplete viral envelope forms. The core particles, or nucleocapsids, which can be released from the virions by detergent treatment, bear the viral core antigen (HBcAg), the viral e antigen (HBeAg) in a cryptic form, the viral DNA, DNA polymerase activity, and a protein kinase activity that phosphorylates HBcAg. The core particle occurs uniquely in the liver early in acute infections. The HBeAg, first detected as a soluble antigen in serum of HBV-infected patients (Magnius and Espmark, 1972), is closely associated with the core and its antigenic reactivity. The HBcAg can be converted into HBeAg by proteolytic degradation under dissociating conditions (MacKay *et al.*, 1981a).

Figure 1.1 Structure of HBV. (A) The Dane particle consists of an envelope (HBsAg and host-derived lipid) and a core particle containing the HBcAg, which surrounds the partially double-stranded viral genome and DNA polymerase/reverse transcriptase. (B) The subviral particles, spherical and filamentous forms, are composed solely of HBsAg and host-derived lipid. (Modified from Murray, 1987).


HBsAg
HBcAg
terminal protein (TP)
RNA oligomer
direct repeat
A. Dane particle
Dane particle 42 nm
Core particle 27 nm
envelope
(-) Strand
(+) Strand
DR1
DR2
5'
5'
B. Subviral particle
sphere
filament
22 nm
various length

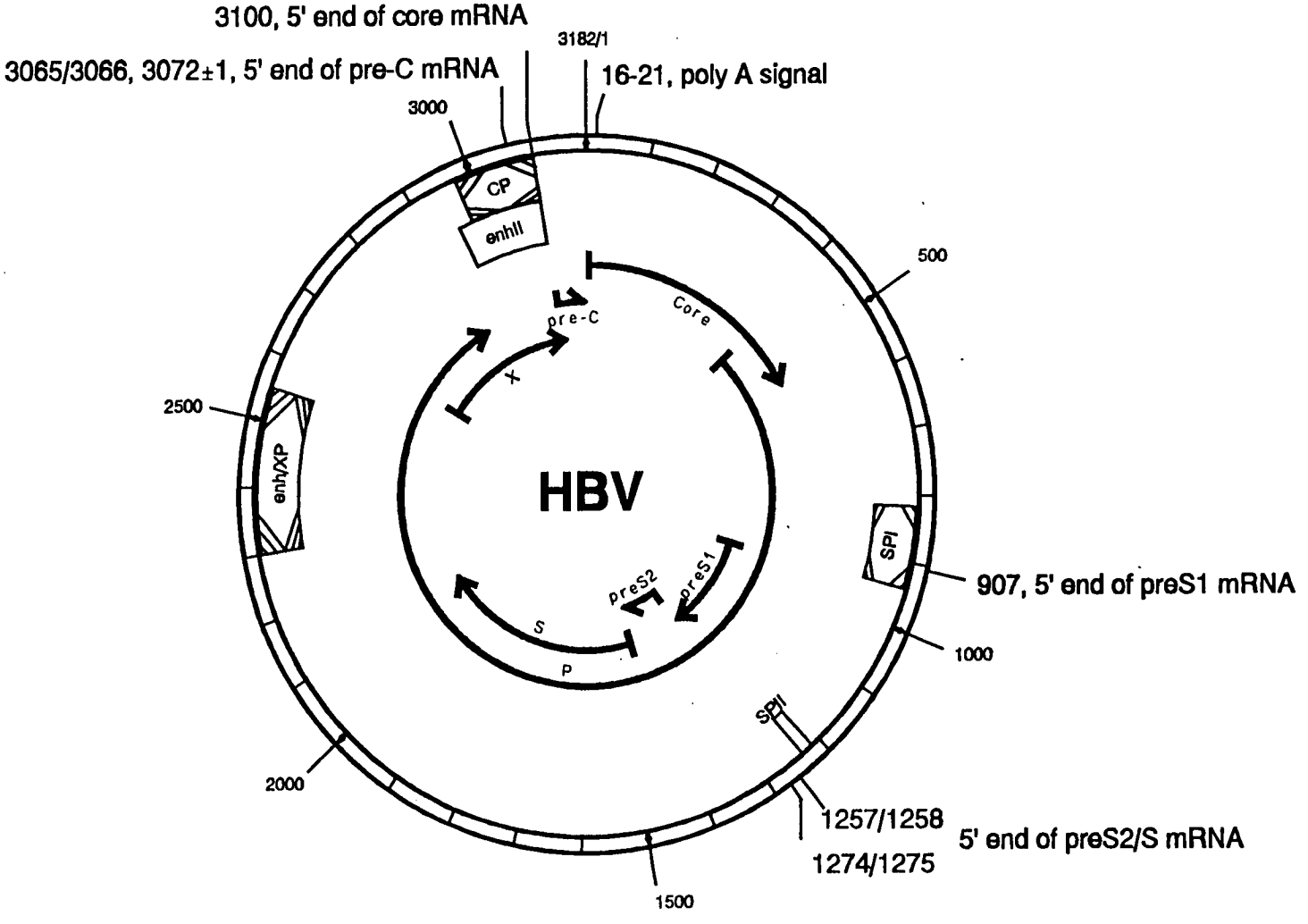
1.3.2 Genome structure

The HBV genome is a partially double-stranded, circular DNA molecule of about 3.2 kb with a molecular mass of about 2 x 10^6 (Figure 1.1; Kaplan *et al.*, 1973; Robinson *et al.*, 1974; Summers *et al.*, 1975). The complete, long strand serves as template for viral transcription and is thus termed the minus strand (Pasek *et al.*, 1979). The long strand is not a closed circle having a nick at a unique site. The position of the 5′ end of the short strand, or termed the plus strand, is fixed, but its 3′ end is variably situated (Summers *et al.*, 1975). The single-stranded gap varies from 20-50% of the circular length. The circular structure is maintained by base-pairing of the 5′ ends of the two strands over a length of 200 to 300 nucleotides (Sattler and Robinson, 1979; Delius *et al.*, 1983). An endogenous DNA polymerase activity can fill in the single-stranded region of the short strand. The 5′ ends of both strands of DNA are not able to be phosphorylated with polynucleotide kinase (Gerlich and Robinson, 1980) because a protein and an oligonucleotide are covalently attached to the 5′ end of the long strand and the short strand, respectively. Within the overlap or "cohesive end" region, there are two 11-base direct repeats, called DR1 and DR2, which play an important role in viral DNA replication (see Section 1.7).

1.4 Genome organisation

The complete nucleotide sequences of cloned DNAs of HBV have been determined (Pasek *et al.*, 1979; Galibert *et al.*, 1979). There are four translational open reading frames (ORFs), utilising all three translational phases, in HBV which are all encoded by the minus-strand DNA (Figure 1.2). The four ORFs are termed C, P, S and X. The P region overlaps within all the other three. In effect, the minus strand is read one-and-a-half times. The HBV isolate (HBV 130) used in this work is 3182 bases in length and the beginning of the coding sequence for HBcAg was selected as number 1 in nucleotide sequence (Burrell *et al.*, 1979; Pasek *et al.*, 1979). The same numbering system is used in this work described here. Galibert *et al.* (1979) used the unique target sequence for restriction endonuclease EcoRI as number 1, which is

Figure 1.2 Genetic organisation of the HBV genome. Open reading frames in three translational phases are indicated by heavy lines. Transcriptional control regions are denoted by boxes and are abbreviated as follows: SPI, $preS_1$ promoter; SPII, $preS_2$/S promoter; CP, core promoter; enh/XP, enhancer/X promoter; enhII, enhancer II. (Data from Pasek *et al.*, 1979; Schaller and Fischer, 1991).


3100, 5' end of core mRNA
3182/1
3065/3066, 3072±1, 5' end of pre-C mRNA
16-21, poly A signal
3000
CP
enhII
500
pre-C
Core
X
2500
enh/XP
HBV
SPI
907, 5' end of preS1 mRNA
preS2
preS1
S
P
1000
SPII
2000
1257/1258
5' end of preS2/S mRNA
1274/1275
1500

located within pre S region. However, in the sequence of HBV130, this EcoRI site does not exist, and in fact of 22 HBV DNA sequences in the EMBL and GENBANK databases, only 5 carry an EcoRI site. To convert the numbering system of Pasek *et al.* to that of Galibert *et al.*, add 1900 to numbers less than 1283 and subtract 1282 from numbers greater than 1282.

The four ORFs of HBV are the following:

1) The C ORF, encompassing nucleotides 1 to 549 of the HBV sequence, encodes HBcAg. The C ORF is also preceded by a short upstream in-phase ORF, termed pre-C ORF, encompassing nucleotides 3096 to 3182 of the HBV sequence, which could encode a larger HBcAg-related polypeptide which is relevent to the formation of HBeAg.

2) The P ORF, encompassing nucleotides 407 to 2902 of the HBV sequence, encodes DNA polymerase, or reverse transcriptase.

3) The S ORF, encompassing nucleotides 1437 to 2114 of the HBV sequence, encodes HBsAg. Upstream of S ORF is an in-phase reading frame (ORF preS) with two conserved in-phase initiation codons that can direct the synthesis of two additional HBsAg-related proteins. These codons subdivide the preS region into two functional subregions, termed $preS_1$ and $preS_2$, encompassing nucleotides 948 to 1271, and 1272 to 1436 of the HBV sequence, respectively.

4) The X ORF encodes HBxAg, encompassing nucleotides 2656 to 3117 of the HBV sequence, which has a transactivating function.

1.5 Viral polypeptides

1.5.1 Core (Nucleocapsid) proteins with HBcAg and HBeAg specificities

The HBcAg, comprising 183 amino acids with a molecular weight of 21 kD, is the single polypeptide found in virion core, which consists of approximately 180 subunits of HBcAg in an icosahedral structure (Onodera *et al.*, 1982). Free HBcAg is confined to infected hepatocytes. In contrast, HBeAg, which has a molecular weight of 16 kD,

is structurally related to HBcAg and is often present in the bloodstream of HBV-infected patients mainly as soluble dimers (Tedder and Bull, 1979). HBeAg was first identified in the serum of individuals infected with HBV (Magnius and Espmark, 1972) and is an important serological marker for HBV infection. High titres of HBeAg in the serum are correlated with high levels of virus replication (Imai *et al.*, 1976; Werner *et al.*, 1977), and therefore high infectivity (Nielsen *et al.*, 1974; Okada *et al.*, 1976). Seroconversion to anti-HBe is indicative of viral clearance. However, the biological role of HBeAg remains enigmatic. HBeAg in the serum exists in free forms as well as in association with IgG and serum albumin (Takahashi *et al.*, 1978) resulting in considerable physical heterogeneity (Tedder and Bull, 1979).

The presence of HBeAg in the core of Dane particles in a cryptic form was demonstrated by treatment of serum-derived core particles with pronase, SDS, chaotropic agents or by sonication or centrifugation in CsCl (Budkowska *et al.*, 1979; Takahashi *et al.*, 1979; Ohori *et al.*, 1979). MacKay *et al.* (1981a) also showed that HBcAg, produced in *E.coli*, can be converted into HBeAg by proteolytic cleavage under dissociating conditions, thereby demonstrating that no other HBV protein was the source of HBeAg. The nature of the conversion of HBcAg to HBeAg was further defined by Takahashi *et al.* (1983) who determined the C-terminus of HBeAg to be 33-35 amino acids upstream of the C-terminus of HBcAg by amino acid sequencing, suggesting that HBeAg represents a part of HBcAg.

HBeAg was detected to contain two subspecificities, HBeAg/1 and HBeAg/2, by immunodiffusion. HBeAg/1 was usually more stable than HBeAg/2 (Tedder and Bull, 1979; Yamada *et al.*, 1979). The monomeric forms of HBeAg displayed only HBeAg/1 antigenicity, while the polymeric forms exhibited HBeAg/2 antigenicity, as treatment of HBeAg with both SDS and 2-mercaptoethanol abolished HBeAg/2 antigenicity (Matsuda and Ohori, 1988). Two distinct antigenic determinants on HBeAg were also demonstrated by monoclonal antibodies (MAbs) designated as "a" and "b" (Imai *et al.*, 1982) or "α" and "β" (Ferns and Tedder, 1984). Salfeld *et al.* (1989) also mapped antigenic determinants of HBeAg, one linear (HBe1) and the other

conformational (HBe2) (see Section 4.5). Whether the two distinct antigenic determinants on HBeAg described by different authors are the same remains unclear.

HBcAg and HBeAg are encoded by two different mRNAs which have slightly different 5′ ends and therefore contain different in-frame initiation codons which are referred to as the core and the pre-core AUGs. Synthesis of the HBcAg starts at the second initiation codon, ie. core AUG, and the resulting protein is located in the cytoplasm and the nuclei of the cells. It self-assembles into virus capsids and is exported from the cells as part of a virus particle. The region between these 2 initiation codons, which are eighty-seven nucleotides apart, is termed pre-core region. The larger "pre-core" protein is encoded by the larger mRNA and therefore contains an extra 29 amino acids encoded by the pre-core region, termed pre-core (pre-C) sequence, at its N-terminus. The first 19 amino acids of the pre-C sequence act as a signal sequence for secretion and therefore directs the pre-core protein into the secretory pathway.

At least two different proteolytic events are involved in the maturation of HBeAg. The first proteolytic event removes the signal peptide from the pre-core protein, which is mediated by the signal peptidase located in the lumen of the endoplasmic reticulum (ER) and results in the formation of a precursor protein, P22 (Bruss and Gerlich, 1988; Garcia *et al.*, 1988). The second proteolytic event removes the C-terminal domain of about 34 amino acids of P22 and converts it to the 16-kD HBeAg which occurs in a post-ER compartment, most likely the Golgi apparatus, and eventually HBeAg is released from the hepatocytes (Ou *et al.*, 1986; Standring *et al.*, 1988; Wang *et al.*, 1991). The processing protease is probably a cellular enzyme, aspartyl protease (Jean-Jean *et al.*, 1989). Suggestions that the protease is of viral origin came from the observation that the sequence near the N-terminal region of HBcAg contains a conserved motif, i.e. LLDTAS for most HBV subtypes, very similar to the protease-like sequences, which is involved in Gag processing in retroviruses (Miller, 1987). On the basis of this finding, a model was proposed that HBeAg would be generated by self-cleavage of HBcAg at the C-terminus by the protease activity of the HBcAg.

However, mutational deletion of the putative active sites of this protease did not impair the processing (Nassal *et al.*, 1989), suggesting that the protease is not of viral origin.

A variety of mutants with defects in pre-core region were detected in the sera from anti-HBe positive patients, but not from HBeAg positive carriers (Carman *et al.*, 1989; Tong *et al.*, 1990; Okamoto *et al.*, 1990). In fact, in the original HBV isolate used for the expression of HBcAg in *E. coli* (Burrel *et al.*, 1979), this pre-core mutant was cloned and sequenced (Pasek *et al.*, 1979). The emergence of pre-core defective mutants also provides evidence to support the view that the pre-core sequence plays an important role in the synthesis and secretion of HBeAg. On the other hand, the detection of these naturally occurring preC-deficient mutants prevailed as hosts seroconvert from HBeAg to anti-HBe, suggesting that pre-core region might not required for HBV replication (Okamoto *et al.*, 1990). Indeed, it has been shown that mutational ablation of pre-core translation has no effect on viral replication, assembly and infectivity in DHBV (Schlicht *et al.*, 1987) or in WHV (Chen *et al.*, 1992a). Since about two thirds of the pre-core sequence spanning amino acids 10-29 is singly coded, mutants in this region would be able to survive, as the coding capacity for the other viral polypeptides is not affected. The pre-core region is conserved in all animal hepadnaviruses. However, the reason why expression of pre-core region has been retained in viral evolution remains an enigma.

Nucleocapsid formation *in vivo* involves the correct assembly of three viral constituents: HBcAg, the polymerase and the pregenome RNA. No major cellular proteins have been detected in the core particle. However, *in vitro*, HBcAg self-assembles to 27 nm core-like particles when expressed in *E. coli* and the recombinant HBcAg is morphologically and immunologically identical to natural HBcAg (Burrell *et al.*, 1979; Stahl *et al.*, 1982; Cohen and Richmond, 1982). HBcAg can also be expressed in a wide range of systems (Gough and Murray, 1982; Kniskern *et al.*, 1986; Roossinck *et al.*, 1986; Clarke *et al.*, 1987). However, the *E. coli* expression system is the simplest and the most popular method, which has been exploited for the

production of large quantities of HBcAg for research and diagnostic uses (Stahl *et al.*, 1982). The nucleic acid and all other viral proteins are not required for this assembly *in vitro*. The addition of heterologous sequences to the N-terminus of HBcAg does not affect such assembly (Clarke *et al.*, 1987; Stahl and Murray, 1989).

HBcAg consists of two structurally and functionally distinct domains. The sequence from the N-terminus to amino acid position 144 is by itself sufficient for particle assembly, at least at high protein concentrations present in heterologous expression systems, eg. *E. coli* (Gallina *et al.*, 1989; Stahl and Murray, 1989; Birnbaum and Nassal, 1990). The C-terminal 34 amino acids contain 16 arginine residues, 14 of which occur in 4 clusters. This C-terminal sequence has been termed the protamine-like region, implying that it is involved in nucleic acid binding.

Addition of heterologous sequences to the N-terminus of HBcAg does not impair particle formation. Stahl *et al.* (1982) synthesized HBcAg in *E. coli* as a fusion protein with the first eight amino acid residues of β-galactosidase fused in frame to HBcAg in which the first two N-terminal residues were deleted. Clarke *et al.* (1987) reported the expression of major immunogenic epitopes from foot and mouth disease virus (FMDV) fused to the N-terminus of HBcAg, which carried a total of 30 amino acid residues from foreign sequences. These fusion proteins still assembled into core-like particles.

The assembly of core-like particle is also unimpeded by the addition of peptide sequences to the C-terminus of the truncated HBcAg consisting of amino acids 3-144, implying that the protamine-like C-terminus of HBcAg is not critical for particle formation (Stahl and Murray, 1989). It has also been shown by deletional analysis that the first 144 amino acids of HBcAg are sufficient for assembly in *E. coli* (Gallina *et al.*, 1989). In addition, the truncated P16 polypeptide linked by disulphide bonds into dimers and assembled into the particle by noncovalent interaction; while in the authentic HBcAg particles, additional disulphide bonds, presumably between the C-terminal cysteines of the protamine-like regions, further stabilise the structure and

convert it into a covalently closed lattice (Gallina *et al.*, 1989). Birnbaum and Nassal (1990) also demonstrated that HBcAg deleted from the C-terminus to amino acid residue 164, 149 or 144 also formed particles when expressed in *E. coli*. However, the presence of the basic C-terminus greatly enhanced encapsidation of nucleic acid and appeared to play a crucial role in capsid stability via protein-nucleic acid interactions (Birnbaum and Nassal, 1990).

Since the basic C-terminal domain of HBcAg is dispensable for assembly and both HBcAg and HBeAg contain the regions which are needed for core particle formation, the failure of HBeAg to form particles may be due to the additional amino acids, ie. pre-core sequence. Indeed, it has been demonstrated that the pre-core sequence not only mediates the secretion but also determines the structural and aggregational properties of HBeAg. When the pre-core sequence was replaced with an unrelated signal sequence in the pre-core protein, it still entered the secretory pathway and was efficiently processed, but secreted as assembled particles (Schlicht and Wasenauer, 1991). Furthermore, the remaining ten amino acids within the pre-core sequence attached to the N-terminus of HBcAg after processing has recently been shown to determine the non-particulate nature and the antigenicity of HBeAg (Wasenauer *et al.*, 1992).

It has been presumed that the C-terminal region of HBcAg has DNA-binding activity because of the resemblance of its amino acid sequence to that of protamine. Indeed, Petit and Pillot (1985) demonstrated that HBcAg purified from liver has nonspecific DNA-binding activity. Furthermore, HBcAg produced in *E. coli* has also been shown to bind both DNA and RNA (Matsuda *et al.*, 1988; Gallina *et al.*, 1989; Hatton *et al.*, 1992). However, there are conflicting reports concerning the importance of the protamine-like domain in mediating nucleic acid binding. HBeAg partially purified from serum bound to HBV DNA (Matsuda *et al.*, 1988), while recombinant HBeAg, consisting of amino acids 1-144 of HBcAg and 6 amino acid residues from a linker sequence, but lacking the ten amino acid residues of the pre-core sequence, did not (Gallina *et al.*, 1989). It is possible that some of the differences in amino acid

sequence between natural HBeAg and the recombinant HBeAg can account for this discordance. In addition, since the HBeAg from serum was only partially purified, the DNA binding might perhaps involve another compartment.

Assembly of replication-competent HBV nucleocapsid required the interaction of the HBcAg, the product of the viral P gene and the RNA pregenome. It has been shown that the protamine-like region of HBcAg is essential for efficient RNA pregenome encapsidation (Birnbaum and Nassal, 1990). Recently, Hatton *et al.* (1992) dissected the nucleic acid-binding capabilities of the protamine-like region of HBcAg and demonstrated that the first arginine repeated sequence motif (Repeat I) spanning amino acids 150-157 was the minimal region required for RNA binding and packaging, but did not bind DNA, while the three other repeats bound DNA much more efficiently than RNA. Nassal (1992) also showed that the arginine-rich region in HBcAg was essential for proper interaction with nucleic acid, but only half of the C-terminal region spanning to amino acid 164 was sufficient for RNA pregenome encapsidation with an efficiency similar to that of the full-length HBcAg. In addition, the C-terminal region from amino acids 165 to 173 was required for proper genome replication (Nassal, 1992). In contrast, sequence beyond amino acid 164 was not required for the formation of enveloped virions, suggesting that nucleocapsid envelopment is independent of the presence of a mature DNA genome (Nassal, 1992).

The arginine-rich C-terminus of HBcAg has also been shown to resemble the nuclear localisation signals for several viral and cellular proteins (Kalderon *et al.*, 1984; Lyons *et al.*, 1987). Viral nucleocapsids can be detected in the nucleus of HBV-infected hepatocytes (Ray *et al.*, 1976; Chu and Liaw, 1987). However, localisation of HBcAg expressed in cultured mammalian cells has been demonstrated in the nucleus (McLachlan *et al.*, 1987), cytoplasm (Ou *et al.*, 1989; Yeh *et al.*, 1990), or both (Roossinck *et al.*, 1986). Ou *et al.* (1989) showed that the HBeAg precursor protein P22, if expressed without the preceding signal sequence, was transported into the nucleus very efficiently; while HBcAg, on the other hand, was detected mostly in the cytoplasm in mammalian cells. Although the pre-core sequence consisting of the C-

terminal ten amino acids, which remains after cleavage of the signal sequence, is important for nuclear transport of P22, it does not contain a nuclear localisation signal (Yeh *et al.*, 1990). Nevertheless, the arginine-rich domain contains a signal for nuclear translocation of P22 (Yeh *et al.*, 1990). Since HBcAg is localised in cytoplasm in their expression system, but contains the arginine-rich domain, the authors argue that the ten C-terminal region of the pre-core sequence, contained in P22, is also required in mediating nuclear localisation. Deletion and point mutational analyses of HBcAg, which is localised in nucleus in this expression system, revealed that the first and the last clusters of the arginine residues within the arginine-rich domain represent distinct and independent nuclear localisation sequences (Eckhardt *et al.*, 1991). The significance of these nuclear localisation sequences in HBcAg to the HBV life cycle is not yet clear.

Core particles purified from mature virions contain a protein kinase activity (Albin and Robinson, 1980). This kinase activity can phosphorylate HBcAg in the core particles. Incubation of core particle with γ-^{32}P ATP leads to phosphorylation of HBcAg on multiple serine and threonine residues, but not of HBeAg (Roossinck and Siddiqui, 1987). The pre-core protein derivative, P22, can also be phosphorylated in cells, while the mature HBeAg, which is converted from P22 by proteolytic cleavage, is not significantly phosphorylated (Yeh and Ou, 1991). Because HBcAg isolated from DHBV-infected hepatocytes is phosphorylated and that isolated from mature DHBV virion is not, it has been speculated that phosphorylation of the core protein regulates the maturation of the virion (Pugh *et al.*, 1989). However, the role of this phosphorylation in HBV infection and replication has not yet been elucidated.

1.5.2 Polymerase

The P ORF occupying 80% of the genome is thought to code for the DNA polymerase, or reverse transcriptase, associated with Dane particles (Kaplan *et al.*, 1973) because its sequence shares homology with sequences in the *pol* genes of retroviruses (Toh *et al.*, 1983). Direct evidence supporting this possibility was shown

by the expression of P-gene-encoded sequences in *E. coli* in the form of fusion proteins. The fusion proteins were found to have reverse transcriptase activity, and cross-reacted with sera from HBV-infected chimpanzees (McGlynn and Murray, 1988), indicating that P gene product is expressed during the viral life cycle. Antibody to synthetic peptides with sequences derived from the P-gene sequence was also shown to immuno-precipitate reverse transcriptase activity in detergent-disrupted HBV virions (Bavand *et al.*, 1989). Furthermore, Weimer *et al.* (1990) demonstrated that antibodies against the RNase H domain of the P protein (see below) are early markers of infection and a signal of ongoing virus replication and decreasing titres indicate the decline or end of active virus production. Due to the low abundance in the virions, this putative protein, however, has not been isolated and fully characterised. The results from Bartenschlager and Schaller (1992) suggest that there are very few, possibly only one, P protein (polymerase) molecules per core particle. Direct evidence has shown that the P-gene product is contained in the nucleocapsid as an unprocessed 90kD protein and it can perform its function as a multidomain polypeptide without requiring proteolytic processing (Barterschlager and Schaller, 1988 and 1992).

From the results of structural and mutational analyses of the P protein, as well as sequence comparison with other reverse transcriptase genes, the P-gene product has been divided into four domains from the N- to the C-terminus: the terminal protein (TP) serves as the primer of the minus DNA strand, a spacer region, the reverse transcriptase, and the RNase H domain (Barterschlager and Schaller, 1988; Radziwill *et al.*, 1990). The P-gene product is required in addition to HBcAg for viral pregenome encapsidation (Bartenschlager *et al.*, 1990; Hirsch *et al.*, 1990). Furthermore, the intact P-gene product, ie. multiple domains of the P protein, is indispensable for RNA encapsidation in HBV (Bartenschlager *et al.*, 1990; Blum *et al.*, 1991) and in DHBV (Chang *et al.*, 1990; Chen *et al.*, 1992b).

Terminal protein (TP) attached to the 5′ end of the minus strand of the genome has been identified to be encoded by the P gene in DHBV (Barterschlager and Schaller, 1988; Bosch *et al.*, 1988) and in HBV (Radziwill *et al.*, 1990). It has been

demonstrated further that the TP domain located in the N-terminus of the P protein is responsible for this DNA linkage (Barterschlager and Schaller, 1988; Bosch *et al.*, 1988). After the TP domain binds to a specific, short target sequence located near the 5′ end of the pregenomic RNA (Bartenschlager *et al.*, 1990), reverse transcription is initiated (Barterschlager and Schaller, 1988; Radziwill *et al.*, 1990). Mutations in the three functional domains within the P gene drastically reduced endogenous DNA polymerase activity (Radziwill *et al.*, 1990). The RNase H domain is responsible for degradation of the RNA component in the hybrid molecule of viral RNA pregenome and minus-strand DNA (Radziwill *et al.*, 1990).

1.5.3 Envelope proteins (surface antigen)

HBsAg (surface antigen) is present both in the envelope of intact virions and as free circulating subviral particles. The 22-nm HBsAg particles isolated from the sera of HBV-infected patients are complex macromolecular assemblies, containing about 75% protein, 25% lipid, and N-linked carbohydrate. One lipoprotein particle contains about 100 HBsAg polypeptide monomers. HBsAg assembly is classically believed to occur in the ER. However, recently, by using immunocytochemical and biochemical approaches, the results support a model in which rapid dimer formation of HBsAg, catalysed by protein disulphide isomerase (PDI), occurs in the ER, and is followed by transport of dimers to a pre-Golgi compartment where the absence of PDI and a different lumenal environment allow the assembly process to be completed (Huovila *et al.*, 1992). HBsAg comprises three envelope polypeptides, the major, or small (S), the middle (M) and the large (L) proteins. All three envelope proteins exist in two forms differing in the extent of glycosylation. They are encoded in a single ORF and initiate at three separate in-phase initiation codons spaced at intervals of 108 (or 119, depending on subtype) and 55 codons, but terminate in the same stop codon. The segments downstream of the three initiation codons are termed the $preS_1$ and $preS_2$ regions and the S gene (Machida *et al.*, 1984; Heermann *et al.*, 1984). The two larger polypeptides could be detected in all three mammalian hepadnaviruses, including HBV, WHV and GSV (Heermann *et al.*, 1984; Wong *et al.*, 1985; Persing

et al., 1986a). The avian hepadnaviruses, DHBV, also contain a preS region with multiple in-frame AUGs, but only one unglycosylated 36-kD preS protein has been detected (Pugh *et al.*, 1987).

Among the three envelope proteins, the major component is the S protein; it is also often called HBV surface antigen (HBsAg), as it was originally thought to be the only viral antigen present on the subviral particle by early immunological studies. The major protein is 226 amino acids in length and is encoded by the S gene. It exists in two forms, non-glycosylated (P24) and glycosylated (GP27), which possesses a complex N-linked glycan at asparagine residue 146 (Peterson, 1981). The middle protein is coterminal with the small protein, having 55 additional amino acids (called $preS_2$) at the amino terminus (Stibbe and Gerlich, 1983; Machida *et al.*, 1984). It is a glycoprotein present in two forms, GP33 and GP36, according to the extent of glycosylation; GP33 contains one glycan at amino acid 4 of the $preS_2$ domain and GP36 is additionally glycosylated within the S domain (Stibbe and Gerlich, 1983). The large protein is also coterminal with the small and the middle proteins, having an additional 108 or 119 amino acids (called $preS_1$) at the N-terminus of the middle protein (Heermann *et al.*, 1984). The large protein is also present in a glycosylated (GP42) and a non-glycosylated (P39) form. In addition to glycosylation, both P39 and GP42 are acylated at their N-terminal glycine residue with myristic acid (Persing *et al.*, 1987). The $preS_1$ and $preS_2$ regions carry specific immunological determinants, which are discussed in detail in Chapter 5.

The S protein is a major component in the virion envelope, the filamentous and the spherical subviral particles. The middle and large proteins are quantitatively minor components of the circulating pool of S-related antigens, with the middle protein accounting for about 5%-15% of the total, and the large protein representing only about 1%-2% or even less of the total (Heermann *et al.*, 1984). The 22-nm spherical particles may consist of the S protein alone or the S and the middle proteins, but without the large protein. The proportions of the three envelope proteins differ in the different forms of HBsAg particles and appear to vary at different stages after

infection.

Which of the three S proteins performs the attachment function to hepatocytes and initiates infection is not clear. It has been proposed that the $preS_2$ domain possessed a polymerised human serum albumin (pHSA) binding site (Machida *et al.*, 1984), which may play a role in the attachment of HBV to hepatocytes using pHSA as a linker molecule (Pontisso *et al.*, 1989a). However, it has not yet been shown experimentally that pHSA mediates uptake of HBV to hepatocytes. It also appears that hepatocyte cultures do not express a pHSA receptor *in vitro*. A further attachment site of HBV has been found in the $preS_1$ domain, located between amino acid residues 21 and 47 (in subtype with 119 residues of the $preS_1$ sequence), suggesting an important function of this attachment site in the viral pathogenesis (Neurath *et al.*, 1986; Pontisso *et al.*, 1989b). Indeed, antibody against this attachment site is virus neutralising (Neurath *et al.*, 1989; Thornton *et al.*, 1989). Nevertheless, the hepatocyte receptor for HBV is not yet identified. Recent findings have suggested a role of interleukin 6 receptor (Neurath *et al.*, 1992), transferrin receptor (Franco *et al.*, 1992), or IgA receptor (Pontisso *et al.*, 1992) in mediating HBV-hepatocyte interaction.

The major S protein contains all the information required for 22 nm particle assembly and secretion. Eukaryotic cells transfected with DNA fragments bearing only the S gene produce morphologically normal 22-nm particles. Effective synthesis of the S protein has been achieved in *S. cerevisiae* (Valenzuela *et al.*, 1982; Murray *et al.*, 1984) and in some animal cells (Dubois *et al.*, 1980; Laub *et al.*, 1983). This property has been exploited to produce highly immunogenic HBsAg particles in yeast as HBV vaccines (McAleer *et al.*, 1984; Murray *et al.*, 1984). Unlike the HBsAg purified from serum, the recombinant S protein is unglycosylated, indicating that glycosylation is not required either for particle assembly or for immunogenicity. However, synthesis of the S protein in prokaryotic cells, eg. *E. coli*, was less successful, as the yields were low (MacKay *et al.*, 1981b). The S protein contains three hydrophobic regions (Stirk *et al.*, 1992). The first and the second domains are known to harbour topogenic signal

sequences, termed signals I and II, that direct the transmembrane orientation (Eble *et al.*, 1987). Mutational analysis of the three hydrophobic domains showed that they also played important roles in the subviral particle assembly and secretion (Bruss and Ganem, 1991a).

Eukaryotic cells expressing the middle protein assemble and secrete 22-nm particles that were indistinguishable from those found in serum (Dubois *et al.*, 1980; Imamura *et al.*, 1987) or reduced in size by 30% compared to those containing only the S protein, suggesting the middle protein can affect subunit packaging and, therefore, the nature of the assembled particle (McLachlan *et al.*, 1987). Mammalian cells transfected with cloned HBV DNA have been reported to express both the middle and the S proteins organised into mixed particles of 22 nm diameter (Persing *et al.*, 1985). Interestingly, the $preS_2$ domain itself lacks functional signal sequence activity and its translocation across the ER membrane is mediated by downstream signals within the S domain (Eble *et al.*, 1990). This unusual behaviour had previously only been identified in experimental fusion proteins constructed *in vitro*.

Unlike the two other envelope proteins, the large protein alone cannot form particles. When expressed alone, it remains bound to the cell membrane (Cheng *et al.*, 1986; Imamura *et al.*, 1987; McLachlan *et al.*, 1987). Moreover, when the large and the S proteins are synthesized in the same cell, the secretion of 22-nm particles is blocked (Persing *et al.*, 1986b; Ou and Rutter, 1987). The over-accumulation of retained large protein aggregates can be harmful to the host cells. It has been demonstrated that overexpression of the large protein gene in transgenic mice caused accumulation of filamentous envelope particles in hepatocytes, followed by cell damage and the development of liver cancer (Chisari *et al.*, 1989). These results suggest that the $preS_1$ domain of the large protein contains signals that promote intracellular retention of the protein. Mutational analysis in the $preS_1$ region of the adw_2 subtype (with 119 amino acid residues) led Kuroki *et al.* (1989) to postulate an ER retention signal located in the $preS_1$ amino acids 6 to 19. Since this region of the large protein is myristylated, ER retention might be attributable to the affinity of this hydrophobic fatty acid for the

ER membrane. Investigation of the role of myristylation in the large protein in secretion has led to divergent results. Single and double mutations of the myristic acid attachment sites did not lead to secretion of the protein (Kuroki *et al.*, 1989). However, similar studies employing the *ayw* subtype (with 108 amino acid residues, lacking 11 residues at the N-terminus) indicated that the block of secretion could be partially overcome by mutation or deletion of the myristylation site, and creation of a myristyl attachment site in the small protein impaired the secretion of the subviral particles but not their intracellular assembly (Prange *et al.*, 1991). The discrepancy of the result might be attributable to the different sequence between the two subtypes at their N-terminus.

The role of the three envelope proteins in the formation of Dane particles has been addressed. By transfecting hepatoma cells with mutant HBV genomes affecting the large, middle or S proteins, Ueda *et al.* (1991) showed that all three proteins were indispensable for the formation of Dane particles. However, the role of the middle protein in virion formation is disputed. Similar studies indicated that the large and S, but not the middle proteins were necessary for virion production (Bruss and Ganem, 1991b). A naturally-occurring HBV mutant unable to produce the middle protein has been shown to be competent in replication, virion formation and secretion (Fernholz *et al.*, 1991). The overexpression of the large protein inhibits virion release, while ER retention of envelope proteins mediated by the large protein and the myristylation of the large protein are not required for Dane particle formation and release (Bruss and Vanem, 1991b). In the virion budding, the envelope proteins must interact, directly or indirectly, with HBcAg. The interaction between the nucleocapsid and the envelope proteins in the formation of the virion and its budding is not yet clear. For example, which regions of HBcAg interact with which region of HBsAg? How do the core particles trigger budding?

1.5.4 HBxAg

The X ORF could encode a polypeptide which contains 154 amino acids and is termed

X (HBxAg) because the function of its product during viral infection is unknown. The X-gene product is expressed during viral infection. A 1-kb or 0.7-kb X-specific mRNA has been detected in mammalian cells transfected with the complete HBV genome or with a fragment of the HBV genome containing X ORF (Gough, 1983; Saito *et al.*, 1986). Antibody to synthetic peptides with sequences derived from the X gene has been used to detect a protein in some HBV-infected liver biopsies, HCC tissues (Moriarty *et al.*, 1985) and in cultured cells transfected with the HBV genome (Pugh *et al.*, 1986). HBxAg has also been detected by indirect immunofluorescence or immunohistochemical staining in hepatocytes from HBV-infected livers (Katayama *et al.*, 1989), hepatoma cell lines containing integrated HBV DNA (Chisaka *et al.*, 1987), and cell lines transfected with the complete HBV genome (Pugh *et al.*, 1986). Synthetic peptides representing segments of HBxAg and fusion proteins synthesized in *E. coli* with X-gene-encoded sequences have also been used to identify anti-X antibodies in the sera of some HBV-infected patients (Kay *et al.*, 1985; Weber *et al.*, 1988), indicating that the X sequence is expressed at some stage during viral infection. HBxAg-specific T-cell response has also been detected in patients with acute, and less frequently with chronic HBV infection (Jung *et al.*, 1990). *In vivo*, HBxAg is probably expressed only in very low amounts and thus far all attempts to visualise it by immunoblotting or other biochemical techniques have failed.

The X genes of HBV, WHV, and GSHV have been shown to encode proteins that transactivate transcription from a very wide array of target promoters (reviewed by Rossner, 1992). HBxAg is capable of stimulating the expression of genes under the control of autologous as well as other viral and cellular regulatory sequences, eg. Simian virus 40 (SV40), Rous sarcoma virus and human immunodeficiency virus (HIV), MHC class I, MHC class II, β-interferon and c-*myc* (Zhou *et al.*, 1990; Hu *et al.*, 1990; Twu and Schloemer, 1987; Seto *et al.*, 1988; Twu and Robinson, 1989; Siddiqui *et al.*, 1989).

Colgrove *et al.* (1989) showed that transient transfection of human hepatoma cells with the complete HBV genome containing a frameshift mutation in the X ORF

resulted in a decrease in the level of all viral mRNAs. Human hepatoma cell lines transfected with a plasmid containing the transcription units for the $preS_2$ and surface ($preS_2$/S) mRNAs and a frame shift mutation in the X mRNA also greatly reduced HBsAg secretion (Rossner *et al.*, 1990). These results suggest that the transactivating activity of HBxAg *in vivo* may contribute to the high level of HBsAg expression observed in the course of natural infection.

Several cellular regulatory sequences have been demonstrated as targets for transactivation by HBxAg, which may explain certain physiological conditions associated with HBV infection. The elevated expression of MHC class I and class II antigens on the surface of hepatocytes during HBV infection may both be attributable to transactivation of the regulatory region of the genes that encode these antigens by HBxAg (Hu *et al.*, 1990; Zhou *et al.*, 1990). As a result, the aberrant expression of MHC class I and class II antigens may influence the clinical course of the HBV infection.

Speculations exist as to the potential role of HBxAg in hepatocarcinogenesis. HBxAg coding sequence isolated from HBV integrates associated with HCC (Wollersheim *et al.*, 1988) or chronic hepatitis (Takada and Koike, 1990) maintained the capacity to transactivate, and a consequent aberration in the expression of a cellular gene involved in regulation of cellular proliferation may contribute to the development and/or maintenance of HCC. The more direct evidence linking HBxAg with HCC has been obtained through the use of HBV X-gene transgenic mice. Kim *et al.* (1991) showed that the induction of HCC in two lines of transgenic mice harbouring the X gene under control of its own promoter/enhancer complex. In contrast, transgenic mice carrying the X gene under control of the human α-1-antitrypsin regulatory region did not develop a pathological reaction (Lee *et al.*, 1990).

Because HBxAg stimulates a striking variety of promoters that do not share a common *cis*-regulatory element and it does not bind to DNA (Siddiqui *et al.*, 1987; Faktor and Shaul, 1990), it has been speculated that HBxAg acts indirectly through cellular

pathways. Recently, Kekule and colleagues (1993) showed that HBxAg uses a tumour promoter signalling pathway for transactivation. HBxAg elicits an elevation of 1,2-diacylglyceral(DAG), the endogenous protein kinase C (PKC) activator, and subsequently activates PKC, which results in activation of the transcription factor AP-1 (Jun-Fos). As a result, HBxAg transactivates through binding sites for AP-1 and other PKC-dependent transcription factors. Since the PKC signalling pathway also mediates cell transformation by tumour-promoting agents, this mechanism of gene regulation might also account for the oncogenic potential of HBxAg. However, the mechanism by which HBxAg causes an increase in DAG is still unclear. The $preS_2/S^t$ may also participate in the transcriptional control of viral gene expression via the same cellular pathway (see Section 1.8.2).

1.6 HBV variants

The adaptive immune response usually protects vertebrate hosts against virus infections either through neutralising antibody responses or virus-specific T cells. Some viruses may persist in the host despite an immune response by hiding in privileged sites, such as brain, ganglion and cornea (eg. herpes simplex virus), or by changing patterns of cytopathogenicity, growth kinetics or tissue tropisms. Other viruses escape by down-modulating viral antigen, MHC gene products, by inducing immunosuppression (eg. HIV), by changing neutralising determinants (eg. influenza virus) or by changing T-cell epitopes recognised by cytotoxic T cells (eg. HIV). Immune selection occurs not only with antibodies, which has been well documented in influenza virus as antigenic drift, but also with cytotoxic T cells. In a transgenic mouse model of lymphocytic choriomeningitis virus (LCMV) infection (Pircher *et al.*, 1990) and in HIV-infected individuals (Phillips *et al.*, 1991), the cells harbouring viruses with mutated T-cell epitopes were not recognised by cytotoxic T cells any more, hence were not destroyed resulting in the survival of the viruses.

The strategy of HBV genome replication involves the reverse transcription of an RNA intermediate. Such a process is highly susceptible to point mutation due to the lack

of proof-reading enzymes for correcting errors in duplication. Therefore, HBV is prone to mutations. In persistent HBV carriers, it is conceivable that mutations could occur at any sequences of HBV DNA, with a frequency proportional to the length of time the host harbours the virus. A mutant HBV may survive so long as the mutation does not interfere with virion formation.

1.6.1 Surface variants

A new type of HBV variant, named HBV_2, has been detected in Senegal (Coursaget *et al.*, 1987). The HBV_2 displays different HBc antigenicity from that of HBV. After the loss of HBsAg, neither anti-HBc nor anti-HBs becomes detectable. Although HBV_2 and HBV share common epitopes of HBsAg, as the HBV_2 reacts with anti-HBs in conventional tests for HBsAg, vaccine-induced anti-HBs does not protect against HBV_2 infection, suggesting that the *a* determinant of HBsAg (see Section 1.9.1) might change in HBV_2. The presence of HBV_2 might partly account for the high proportion of HBsAg(+) and anti-HBs(-) individuals in some chronic carriers. Wands *et al.* (1986) also found a variant which shares some HBsAg epitopes with HBV, but is only detected by a particular monoclonal anti-HBs antibody radioimmunoassay. Chimpanzees infected with this long-incubation variant were not protected by prior immunisation with HBsAg. Another mutant with a single amino acid change in the C-terminus of HBsAg has been reported in a patient in whom the only HBV marker was HBV DNA detected by polymerase chain reaction (PCR) (Thiers *et al.*, 1988). Whether these mutants are capable of infecting hepatocytes or/and influencing the severity of the liver disease is not known.

In HBV vaccination programmes, infants born to HBV mothers receive anti-HBs immunoglogulins and HBsAg vaccines. Approximately 95% of the vaccinees were protected, while the others did not develop an anti-HBs response or became infected in spite of adequate passive and active immunisation. Recently, a vaccine-induced escape mutant that expresses an altered *a* determinant of HBsAg has been reported independently in such infected children who responded well to the vaccine (Carman

et al., 1990; Harrison *et al.*, 1991; Fujii *et al.*, 1992; Okamoto *et al.*, 1992). In most cases, there was only a transient appearance of HBsAg in the patient's serum followed by the appearance of anti-HBc and anti-HBe, as would be expected in acute infection. However, acute hepatitis occurred in a few cases, as persistent rises in transaminases were detected (Carman *et al.*, 1990; Fujii *et al.*, 1992).

Molecular analyses of the mutant revealed a point mutation from G to A resulted in an amino acid substitution from glycine to arginine at amino acid 145 of HBsAg (Carman *et al.*, 1990; Harrison *et al.*, 1991; Fujii *et al.*, 1992). HBsAg in patient's serum may be detected by some but not other commercial kits (Fujii *et al.*, 1992). The mutation site is located within the immunodominant *a* region. The binding of the mutant to MAbs specific to this region was much reduced (Carman *et al.*, 1990). These results suggest that the mutation has resulted in a conformational change affecting one or several epitopes in the region of the *a* determinant. These antigenic changes are not surprising, as arginine is a much larger residue than glycine and is charged. The mutant, therefore, was more hydrophilic than the wild type and a new HBsAg specificity may be present. Interestingly, the same mutant has also been found in two of three liver transplant recipients who were given monoclonal anti-HBs to prevent reinfection of the homograft but who became viraemic during therapy (McMahon *et al.*, 1992). Although the children bearing this gly_{145} HBsAg mutant seldom have disease, the mutant is infectious and can cause hepatitis in chimpanzees (Ogata *et al.*, 1993). Recombinant HBsAg carrying $gly_{145} \rightarrow$arg produced in yeast is not able to bind to anti-HBs (Waters *et al.*, 1992). Thus, it is conceivable that current HBsAg vaccine might not protect against infection with this mutant. The emergence of the gly_{145} mutant has implications for vaccine development.

Although other mutations have been found in the *a* determinant, these do not seem to be important (Moriyama *et al.*, 1991; McMahon *et al.*, 1992). The substitution of threonine or isoleucine for asparagine at codon 126 of the S gene has been found in HBV-vaccinated children in a family (Okamoto *et al.*, 1992). The mutation was associated with a significant decrease in the antigenicity of some determinants on the

S protein as measured by MAbs. The same mutation was observed in 12 of 17 clones of DNA obtained from their mother before the mother-to-child transmission. This suggests a selective advantage of the mutant over the wild-type HBV under immune pressure.

The gly_{145} mutant has emerged under pressure generated by anti-HBs antibody, both vaccine induced and therapeutic. Although first found in Italy (Carman *et al.*, 1990), this escape mutant has since been reported elsewhere, eg. the Far East, England, and the USA. However, there are no data on how far such a virus has spread within the vaccinated population or the general population. The prevalence of such mutation is also little known. This information is necessary to assess the need to change vaccine components. In addition, mutants unable to produce the middle S protein have been documented (Fernholz *et al.*, 1991; Raimondo *et al.*, 1991; Santantonio *et al.*, 1992). These findings may have important implications for the usefulness of diagnostic preS protein assays and for the efficacy of new vaccines containing preS proteins.

1.6.2 Pre-core variants

The observation that some patients in the South Mediterranean and the Far East had HBV DNA measurable in their sera by dot-dot hybridisation but were negative for HBeAg (Bonino *et al.*, 1981; Chu *et al.*, 1985) led to the discovery of a mutant that was incapable of synthesizing the pre-core protein from which HBeAg is derived (Carman *et al.*, 1989). A mutant with a single substitution in the second last codon (codon 28, TGG→TAG) of the pre-core region, converting tryptophan 28 to a stop codon, hence preventing translation of the pre-core protein, was found in sera from anti-HBe(+) and HBV DNA(+) patients with chronic hepatitis. The translation of HBcAg is not affected. Thus these patients can have continuing viraemia in the absence of circulating HBeAg. The emergence of the pre-core defective mutant has implications for the use of anti-HBe as a serological marker for viral clearance.

Okamoto *et al.* (1990) also reported that various mutants occurred in the pre-core

sequence, including point mutations to convert tryptophan 28 to a stop codon or abort the initiation codon, as well as deletion and insertion to induce frameshifts, were found in sera of persistent carriers, along with seroconversion to anti-HBe(+). Among them, the same pre-core mutant changing trp_{28} to a stop codon as described by Carman *et al.* (1989) was the commonest and was found in 16 of 18 (89%) anti-HBe(+) carriers studied (Okamoto *et al.*, 1990). These mutations leading to pre-core region defects were rarely observed in persistently infected HBe(+) individuals or in patients with acute hepatitis after they had seroconverted to anti-HBe (Okamoto *et al.*, 1990). Santantonio *et al.* (1991) also indicated high prevalence, 33 out of 42 (79%) cases, and heterogeneity of the pre-core mutants in anti-HBe(+)/HBV DNA(+) patients with chronic hepatitis in the Mediterranean areas. Patients may also harbour a mixture of the wild-type and the mutant viruses during the early seroconversion phase (Okamoto *et al.*, 1990; Brunetto *et al.*, 1991; Santantonio *et al.*, 1991). Gunther *et al.* (1992) showed that several different pre-core mutants emerged from HBe(+) carriers who seroconverted to anti-HBe(+) during interferon treatment. However, the emergence of these pre-core mutants was not correlated to the outcome of treatment with interferon (Gunther *et al.*, 1992; Xu *et al.*, 1992).

It is believed that hepatitis is due to immune-mediated killing of hepatocytes and that HBcAg/HBeAg is an important target. As the host develops immune responses to HBeAg, hepatocytes secreting HBeAg would be subjected to immune elimination, whereas hepatocytes incapable of secreting HBeAg would escape it. This kind of immune selection may explain how pre-core-defective HBV replaced non-defective, wild-type HBV along with seroconversion to anti-HBe in persistent carriers. The studies of the relations between HBV heterogeneity and course of chronic infection in 145 HBsAg(+) carriers indicated that the pre-core defective mutant could be found in any phase of HBV infection (Brunetto *et al.*, 1991): in 27% of immunotolerant carriers, in 67% of patients with chronic hepatitis (immunoelimination phase) and in 17% of carriers with latent infection. These results suggest that the presence of mutant HBV can be independent from the pressure of immunoelimination. However, immunoelimination appears to determine a positive selection of the mutant.

The pre-core defective mutants have been suggested to cause severe forms of hepatitis in several clinical settings. This mutant was described in diagnosed sera of 8 out of 9 HBeAg(-)/anti-HBe(+) patients but in none of 6 HBeAg(+) patients who had fulminant hepatitis B (Carman *et al.*, 1991). However, whether patients with fulminant hepatitis were infected, *ab initio*, with the pre-core defective mutant or have this mutant early in the course of the infection under an unusually potent immune selection could not be determined. In Japan, the pre-core defective mutant was found in all 7 patients with fulminant hepatitis but in none of 10 patients with acute, self-limited hepatitis B (Omata *et al.*, 1991). In addition, the outbreaks of fulminant hepatitis in nosocomial infections (Liang *et al.*, 1991) as well as in maternally and sexually transmitted cases (Terazawa *et al.*, 1990; Yotsumoto *et al.*, 1992) have also been ascribed to pre-core defective mutants. In these cases, it has been suggested that infection *ab initio* with the pre-core defective mutants causes a more severe hepatitis than that associated with the emergence of the mutant during HBeAg/anti-HBe seroconversion (Kosaka *et al.*, 1991; Liang *et al.*, 1991). Yoshiba *et al.* (1992) reported reactivation of the pre-core defective mutant leading to fulminant hepatitis in three HBeAg(-)/anti-HBe(+) carriers following intensive cytotoxic chemotherapy for tumours.

It seems too early to claim that the pre-core defective mutant causes fulminant hepatitis, as a cause-effect relationship has not been established yet. The humoral response to HBcAg, particularly to HBeAg, is capable of inhibiting the cytotoxic T-cell killing of hepatocytes (Mondelli *et al.*, 1982; Pignatelli *et al.*, 1987). Therefore, in those patients infected *ab initio* with a pre-core defective mutant, the absence of HBeAg would allow the cytotoxic T cells (CTL) to destroy infected hepatocytes in an unrestricted way. However, this hypothesis does not explain the finding that fulminant hepatitis also occurs in HBe(+) patients infected with wild-type HBV. The alternate hypothesis is that the patient may be genetically predisposed to mount a very strong immune response against the HBe(+) wild-type virus, resulting in fulminant hepatitis and rapid selection of the mutant.

The extremely high prevalence of the pre-core defective mutant bearing the codon 28 non-sense mutation may be attributable to the following reasons. First, the mutation occurs in the sequence TGGGG; all of the Gs have been documented to be mutated to A, an example of mutation at hot-spots. Secondly, Tong *et al.* (1992) showed that the two naturally occurring pre-core mutation with stop codons in the 28th or 2nd codon had no perceptible effect on viral DNA replication, while the other eight artificial pre-core stop codon mutants were partly or completely defective in DNA replication. Because the pre-core sequence is largely overlapped by the pregenomic encapsidation signal (Junker-Niepmann *et al.*, 1990) which is required for RNA packaging, the nonsense mutation in the other codons caused the impairment of this signal resulting in lack of packaged pregenomic RNA. The replication capability of the HBV pre-core defective mutant with a stop codon in the 28th codon has been demonstrated (Tong *et al.*, 1991a and 1991b). Furthermore, DHBV mutants defective in the pre-core region can also infect susceptible ducks (Chang *et al.*, 1987; Schlicht *et al.*, 1987).

1.6.3 Core variants

Wakita *et al.* (1991) described a long span of in-frame deletions (105-183 bp) in the middle of the C gene detected in 7 out of 11 chronic hepatitis patients but not in asymptomatic carriers of HBV. The expected HBcAg or HBeAg carries deletions of amino acids located within 63-132 amino acids from the N-terminus of HBcAg. Recently, a cluster of changes in 18 amino acids (codons 84-101 from the start of the C gene) has also been found in 15 (with CAH and cirrhosis) of the 20 chronic hepatitis cases, whereas only mild liver disease (CPH) was found in the 5 patients without mutation (Ehata *et al.*, 1992).

The mutation region may contain antibody binding sites (Colucci *et al.*, 1988; Salfeld *et al.*, 1989) and T helper (Th)-cell epitopes which have been identified in mice (Milich *et al.*, 1987b). Although CTL epitopes within HBcAg/HBeAg have not been precisely identified, they could be located close the antibody binding sites since anti-

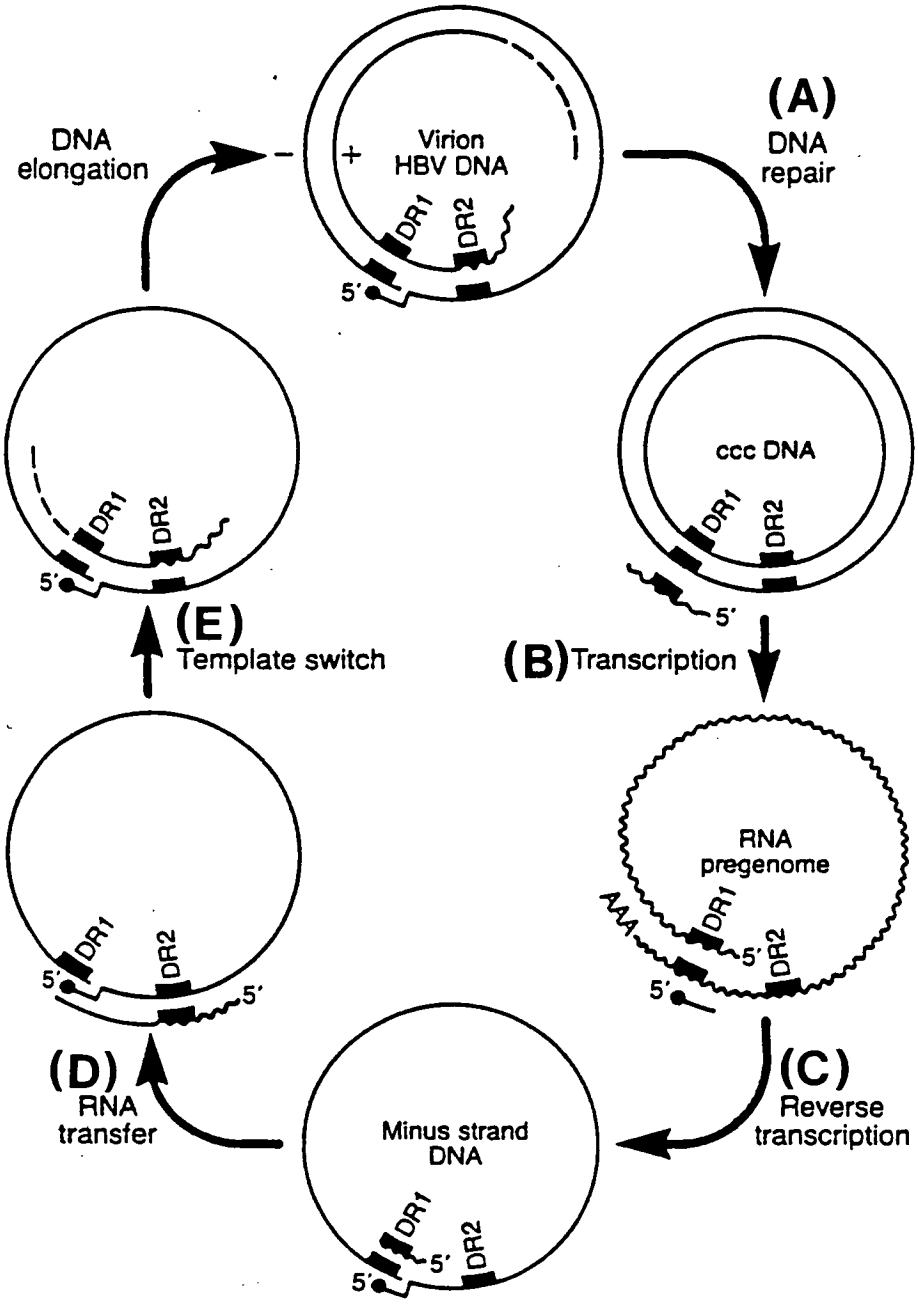
HBc and anti-HBe have been shown to inhibit the CTL response *in vitro* (Mondelli *et al.*, 1982; Pignatelli *et al.*, 1987). These results suggest that the region with substitution or deletion found in these core mutants contains the major target of CTL. However, the question may be raised whether the mutations are the result of immunological pressure or they affect the pathogenesis of hepatocellular injury in chronic hepatitis or primarily evoke lymphocyte attack. The association of these core mutants with hepatocellular injury awaits to be clarified.

1.7 Genome replication

The mechanism of the HBV genome replication is strikingly different from that of other DNA viruses. It involves a reverse transcriptase step first indicated by the findings of Summers and Mason (1982) with DHBV-infected duck livers, and confirmed later for HBV (Miller *et al.*, 1984a and 1984b) and other hepadnaviruses (Lien *et al.*, 1986; Seeger *et al.*, 1986). The model for replication of the hepadnaviruses is based on that proposed by Summers and Masòn (1982), Lien *et al.* (1986) for DHBV and by Seeger *et al.* (1986) for GSHV replication. The replication of the hepadnaviral genome involves several steps (Reviewed by Robinson and Marion, 1988; by Blum *et al.*, 1989; and by Seeger *et al.*, 1991) as illustrated in Figure 1.3.

1) Conversion of open circular DNA to covalently closed circular DNA (cccDNA) within the nucleus of infected hepatocytes. In Peking ducks 6-16 hr after DHBV infection, cccDNA can be found in the liver, which precedes other viral RNA and DNA intermediates (Mason *et al.*, 1983; Tagawa *et al.*, 1986). The formation of cccDNA further involves several steps: a) removal of the covalently bound terminal protein (TP) and the terminal redundant sequence, termed "r", from the 5′ end of the minus-strand DNA; b) ligation of the 5′ and 3′ ends of minus-strand DNA; c) removal of the RNA primer from the 5′ end of plus-strand DNA; d) completion of the plus-strand gap; e) ligation of the 5′ and 3′ ends of plus-strand DNA. The cccDNA accumulates in the nucleus where it serves as a template for transcription of mRNAs

Figure 1.3 Replication of the HBV genome. (A) Relaxed circular virion DNA is converted into ccc DNA. (B) Pregenomic RNA initiates ca. 5 nucleotides upstream of DR1 and terminates near a polyadenylation signal downstream of DR1, creating terminally redundant ends. (C) Primed by terminal protein (TP), minus-strand DNA synthesis begins at the copy of DR1 close to the 3′ end of pregenomic RNA and proceeds to the 5′ end of the RNA template, leading to a ca. 9 nucleotide-long terminal redundancy, termed "r", while at the same time the pregenomic RNA is degraded by RNase H. (D) Plus-strand DNA synthesis is primed by an RNA oligomer bearing a copy of DR1. The RNA primer derived from the 5′ end of pregenomic RNA transported to the DR2 region of the minus-strand DNA. (E) Plus-strand DNA synthesis proceeds to the 5′ end of minus-strand DNA is necessary for the continuation of DNA synthesis. (Adapted from Blum *et al.*, 1989).


DNA
elongation
Virion
HBV DNA
DR1
DR2
5′
(A)
DNA
repair
ccc DNA
(B) Transcription
RNA
pregenome
AAA
(C)
Reverse
transcription
Minus strand
DNA
(D)
RNA
transfer
(E)
Template switch

and pregenome RNA, the template of new DNA genomes by reverse transcription (Summers and Mason, 1982; Miller and Robinson, 1984; Tagawa *et al.*, 1986). cccDNA is then amplified not only from infecting viral DNA as described above but also through *de novo* synthesis by reverse transcription (Tuttleman *et al.*, 1986; Wu *et al.*, 1990). cccDNA levels in persistently infected hepatocytes *in vivo* have been estimated at various values, ranging from 3 to 50 copies per nucleus (Summers *et al.*, 1990). The low copy numbers suggest that amplification of cccDNA in natural infections occurs at the early stage of the infection and the size of the pool of cccDNA may be limited by specific control mechanism. Summers and colleagues (1990) showed that the viral envelope proteins are required for regulation of cccDNA amplification.

2) Transcription of minus-strand cccDNA by host RNA polymerase to generate a 3.5-kb RNA, the pregenomic RNA. That cccDNA prepared from cloned viral DNAs are infectious *in vivo* supports the notion that cccDNA is the template for the transcription of the pregenomic RNA (Will *et al.*, 1982; Seeger *et al.*, 1984). This RNA contains terminally redundant ends (termed "R") and three copies of an 11-bp direct repeat (DR), which contains signals important for minus- and plus-DNA synthesis. DR1 is located at both the 5′ end and near the 3′ end, while a third copy of the repeat, termed DR2, is located upstream of DR1 close to the 3′ end of pregenomic RNA (Buscher *et al.*, 1985; Enders *et al.*, 1985).

3) Encapsidation of pregenomic RNA. The RNA pregenome is transferred to the cytoplasm and packaged along with the newly-synthesized viral DNA polymerase (reverse transcriptase) into core particles in which reverse transcription occurs. A sequence located near the 5′ end of the RNA pregenome encompassing the last two-thirds of the pre-core region and the beginning of the C gene has been identified as a *cis*-acting element, termed pregenome encapsidation signal (ε), for RNA packaging (Junker-Niepmann *et al.*, 1990). The P protein containing all functional domains is required as a structural component for RNA pregenome packaging, as known enzymatic activities of the P protein are not involved in the packaging reaction

(Bartenschlager *et al.*, 1990). In addition, the protamine-like C-terminus of HBcAg is important for RNA packaging (Gallina *et al.*, 1989; Birnbaum and Nassal, 1990).

4) Synthesis of minus-strand DNA by reverse transcription of pregenomic RNA (Mason *et al.*, 1982; Summers and Mason, 1982). DNA synthesis is initiated at DR1 near the 3′ end of the RNA template, as the 5′ end of minus-strand DNA is mapped to the DR1 sequence at the 3′ end of pregenomic RNA (Seeger *et al.*, 1986; Seeger and Maragos, 1990). Terminal protein (TP) covalently attached to the 5′ end of minus-strand DNA is believed to serve as a primer for synthesis of minus-strand DNA (Gerlich and Robinson, 1980; Molnar-Kimber *et al.*, 1983), and is encoded by the P gene (Barterschlager and Schaller, 1988; Bosch *et al.*, 1988; Radziwill *et al.*, 1990). The RNA template is degraded by viral P protein (RNase H domain) as DNA synthesis proceeds (Radziwill *et al.*, 1990).

5) Synthesis of plus-strand DNA by copying the minus-strand DNA. Synthesis of plus-strand DNA is primed by an RNA oligomer (with 17-18 nucleotides), which is donated from the 5′ end of the RNA pregenome and bears a copy of DR1 (Lien *et al.*, 1986; Seeger *et al.*, 1986). The RNA primer is transposed to the DR2 region of the minus strand and primes the synthesis of plus-strand DNA (Lien *et al.*, 1986; Seeger *et al.*, 1986). When the 3′ end of the elongating plus-strand DNA reaches the 5′ end of the minus-strand DNA template, it must switch template to the 3′ end of the minus-strand DNA forming a circular molecule. The 3′ end of the new plus strand dissociated from the 5′ end of the minus strand contains a sequence complementary to the short terminal redundancy (r) of the minus strand. This complementary sequence in the plus strand could then base-pair with r at the 3′ end of the minus strand, resulting in circularisation of the DNA and positioning of the 3′ end of the minus strand for use as the template for continued elongation of the plus strand. The failure of plus strand to proceed to full-length may be attributable to the constraining of viral polymerase within the viral core.

During the process of minus- and plus-strand DNA synthesis, the core particle are

assembled into mature virions by coating with post-translationally modified HBsAg and then exported from the hepatocytes in the blood stream.

1.8 Viral transcription

HBV genome replication involves the reverse transcription of an RNA intermediate, termed pregenomic RNA. Therefore, viral transcription has a dual function: the synthesis of the RNA pregenome, which serves as a template for genome replication, and the synthesis of mRNAs for viral protein synthesis. It appears that the cccDNA, the first viral DNA form detected after infection and the only form found in the nucleus, is the template for viral transcription which is carried out by host RNA polymerase II.

1.8.1 The HBV transcripts

Four different transcripts which can be divided into two species, genomic and subgenomic, have so far been identified (reviewed by Blum *et al.*, 1989; and by Schaller and Fischer, 1991). All of these RNAs are unspliced with the same polarity and are polyadenylated at a common 3′ terminus (Figure 1.2). The genomic transcripts are greater than one genome-unit in length (3.5 kb) and serve as template for viral DNA synthesis as well as for the pre-C, C and P gene products (Will *et al.*, 1986 and 1987). Their synthesis is liver-specific (Will *et al.*, 1987) and controlled by the core promoter (or genomic promoter), which has been identified within the 150 nucleotides upstream of the genomic RNA start site with no "TATA-box" homology (Yaginuma and Koike, 1989). The core promoter directs the synthesis of two closely related subsets of genomic transcripts which have been designated according to their functions as pre-core mRNA and C mRNA/pregenome. The slightly shorter C mRNA/pregenome is synthesized more abundantly than the pre-core mRNA and is the messenger for HBcAg and the DNA polymerase (Ou *et al.*, 1990). Furthermore, after assembly into nucleocapsid containing pregenomic RNA and viral DNA polymerase surrounded by the core proteins, this RNA becomes the template for reverse

transcription to synthesize the viral DNA genome. The slightly longer pre-core mRNA is the messenger for the pre-core protein which is the precursor of HBeAg. The pre-core mRNAs are not packaged into nucleocapsids (Enders *et al.*, 1987; Will *et al.*, 1987; Nassal *et al.*, 1990). Expression of the 3.5-kb transcript has been observed only in hepatic cell lines infected with HBV or transfected with its DNA (Will *et al.*, 1987). Therefore, it seems likely that liver-specific host factors are required for genomic RNA transcription.

The subgenomic transcripts consist of three species with 2.4, 2.1 and 0.9 kb in length, among which the 2.1-kb mRNA is the most abundant detected in the acutely infected liver. The 2.1-kb mRNAs (or the $preS_2/S$ transcripts) are heterogeneous in size and are the templates for the synthesis of the S and the middle proteins specified by the same reading frame (Cattaneo *et al.*, 1983 and 1984). A longer RNA is the messenger for the middle protein, while several shorter RNAs with similar length, which initiate from different position, are the messengers for the S protein. Synthesis of these 2.1-kb mRNAs is also controlled by a single promoter, termed the $preS_2/S$ promoter (Raney *et al.*, 1989). This promoter also lacks a "TATA-box" sequence, instead, the DNA sequence upstream of the $preS_2/S$ mRNA start sites contains homology to GC-rich elements of the SV40 late promoter (Cattaneo *et al.*, 1983). The 2.1-kb $PreS_2/S$ transcripts have been detected in several transfected cell lines derived from non-hepatic tissues, suggesting that expression of these transcripts is not particularly tissue- or species-specific (Laub *et al.*, 1983). However, it is possible that expression of the transcripts might be substantially more efficient in hepatocytes.

The 2.4-kb mRNAs (or the $preS_1$ transcripts) are the templates for the synthesis of the large protein (Will *et al.*, 1987). The $preS_1$ promoter contains a "TATA box" sequence found in most eukaryotic promoters, which is highly liver-specific (Antonucci and Rutter, 1989) and displays much lower activity than the $preS_2/S$ promoter (Cattaneo *et al.*, 1984; Yaginuma *et al.*, 1987; Raney *et al.*, 1990).

From its map position, a polyadenylated transcript of about 0.9-kb in length is a

candidate for a mRNA for X-gene expression. Gough (1983) demonstrated a 1.0-kb transcript which appeared to map to the X region in HBV-transfected rat cells. In human cells transfected with HBV, a 0.7-kb transcript which coded for X protein was also identified (Saito *et al.*, 1986). In woodchucks with acute or chronic WHV infections, an X-region-specific transcript of approximately 0.65 kb was found primarily in the nucleus of hepatocytes (Kaneko and Miller, 1988). However, the X transcript has not been identified during the course of HBV infections. The production of this mRNA seems to be drastically repressed under conditions supporting viral replication. Apart from the core, $preS_2/S$, and $preS_1$ promoters, the fourth promoter element, the X promoter, located upstream from the X gene which directs transcription of a heterologous reporter gene has been identified (Siddiqui *et al.*, 1987; Treinin and Laub, 1987).

1.8.2 The regulation of viral transcription

Besides the four promoters, which direct the synthesis of the four different transcripts, and the single polyadenylation site, two enhancer elements have been identified in the HBV genome (Figure 1.2). One is located as a part of the X promoter overlapping the 3′ end of the P gene, which is generally known as the enhancer and has been recently designated enhancer I (Shaul *et al.*, 1985), and the other is located downstream of the enhancer I and immediately upstream of the core promoter overlapping the 3′ end of the X gene, and is designated enhancer II (Yee, 1989). Transcription from all HBV promoters is increased in the presence of the enhancer I (Hu and Siddiqui, 1991). The enhancer I is also able to stimulate heterologous promoters, eg. the herpes simplex virus thymidine kinase promoter (Antonucci and Rutter, 1989; Yee, 1989) and the SV40 early promoter (Shaul *et al.*, 1985; Chang and Ting, 1989). There are disparate results concerning the liver specificity of the enhancer I (Elfassi, 1987; Chang and Ting, 1989; Patel *et al.*, 1989; Yee, 1989). In contrast, the enhancer II functions exclusively in a liver-specific manner, thus it is also termed liver-specific enhancer (Yee, 1989; Yuh and Ting, 1990).

Negative regulatory elements within the HBV genome have been detected. It has been shown that a negative regulatory element within the $preS_1$ coding region could down-regulate transcription from the $preS_1$/S promoter (De-Medina *et al.*, 1988; Bulla and Siddiqui, 1989). Another negative regulatory element, a silencer element, located within the X ORF and near the core promoter has also been found to down-regulate the activity of the core promoter, and the SV40 early promoter in an orientation-independent, but a position-dependent, manner (Gerlach and Schloemer, 1992).

Viral transcription is also regulated by viral gene products. It has been shown that HBxAg is capable of acting as a transcriptional transactivator of a wide range of homologous and heterologous transcriptional regulatory sequences of viruses and various cellular genes (Reviewed by Rossner, 1992). HBxAg regulates transcription from the HBV core promoter/enhancer (Colgrove *et al.*, 1989; Siddique *et al.*, 1989), the $preS_2$/S promoter/enhancer (Colgrove *et al.*, 1989; Rossner *et al.*, 1990), the $preS_1$ promoter/enhancer (Raney *et al.*, 1990; Rossner *et al.*, 1990), and the X promoter/enhancer (Siddiqui *et al.*, 1989). In addition, it has been demonstrated that a 3′-truncated $preS_2$/S region of integrated HBV DNA expressed in a human hepatoma cell line and tissue encoded a C-terminally truncated middle surface protein ($MHBs^t$), which exerted a transcriptional transactivator function (Kekule *et al.*, 1990; Lauer *et al.*, 1992). This transactivation phenomenon is at least in part linked to the protein kinase C signalling pathway (Natoli *et al.*, 1992). This mechanism has recently been demonstrated to be used by HBxAg for transactivation (Kekule *et al.*, 1993).

1.9 Immune response to HBV

Immune response to HBV antigens plays a major role in determining pathological sequelae in acute and chronic HBV infection. The serological profiles of acute and chronic disease states are presented in Figure 1.4. Termination of the HBV infection occurs with the disappearance of HBsAg and the appearance of anti-HBs. The presence of HBsAg (and perhaps preS)-specific cell-mediated immunity might be crucial for the removal of replicating virus in hepatocytes during acute hepatitis. In

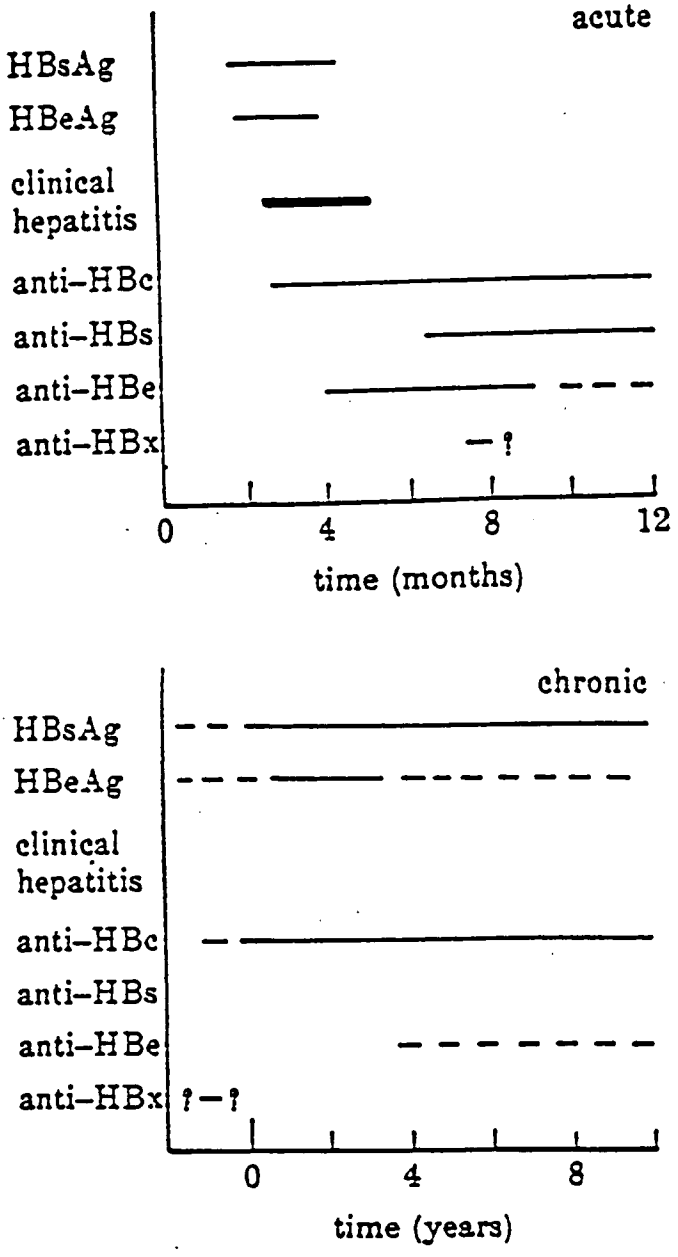


Figure 1.4 The serological profiles during acute and chronic HBV infection. Variations in the time at which the various markers appear in individual cases are indicated by dash lines. (Adapted from Murray, 1987).

addition to the cellular immune responses for clearance of infected hepatocytes, neutralising antibodies are required to prevent spread of released virions to uninfected liver cells. The establishment of the chronic carrier state could be regarded as a failure to mount appropriate immune responses to HBV infection. It is generally accepted that the pathogenesis of chronic HBV infection is immunologically mediated and not due to direct cytopathic effects of the virus. T-cell responses to HBcAg on the surface of infected hepatocytes play an important role in the pathogenesis of liver damage in chronic HBV infection (Mondelli *et al.*, 1982).

1.9.1 Immunology of HBsAg

The antigenic specificities of HBsAg are complex. All HBsAg preparations possess the dominant, group-specific determinant *a* and two pairs of subtype determinants *d* or *y* and *w* or *r* which are, for the most part, mutually exclusive. Therefore, four HBsAg serological subtypes are generally encountered, namely, *adw, adr, ayw* and *ayr*. However, isolated cases of unusual HBsAg subtype determinants, such as *awr, adwr, adyw, adyr* and *adywr*, have been reported (Okamoto *et al.*, 1987; Yamanaka *et al.*, 1990). The "compound" subtype may be due to phenotypic mixing either by co-infection of hepatocytes with one original subtype and the mutant strain induced by point mutation which leads to subtype conversion or by double infection of hepatocytes with two HBV strains of different subtypes, which might be introduced to HBV carriers in early stages while immune responses are immature, or to those who are immunocompromised (Yamanaka *et al.*, 1990). The HBsAg subtypes are unevenly distributed around the world and have provided useful markers for epidemiologic studies.

The *a* determinant of the S region has been located between amino acid residues 138 and 149 (Prince *et al.*, 1982), as well as 139 and 147 (Bhatnagar *et al.*, 1982) by peptide epitope mapping and antigenic prediction approaches. An additional *a* determinant has also been identified with a disulphide induced cyclic peptide corresponding to residues 122 to 137 of HBsAg (Dreesman *et al.*, 1982; Ionescu-Matiu

et al., 1983). The *a* determinant is composed of more than one epitope since panels of non-crossreacting *a*-specific monoclonal antibodies exist (Swenson *et al.*, 1988). The majority of the *a* epitopes are conformationally sensitive and likely to be discontinuous. The spatial arrangement of the antigenic region appears to be stabilised by cystine bridges. Cyclisation of peptides corresponding to the *a* region through cysteine residues 124 and 137 (Dreesman *et al.*, 1982) or 139 and 147 (Brown *et al.*, 1984) results in increased antigenicity. It was also shown that the replacement of cysteines 124 and 147 by serines drastically reduced or eliminated antigenicity and proline residue 142 was required for the display of full antigenicity (Ashton-Rickardt and Murray, 1989a). In contrast, cysteine 121 can be changed to serine without affecting antigenicity (Antoni and Peterson, 1988). Antibodies to the *a* determinant provide protection against infection by any of the HBV subtypes (Szmuness *et al.*, 1982). Some of the antibodies to the *a* determinant have been shown to be capable of neutralising both *ad* and *ay* subtypes of HBV (Iwarson *et al.*, 1985a).

Extensive studies on the immunogenicity of three forms of HBsAg and on the regulation of the immune response to them have been carried out in inbred mice by Milich and his colleagues. Antibody production and T-cell response, including HBsAg-specific T cell proliferation and interleukin-2 (IL-2) production, to the S protein are regulated by at least two immune response (Ir) genes mapping in the I-A and I-C subregions of the H-2 locus (Milich and Chisari 1982; Milich *et al.*, 1985a). HBsAg high-responder and non-responder haplotypes have been identified. Multiple T cell recognition sites on HBsAg, dependent on the H-2 haplotype of the responder strain, have also been recognised (Milich *et al.*, 1985c). Furthermore, the T cell recognition sites do not necessarily overlap with B cell recognition sites (Milich *et al.*, 1985c). Different B-cell as well as Th-cell epitopes of S, $preS_1$ and $PreS_2$ regions are recognised in different inbred strains (Milich and Chisari, 1982; Milich *et al.*, 1986b and 1987a).

The $preS_2$ antigen is more immunogenic than the S antigen in terms of antibody production *in vivo* with respect to the effective immunisation dose and the magnitude

as well as the time of onset of the primary immune response (Milich *et al.*, 1985d). The $preS_2$ antigen also appears to be more immunogenic than the S antigen at the T-cell level in terms of *in vitro* T-cell activation, including the effective priming dose, the magnitude of the T-cell response, and the quantitative *in vitro* dose-response curve (Milich *et al.*, 1985b). Furthermore, the immune response to $preS_2$ antigen is regulated by H-2 linked genes distinct from those regulating the immune response to S antigen, such that immunisation with $preS_2$ antigen of mice responsive to $preS_2$ antigen but non-responsive to S antigen can circumvent non-responsiveness to the S antigen. Therefore, the $preS_2$ antigen appears capable of generating Th-cell activity that can induce S-region as well as $preS_2$-region antibody production, thereby circumventing non-responsiveness to the S region (Milich *et al.*, 1985b and 1985d). Although the antibody response to the $preS_2$ region is largely group-specific (Milich *et al.*, 1986b and 1990c), the $preS_2$-specific T-cell response is highly subtype-specific (Milich *et al.*, 1990a).

Similar characteristics have been described for the immune response to $preS_1$ antigen. The genetic regulation of anti-$preS_1$ antibody production can be independent of the anti-S and anti-$preS_2$ regions, as immunisation with HBsAg/P39 particles containing the $preS_1$ region can bypass non-responsiveness to the S and $preS_2$ regions in terms of antibody production (Milich *et al.*, 1986a). Therefore, although the production of antibody to the three regions of HBsAg is independently H-2-restricted, $preS_1$- and $preS_2$-specific T cells can provide functional help not only to B cell clones of the corresponding antigen specificity, but also to S region-specific B cell clones. Other results suggest that antibodies to $preS_1$ and $preS_2$ are virus-neutralising (Neurath *et al.*, 1985). These results argue that inclusion of the preS region may augment the effectiveness of future HBV vaccines by providing a more immunogenic HBsAg particle and by decreasing the incidence of genetic non-responsiveness. If the human T cell response to $preS_2$ is focused on the subtype variable C-terminal region, which is the case in murine T cell recognition (Milich *et al.*, 1990a), there may be a case for including both major subtypes of HBsAg/P33 in $preS_2$-containing vaccines, whereas the $preS_2$-containing vaccines currently in clinical trials are composed of a single

HBsAg subtype (Tron *et al.*, 1989). However, so far there is no clinical evidence to prove that the $preS_2$-containing vaccines are superior to the S protein-containing vaccines.

The S protein bears B-cell determinants important for the induction of a protective immune response in man. Both individuals who recover from an acute HBV infection and those who respond properly to the HBsAg vaccine produce significant amounts of anti-HBs antibodies. On the other hand, patients with chronic HBV infections and non-responders to the vaccine produce little, if any anti-HBs. Indeed, the HBV vaccine produced from plasma-derived HBsAg and treated with pepsin in its manufacturing contains no $preS_1$ or $preS_2$ region but only S region, as does the vaccine produced in yeast containing only the S coding sequence (Murray *et al.*, 1984).

In the course of acute HBV infection, the appearance of anti-preS antibodies precedes anti-S antibody. The antigenic determinants of $preS_2$ appear to be linear, as they are resistant to reduction of disulphide bonds (Neurath *et al.*, 1984). The disulphide bond-independent determinants on the $preS_2$ region may be more easily mimicked by peptide analogues than "conformational" determinants on the S region. The protective role of anti-preS antibody is supported by chimpanzee-challenge experiments. The complete $preS_2$ region or a synthetic peptide derived from it has been shown to confer protective immunity in chimpanzees (Itoh *et al.*, 1986; Emini *et al.*, 1989; Thornton *et al.*, 1987 and 1989). A synthetic peptide derived from the $preS_1$ region (preS 12-47) also elicits antibodies protective against HBV infection in chimpanzees (Neurath *et al.*, 1989; Thornton *et al.*, 1989).

In humans, T cell sensitisation to HBsAg in acute and chronic HBV infection is usually undetectable and when measurable is expressed transiently and at low level (Ferrari *et al.*, 1986 and 1990). Vento *et al.* (1987) evaluated prospectively the T cell sensitisation to HBV antigens from the preclinical phase of acute hepatitis. The first detectable response in terms of T lymphocyte migration inhibitory factor (T-LIF) production (about 1 month before the first evidence of liver damage) was directed to

$preS_2$ determinant, followed by the appearance of T cell sensitisation to HBcAg and finally to HBsAg. These T cell responses became undetectable shortly after the clinical symptoms. After recovery, T cells were again responsive to HBsAg and $preS_2$ antigen, whereas sensitisation to HBcAg was no longer detectable. Mishra *et al.* (1992) also showed the presence of T cell response in terms of lymphocyte proliferation against the $S_{(124\text{-}147)}$ peptide during early acute hepatitis. This response was suppressed during late stages followed by partial reversal during recovery. The decline in T cell responsiveness during the late acute phase of hepatitis may be due to immunosuppression (Barnaba *et al.*, 1985) and persistence of the same may also be involved in the development of chronicity. Ferrari *et al.* (1990) found positive lymphocyte responses to the envelope proteins, which although low, were almost equally distributed in the different groups of patients studies [HBeAg(+) CAH, anti-HBe(+) CAH, or chronic asymptomatic carriers] and the S-, $preS_1$-, and $preS_2$-specific T cells were simultaneously elicited. The T cell response to HBsAg in vaccine recipients has been investigated. Celis *et al.* (1984) showed that the PBLs from most of the vaccinees did not proliferate to a great extent to HBsAg *in vitro*. However, the lymphocyte proliferation to HBsAg could be substantially increased with some of the non-responding individuals if their peripheral blood lymphocytes (PBLs) were maintained in culture in the presence of HBsAg and IL-2. Since production of anti-HBs antibody is T-cell dependent (Roberts *et al.*, 1975), T cell sensitisation to HBsAg plays an important role in the clearance of HBV.

1.9.2 Immunology of HBcAg and HBeAg

Despite the fact that HBcAg is an internal component of the virion, high-titred anti-HBc antibodies are regularly produced by virtually 100% of HBV-infected individuals. Furthermore, anti-HBc of IgM class appears early in acute hepatitis B, before anti-HBs, and IgM and IgG anti-HBc can persist with slowly decreasing titres for many years. In contrast, anti-HBe antibody may not develop or may appear at various times after the appearance of anti-HBc, and its presence is correlated with viral clearance. Although HBcAg and HBeAg are structurally related and highly cross-reactive at the

T-cell level (Milich and McLachlan, 1986; Milich *et al.*, 1988b), they are serologically distinct and the immune responses to them appear to be regulated independently.

In the murine system, Milich and McLachlan (1986) demonstrated that HBcAg can induce antibody response via both T-cell-dependent and T-cell-independent pathways. In contrast, anti-HBe production is T-cell-dependent. Anti-HBc antibody production and the HBcAg-specific T cell response are all influenced by similar H-2-linked-genes (Milich *et al.*, 1987b). The anti-HBc response is significantly greater than the anti-HBsAg response in all strains of mice and no non-responder strains have been identified. Multiple, but distinct, T-cell sites on HBcAg are also recognised by individual inbred strains of mice (Milich *et al.*, 1987b). Agretopic (residues binding to MHC molecules) and epitopic (residues binding to T-cell receptors) residues within a dominant T-cell determinant of the HBcAg have been demonstrated in a certain haplotype of mice (Milich *et al.*, 1989). This murine model predicts that a human outbred population would exhibit similar complexity, and individuals may recognise distinct T-cell sites in the context of their specific HLA genotypes. Although neither anti-HBc nor anti-HBe antibody is virus-neutralising, vaccination with HBcAg can confer protection against HBV infection in chimpanzees (Murray *et al.*, 1984 and 1987; Iwarson *et al.*, 1985b). Milich *et al.* (1990b) investigated the consequences of *in utero* exposure to HBeAg on HBcAg/HBeAg-specific immune responses by using HBeAg-expressing transgenic mice as a model. The HBeAg transgenic mice were tolerant to both HBeAg and HBcAg at the T-cell level. They did not produce antibody to HBeAg, but did produce anti-HBc *in vivo* and *in vitro*. The finding that *in utero* exposure to HBeAg renders T cells non-responsive to HBeAg as well as to HBcAg may be relevant to the mechanisms of T-cell tolerance in human HBV infection because HBeAg is more likely than HBcAg to traverse the placenta.

The studies by Milich *et al.* (1987c) demonstrated that HBcAg-specific T cells are capable of providing Th-cell activity for anti-S, anti-preS_1 and anti-preS_2 antibody production provided HBcAg and these envelope proteins are present within the same particle or entity. This fact may explain the mechanism of protection by HBcAg in

chimpanzees (Murray *et al.*, 1984). Furthermore, these HBcAg-specific Th cells were shown to induce anti-HBs production in HBsAg-non-responder mice (Milich *et al.*, 1987c). These studies describe a mechanism for circumventing HBsAg non-responsiveness in which the only requirement was that HBcAg and HBsAg be present within the same particle, eg. HBV. A similar result was obtained by using a Th-cell epitope from HBcAg, instead of HBcAg itself, as a T-cell carrier moiety. A synthetic peptide composed of a Th-cell epitope from HBcAg and a B-cell epitope from HBsAg can elicit production of anti-peptide antibody, that is cross-reactive with the native HBsAg (Milich *et al.*, 1988a). These results indicate the potential use of HBcAg or HBcAg-specific Th-cell epitopes as carrier moieties for a multivalent vaccine against HBV and other infectious agents. Moreover, HBcAg was shown to function efficiently as an immunologic carrier moiety for DNP (dinitrophenyl) hapten in euthymic as well as athymic mice in contrast to conventional carrier proteins (Milich *et al.*, 1988a). Therefore, the T-cell-independent nature of HBcAg may be exploited to advantage for vaccine development for use in individuals who are T-cell compromised.

In chronic HBV infection, Vento *et al.* (1985) showed that T lymphocytes from patients with chronic HBV infection were able to produce T-LIF in response to HBcAg but not to HBsAg. Ferrari *et al.* (1986) demonstrated that peripheral blood T cells bearing the $CD4^+$ (helper/inducer) phenotype from patients with chronic HBV infection were sensitised by HBcAg. The intrahepatic, infiltrating lymphocytes, which may be more representative of events occurring at the site of HBV antigen synthesis and cellular injury, contain functionally competent Th cells and CTLs that are specific for HBcAg and are capable of regulating each other's activities (Ferrari *et al.*, 1987a and 1987b).

There is accumulating evidence that HBcAg is a major target for a specific cell-mediated immune response during chronic HBV infection. HBV-infected hepatocytes may be destroyed *in vitro* by autologous peripheral blood T cells and that killing is inhibited by anti-HBc but not by anti-HBs antibodies (Mondelli *et al.*, 1982; Naumov

et al., 1984). A reduction of T cell cytotoxicity against autologous hepatocytes similar to that obtained with anti-HBc antibodies has also been observed using monoclonal anti-HBe antibodies (Pignatelli *et al.*, 1985). Further evidence in support of HBcAg as the primary target antigen comes from immunofluorescence studies showing that cytoplasmic/membranous distribution of HBcAg is associated with CAH compared to a nuclear distribution of HBcAg in patients with CPH, while membranous HBsAg is present in both CAH and CPH (Chu *et al.*, 1987), suggesting that the immune system may recognise HBcAg and thereby contribute to pathology by eliminating some cells expressing HBcAg and replicating virus. However, it is not clear whether HBcAg is also the target for recognition by CTL during acute hepatitis B. It was shown that monoclonal anti-HBc antibodies were able to inhibit cytotoxicity by approximately 50% in acute hepatitis infection (Mondelli *et al.*, 1987). The remaining activity which was not inhibited by anti-HBc antibodies presumably was due to natural killer cells which have been shown to be active during the course of acute HBV infection (Chemello *et al.*, 1986). These results suggest that HBcAg can also be a major target for recognition by CTL during acute infection.

1.10 Vaccine development

Conventional vaccines based on inactivated or attenuated infectious agents have some disadvantages. With inactivated vaccines there is always the need to ensure that the product no longer contains any live organisms. Moreover, the handling of large volumes of virulent organisms is a hazard to the personnel involved. With attenuated vaccines the disadvantages include the possible presence of adventitious agents in the cells or medium used for production, the hazard of reversion to virulence, and the need for storage at low temperature. These factors encouraged the development of new vaccines based on recombinant DNA technology, the best example of which is the HBV vaccine based on the S protein produced in yeast. In fact, the first hepatitis B vaccines were prepared from plasma of asymptomatic HBV carriers using purified 22 nm HBsAg particles. This is a unique feature among various vaccines available at present. The drawback of the difficulty to propagate HBV *in vitro* has prevented

the development of conventional vaccines from virus propagated in cell culture. Since HBsAg has been proved to elicit neutralising or protective antibody as shown by serological surveys and experimental transmission studies in human volunteers and chimpanzees, purified HBsAg particles isolated from HBV carriers have been developed as vaccines. The plasma-derived vaccines have been shown to be safe and effective (Szmuness *et al.*, 1980) and have been widely used throughout the world. However, the source of HBsAg is relatively limited and is not uniform, and extensive purification procedures and safety testing are required, but HBsAg can now be produced in yeast on a large scale for use as a vaccine in humans (Murray *et al.*, 1984).

The use of synthetic peptides for vaccination is one alternative which is vigourously being explored. The synthetic vaccines contain a relatively small peptide or peptides shown to constitute epitopes of the organisms which elicit a protective immune response. Since they can be chemically synthesized in unlimited quantities and do not contain infectious agents, they might prove to be the vaccines of choice if found sufficiently efficacious. Since the dominant B-cell epitopes on the S protein are discontinuous, it has proved difficult to mimic them by linear synthetic peptides. This problem is partially overcome by using cyclic peptides generated by intramolecular disulphide bond formation between cysteine residues present in the peptides (Dressman *et al.*, 1982; Brown *et al.*, 1984). Successful mimicking of determinants of the S protein using synthetic peptides has been reported (Prince *et al.*, 1982; Dressman *et al.*, 1982; Brown *et al.*, 1984). Since enhancement of the immunogenicity of the preS region of HBsAg has been demonstrated in mice (Milich *et al.*, 1985b and 1985d), and the peptides derived from the $preS_1$ or $preS_2$ are protective against HBV infection in chimpanzees (Itoh *et al.*, 1986; Emini *et al.*, 1989; Neurath *et al.*, 1989; Thornton *et al.*, 1987 and 1989), it is suggested that the inclusion of the B-cell epitopes from the preS region into S protein-based HBV vaccines might broaden the repertoire of anti-HBV-specific antibodies elicited by active immunisation.

The first crucial step in raising protein-reactive anti-peptide antibodies that can provide protective immunity is identification of the protein fragment(s) comprising B-cell epitope(s). The only exact way to identify B cell epitopes is to understand the three-dimensional structure of antigens or of antigen-antibody complexes. However, because such studies are difficult to carry out, many attempts to predict B-cell epitopes have been made. Because the antigenic determinants recognised by B cells can be either continuous or assembled topographic sites (discontinuous) and their location has been related to accessible, hydrophilic and mobile structures on the antigen, most predictive methods for B-cell epitopes are based on selecting amino acid sequences typical of such features (Hopp, 1986), such as hydrophilicity (Hopp and Woods, 1981), β-turn propensity (Chou and Fasman, 1978), and acrophilicity (Hopp, 1986). The acrophilicity scale was derived by visual observations to determine the frequency of occurrence of the amino acids in highly exposed locations on the surface of proteins. In contrast to the hydrophilicity scale, which is based on the water solubility of the amino acids, the acrophilicity scale is, for the most part, a size scale. Some computer programmes containing relevant algorithms for prediction of B-cell epitopes have also been developed (Menendez-Arias and Rodriguez, 1990). Practical approaches to defining B-cell epitopes on a specific protein have concentrated largely on immunising laboratory animals with synthetic peptides predicted as described above, and seeking antibody reactivity with the native protein. Recently, libraries of random peptide sequences were constructed in filamentous phages and screened to identify peptides that specifically bind to known antibodies or other proteins (Scott and Smith, 1990; Devlin *et al.*, 1990). The epitopy library may provide a simple, inexpensive alternative to chemical synthesis of peptides for mapping B-cell epitopes, and has the advantage of being completely unbiased.

It was generally believed that, because of their small size, peptides would behave like haptens and would therefore require coupling to a large "foreign" protein carrier, such as bovine serum albumin, keyhole limpet haemocyanin (KLH) or thyroglobulin, to enhance their immunogenicity. The choice of appropriate carriers and the method of peptide-carrier linkage are of primary importance. Tetanus toxoid (TT) has been

employed in many studies because it has been used for human vaccination for many years without side effects. Moreover, because most humans have been immunised with TT, the response against a synthetic peptide conjugated to TT carrier should be improved by the pre-existence of an anti-carrier immunity. However, epitopic suppression could occur as demonstrated by Schutze *et al.* (1985). A peptide with helper, but not suppressor function within TT has been identified and may circumvent epitope-specific suppression (Etlinger *et al.*, 1990). Other carrier systems have been explored, such as liposomes (Garcon and Six, 1991), lipids (Goodman-Snitkoff *et al.*, 1991; Schild *et al.*, 1991) and some integral membrane proteins isolated from *E. coli* (Croft *et al.*, 1991).

In fact, synthetic peptides can be highly immunogenic in their free form if they contain not only a B-cell epitope but also a Th-cell epitope. The 141-160 peptide from VP1 of FMDV is a good example of such a peptide. In its unconjugated form, this peptide is capable of eliciting a neutralising antibody response that will protect experimental animals against infection (Francis *et al.*, 1985 and 1987a). Mice immunised with a peptide from Sendai virus nucleoprotein, which was recognised by the CTLs of the mice were protected against lethal virus challenge (Kast *et al.*, 1991). Similarly, when Schulz *et al.* (1991) injected a T-cell epitope from the nucleoprotein of LCMV as a free synthetic peptide, protection was demonstrated by inhibition of viral replication in mice spleens, which correlated with CD8(+) LCMV-specific CTLs.

If, however, a free peptide is a poor immunogen or produces an immune response that is genetically restricted, an appropriate Th-cell epitope, either from a foreign protein or from the same molecule as the B-cell epitope, may be added. Francis *et al.* (1987b) demonstrated that H-2^d mice, which are poor or non-responders to the 141-160 peptide of FMDV, can be converted into responders by adding foreign Th-cell epitopes from ovalbumin or sperm whale myoglobin to the FMDV peptide sequence. A synthetic immunogen comprising an HBcAg Th-cell epitope (P120-140) and an HBsAg B-cell epitope from the $preS_2$ region (P14-21) elicited anti-peptide antibody production which was cross-reactive with the native envelope protein (Milich *et al.*,

1988a). It was also shown that the peptide consisting of a Th-cell epitope and a B-cell epitope from human rhinovirus also elicited good anti-peptide and antiviral response and may overcome the non-responsiveness of mice to the B-cell epitope alone (Francis *et al.*, 1989). The drawback of inclusion of Th-cell epitope is that it is necessary to characterise and incorporate additional Th-cell epitopes in order to broaden the potential of Th-cell recognition in an outbred population. In identification of Th-cell epitopes, generally, *in vitro* assays of T-cell proliferation in response to component proteins, protein fragments and peptides are required to identify appropriate sequences (Livingston and Fathman, 1987). Furthermore, it seems that universal Th-cell epitopes for each species should be sought.

However, there are two published algorithms that appear to improve the chances of selecting appropriate peptide sequences with T-cell epitopes from the primary sequence of a protein. The first, proposed by DeLisi and Berzofsky (1985), suggests that T-cell epitopes tend to be amphipathic structures, i.e. molecules that possess opposed hydrophilic and hydrophobic domains, which are frequently on opposite faces of an α-helix. The second method, proposed by Rothbard and Taylor (1988), is based on amino acid sequence patterns found to be associated with T cell antigenicity. Computer programmes for prediction of T-cell epitopes are now available (Menendez-Arias and Rodriguez, 1990).

An alternative approach to presentation of peptide epitopes to the immune system is the linkage of the potential B-cell epitopes to larger carrier molecules which may, or may not, possess their own Th-cell epitopes. Research has focused on methods of polymerising peptides or conjugating them to large immunogenic carrier proteins. Problems with these methodologies include the introduction of chemicals that are undesirable in vaccine preparations. A number of approaches have been developed to overcome these drawbacks. Tam (1988) has shown that, by building the peptide of interest onto a framework of lysine residues so that eight copies instead of one are present, the product elicits a much greater response than the monomeric form. This system, termed multiple antigenic peptide (MAP) system has been proved useful for

synthetic peptides derived from FMDV (Francis *et al.*, 1991), *Schistosoma mansoni* (Wolowczuk *et al.*, 1991) and HIV (Nardelli *et al.*, 1992) etc. The diepitope MAPs containing both the *a* and the $preS_2$ determinant of HBV induced high-titre antibodies reactive to the *a* synthetic peptide and the S protein, as well as to the middle proteins (Tam and Lu, 1989). Another system has been developed for the efficient attachment of a synthetic peptide to a reassembled rotavirus nucleocapsid without using additional chemical reactions (Frenchick *et al.*, 1989). Yet another promising approach is the use of recombinant DNA technology to engineer coding sequences for small peptide sequences fused to the genes for larger proteins. The presentation of viral epitopes on the surface of particulate structures has been explored, so that the epitopes are repeated over the entire surface of the particle and may therefore resemble the virion structure from which they were derived. Several approaches have been made in this direction, particularly the use of self-assembling particles from either Ty protein from yeast (Adams *et al.*, 1987), HBsAg (Delpeyroux *et al.*, 1986) or HBcAg (Clarke *et al.*, 1987; Stahl and Murray, 1989; Francis *et al.*, 1990).

The use of HBcAg to present peptide epitopes offers several advantages. HBcAg can be synthesized in *E. coli*, where it spontaneously self-assembles into characteristic 27 nm particles (Burrell *et al.*, 1979) and can be expressed in a wide range of systems (Clarke *et al.*, 1987; Kniskern *et al.*, 1986; Roossinck *et al.*, 1986). From the studies by Milich and his colleagues as discussed earlier, HBcAg is known to be highly immunogenic, due to the presence of a number of well-defined Th-cell epitopes, its ability to function as a T-cell independent antigen, and its polymeric nature. Furthermore, both the N- and the C-termini of HBcAg can be replaced, to some extent, by foreign epitopes without affecting the self-assembly properties. A study using FMDV VP1 peptide 141-160 fused to the N-terminus of HBcAg and expressed via vaccinia virus demonstrated that the immunogenicity could approach that of inactivated FMDV particles (Clarke *et al.*, 1987). Stahl and Murray (1989) reported construction of several fusion proteins in which coding sequences for different epitopes from HBV S and preS proteins and from the envelope protein of HIV were linked to C-terminus of the first 144 residues of HBcAg. These fusion proteins are

produced in high yields in *E. coli*, assemble into core-like particles, and are good immunogens.

1.11 Aims of this study

Although currently used HBV vaccines based on HBsAg have proved to be effective and safe, 2-5% vaccinees fail to respond to them, or show little or no seroconversion. In addition, in patients on routine haemodialysis and those with immunodeficiencies, the efficiency of the vaccine was as low as 50% (Sanchez *et al.*, 1983; Stevens *et al.*, 1984). The emergence of a vaccine escape mutant, which carries an amino acid substitution from glycine to arginine at amino acid 145 of HBsAg (Carman *et al.*, 1990; Harrison *et al.*, 1991; Fujii *et al.*, 1992) suggests that it may be necessary to modify future HBV vaccines. An ideal HBV vaccine should provide protection in those who do not respond to HBsAg or become infected with antigenic variants. Since preS regions are believed to play a role in viral entry into the hepatocytes (Machida *et al.*, 1984; Neurath *et al.*, 1986; Pontisso *et al.*, 1989a) and anti-preS antibodies are virus-neutralising (Itoh *et al.*, 1986; Emini *et al.*, 1989; Neurath *et al.*, 1989; Thornton *et al.*, 1987 and 1989), one approach would be the inclusion of the neutralising epitopes from $preS_1$ and $preS_2$ domains. Another approach would be the addition of the sequence of HBsAg from the mutant virus to induce antibodies with a different specificity to confer immunity against the mutants.

HBcAg was shown to be an extremely efficient immunogen in terms of T cell activation and antibody production (Milich *et al.*, 1987b) and its T-cell independent nature may be exploited to advantage for vaccine development for use in T-cell compromised individuals (Milich and McLachlan, 1986). Although anti-HBc antibody is not virus-neutralising, vaccination with HBcAg can confer protection against HBV infection in chimpanzees (Murray *et al.*, 1984 and 1987; Iwarson *et al.*, 1985b). Murine experiments showing that HBcAg-primed T cells can provide Th-cell recall memory for antibody production to envelope proteins may well explain the protection of chimpanzees against HBV infection by HBcAg vaccination. Furthermore, HBcAg-

specific Th cells were shown to induce anti-S antibody production in mice that did not respond to HBsAg (Milich *et al.*, 1987c). Studies in mice also indicated that the production of antibody to $preS_1$, $preS_2$ and S regions of HBsAg is independently H-2-restricted, $preS_1$- and $preS_2$-specific T cells can provide functional help not only to B-cell clones of the corresponding antigen specificity, but also to B-cell clones specific for S region; thus attachment of the Th-cell epitopes from $preS_1$ or $preS_2$ to the S protein can circumvent non-responsiveness to the S region (Milich *et al.*, 1985b, 1985d and 1986a). Therefore, the problem that some vaccinees and patients do not respond to the S protein could perhaps be circumvented using the response to HBcAg, $preS_1$ or $preS_2$ through multivalent vaccines. The use of HBcAg to present peptide epitopes to the immune system has been shown to enhance immunogenicity of peptide epitopes (Clarke *et al.*, 1987; Stahl and Murray, 1989). HBcAg could be exploited to serve as a carrier moiety to present S, alone or with mutant sequences, $preS_1$ and $preS_2$ epitopes from the envelope proteins with the aims of creating a multivalent vaccine which may elicit a broader repertoire of protective antibodies.

In this study, epitopes from $preS_1$, $preS_2$ and S regions of the envelope proteins were fused to truncated HBcAg to create HBcAg fusion proteins carrying $preS_1$ and $preS_2$ epitopes with or without the immunodominant *a* determinant of HBsAg, in the N- or C-terminal regions of HBcAg. Another series of HBcAg fusion proteins carrying the immunodominant epitope of HBsAg were also constructed in which gly_{145} was replaced by arginine, to mimic the reported escape mutant, glutamic acid or lysine. In addition, the wild-type *a* region was combined with the gly_{145} mutant sequence of HBsAg in HBcAg fusion proteins carrying in addition to one mutant and one wild-type sequence of $HBs_{(111-156)}$, the $preS_1$ and $preS_2$ epitopes.

The antigenicities of these HBcAg fusion proteins were examined in terms of their reaction with anti-HBc and anti-HBs antisera. The humoral and cellular immune responses of rabbits and mice to these HBcAg fusion proteins were compared. To explore further the specificity of the antibody to the HBcAg fusion proteins, antisera to the native HBsAg and to the HBcAg fusion proteins were compared. The results

not only provide information on the immunological aspects of the HBcAg fusion proteins carrying the envelope epitopes from HBV, but also shed light on the design of HBV vaccines based on HBcAg particles.

CHAPTER 2: MATERIALS AND METHODS

2A MATERIALS

2A.1 Suppliers of laboratory reagents

Restriction endonucleases:

Boehringer Mannheim GmbH: Mannheim, Germany

GIBCO BRL Life Technologies: Paisley, Scotland

New England Biolabs Inc.: Beverly, Massachusetts, U.S.A.

Pharmacia LKB Biotechnology: Milton Keynes, U.K.

Modifying enzymes:

Alkaline phosphatase, *E. coli* DNA polymerase I (Klenow large fragment), Mung bean nuclease, Sequenase™ version 2.0 DNA polymerase, *Thermus aquaticus* (*Taq*) DNA polymerase, T4 DNA ligase, T4 DNA polymerase, T7 DNA polymerase.

Boehringer Mannheim GmbH

GIBCO BRL Life Technologies

Northumbria Biologicals Ltd: Cramlington, U.K.

Pharmacia LKB Biotechnology

United States Biochemical Co.: Cleveland, U.S.A.

Standard laboratory reagents:

BDH Chemicals Ltd: Poole, U.K.

Calbiochem-Novabiochem Co.: La Jolla, U.S.A.

Fisons Chemicals: Loughborough, U.K.

GIBCO BRL Life Technologies

ICN Flow Limited: Rickmansworth, U.K.

Sigma Chemical Co.: Poole, U.K.

Reagents for bacterial media:

Becton-Dickinson U.K. Limited: Oxford, U.K.

Difco Laboratories: East Moseley, U.K.

Reagents for lymphocyte culture:

GIBCO BRL Life Technologies

ICN Flow Limited

Pharmacia LKB Biotechnology

Sera-lab: Sussex, U.K.

Sigma Chemical Co.

Radioactive reagents:

	Specific activity
[α-^{32}P]-dCTP	~ 3,000 Curies/mmole
[α-^{35}S]-dATP	1,000 Curies/mmole
[methyl-^{3}H]-thymidine	5 Curies/mmole
Sheep anti-mouse Ig, ^{125}I-labelled whole antibody	~ 2,400 Curies/mmole
Donky anti-rabbit Ig, ^{125}I-labelled whole antibody	~ 1,800 Curies/mmole

Amersham International plc.: Aylesbury, U.K.

Antibiotics:

ampicillin - Beecham Research Laboratories: Brentford, U.K.

penicillin G - Sigma Chemical Co.

streptomycin - Sigma Chemical Co.

tetracycline - Sigma Chemical Co.

Sera:

Anti-mouse IgG (sheep) precipitating serum

Anti-rabbit IgG (donkey) precipitating serum

Donkey serum

Rabbit serum

Sheep serum

Scottish Antibody Production Unit (SAPU): Lanarkshire, U.K.

Detection kits for radioimmunoassays (RIAs):

AUSRIA II 125 diagnostic kit

AUSAB diagnostic kit

Abbott Laboratories: Chicago, U.S.A.

2A.2 Media

2A.2.1 Bacterial media

Luria broth (LB):

Difco Bacto-tryptone, 10 g

Difco Bacto-yeast extract, 5 g

NaCl, 5 g

per litre adjusted to pH 7.2.

Luria agar:

As Luria broth with 15 g per litre Difco agar.

Terrific broth:

Difco Bacto-tryptone, 12 g

Difco Bacto-yeast extract, 24 g

glycerol, 4 ml

per 900 ml mixed with 100 ml sterile solution of 0.17 M KH_2PO_4, 0.72 M K_2HPO_4 after autoclaving

M9 Minimal agar:

Difco Bacto Agar, 15 g

10 x M9 salts, 100 ml

glucose, 20% (v/w), 20 ml

Vitamin B_1, 1 mg/ml, 10 ml

$MgSO_4$, 0.1 M, 10 ml

$CaCl_2$, 0.1 M, 10 ml

per litre.

10 x M9 Salts:

Na_2HPO_4, 60 g (0.423 M)

KH_2PO_4, 30 g (0.220 M)

NaCl, 5 g (0.086 M)

NH_4Cl, 10 g (0.187 M)

per litre.

Top-layer agar:

Difco Bacto agar, 10 g

Trypticase, 10 g

NaCl, 5 g

per litre.

2 x YT:

Bacto-tryptone, 16 g

Bacto-yeast extract, 10 g

NaCl, 5 g

per litre.

Antibiotics:

Ampicillin to a final concentration of 100 μg/ml or tetracycline to 10 μg/ml was added to media immediately prior to use when required.

2A.2.2 Lymphocyte culture medium

RPMI-1640 medium was supplied by GIBCO BRL Life Technologies as 10 x concentrate medium and buffered with $NaHCO_3$ (10 mM) and HEPES (20 mM). It was supplemented with L-glutamine (2 mM), β-mercaptoethanol (2-ME, 5 x 10^{-5} M) and foetal calf serum (FCS, 5%) for lymphocyte culture.

2A.3 Solutions

20 x SSC: NaCl, 3 M; tri-sodium citrate, 0.3 M; pH 7.0

50 x TAE: Tris-HCl, 2 M; glacial acetic acid, 1 M; EDTA, 50 mM; pH 8.0

10 x TBE: Tris-HCl, 0.9 M; boric acid, 0.9 M; EDTA, 20 mM; pH 8.0

TE: Tris-HCl, 10 mM, pH 8.0; EDTA, 1 mM

Sequencing TE: Tris-HCl, 10 mM, pH 8.0; EDTA, 0.1 mM

STE: Tris-HCl, 10 mM, pH 8.0; EDTA, 1 mM; NaCl, 150 mM

PBS: K_2HPO_4, 8 mM; KH_2PO_4, 1.5 mM; NaCl, 150 mM

Low-Tris buffer (LTB): Tris-HCl, 20 mM, pH 7.9; NaCl, 20 mM; EDTA, 1 mM

PCA: 25 parts redistilled phenol: 24 parts chloroform: 1 part *iso*-amylalcohol

CA: 24 parts chloroform: 1 part *iso*-amylalcohol

Scintillation fluid: 2,5-diphenyloxazole (PPO), 6 g;
1,4-*bis*(5-phenyloxazol-2-yl)benzene (POPOP), 0.05 g;
toluene, 1 litre

2A.4 Bacterial strains

NAME	GENOTYPE	REFERENCE
RB791	*ara, Δ(lac-pro), strA, thi, lacZΔM15*	Brent & Ptashne (1981)
DH 5α	*supE44, ΔlacU169(ϕ80lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1*	Hanahan (1983)
NM522	*hsdΔ5, Δ(lac, pro), supE, thi, F´[proAB$^+$, lacZΔM15, lacI^q]*	Gough & Murray (1983)
BMH 71-18 *mutL*	*mutL::Tn10, Δ(lac, pro), supE, thi, F´[proAB$^+$, lacZΔM15, lacI^q]*	Kramer *et al.* (1984)
XL1-Blue^r	*supE44, hsdR17, recA1, endA1, gyrA46, thi, relA1 lac, F´[proAB$^+$, lacI^q, lacZΔM15, Tn10(tet^r)]*	Bullock *et al.* (1987)
TG1	*supE, hsdΔ5, thi, Δ(lac-proAB), F´[traD36, proAB$^+$, lacI^q, lacZΔM15]*	Gibson (1984)

2A.5 Plasmids

NAME	DESCRIPTION	REFERENCE
$pHBcS_{(111-156)}$	This plasmid contains the coding sequence for a fusion protein of β-galactosidase$_{(1-8)}$/HBc$_{(3-144)}$ /HBs$_{(111-156)}$ under control of the *tac* promoter.	Stahl & Murray (1989)
$pHBcS_{(111-165)}$	Similar to $pHBcS_{(111-156)}$. Contains $HBs_{(111-165)}$ instead of $HBs_{(111-156)}$.	Stahl & Murray (1989)
$pHBcPreS_{1(1-20)}$	Similar to $pHBcS_{(111-156)}$. Contains $PreS_{1(1-20)}$ instead of $HBs_{(111-156)}$.	Stahl & Murray (1989)
$pHBcPreS_{1(1-36)}$	Similar to $pHBcS_{(111-156)}$. Contains $PreS_{1(1-36)}$ instead of $HBs_{(111-156)}$.	Stahl & Murray (1989)
$pHBcPreS_2$	Similar to $pHBcS_{(111-156)}$. Contains $PreS_{2(1-26)}$ instead of $HBs_{(111-156)}$.	Stahl & Murray (1989)
pHBcX	Similar to $pHBcS_{(111-156)}$. Contains $HBs_{(111-120)}$ and $HBx_{(1-154)}$ instead of $HBs_{(111-156)}$.	Rossner (1991)
pHinG2	*E. coli/S. cerevisiae* shuttle vector. Allows expression of HBsAg in *S. cerevisiae* under control of yeast PHO5 promoter.	Murray *et al.* (1984)
$pHinG2_{145R}$	Similar to pHinG2. Carries an amino acid substitution from glycine to arginine at amino acid 145 of HBsAg.	K. Murray (personal communication)

2A.6 Oligonucleotides

NAME	SEQUENCE 5′-3′	COMMENTS
M13-20	GTA AAA CGA CGG CCA GT	Bacteriophage M13 universal sequencing primer (20 nucleotides from the polylinker site).
M13-40	GTT TTC CCA GTC ACG AC	Bacteriophage M13 universal sequencing primer (40 nucleotides from the polylinker site).
991H	CCT GCA GAA CCT GCA CG	HBV sense strand, nucleotide positions 1795-1811.
405J	CCT GGG TGG GTA CTA ATT	HBV sense strand, nucleotide positions 209-226.
705C	TCA GGA GAC TCT AAG GC	HBV anti-sense strand, nucleotide positions 137-121.
212S	GGA ATT CAT CCA ACT GGT GGT CGG G	HBV anti-sense strand, nucleotide positions 1007-990 with 7 additional nucleotides at the 5′ end including a cleavage site for EcoRI.

010H	GGA ATT CCC AGG AAC ATC AAC CAC	HBV sense strand, nucleotide positions 1767-1783 with 7 additional nucleotides at the 5′ end including a cleavage site for EcoRI.
011H	GGA ATT CCC AGG ATG ATG GGA TG	HBV anti-sense strand, nucleotide positions 1905-1889 with 6 additional nucleotides at the 5′ end including a cleavage site for EcoRI.
412G	ACC TTC GGA TAG AAA CTG CAC CT	HBV sense strand, nucleotide positions 1859-1881 with 1869 G→A.
411G	ACC TTC GGA TGA AAA CTG CAC CT	HBV sense strand, nucleotide positions 1859-1881 with 1870 G→A.
359G	AAA CCT TCG GAT AAA AAC TGC ACC TG	HBV sense strand, nucleotide positions 1857-1882 with 1869 G→A and 1870 G→A.

Oligonucleotides were synthesized by the OSWEL DNA Service, Department of Chemistry, University of Edinburgh.

2A.7 Antibodies

Antibody	Description	Source
R82 anti-HBs antiserum	Rabbit antiserum raised against HBsAg produced in yeast.	K. Murray
R87 anti-HBc antiserum	Rabbit antiserum raised against HBcAg produced in *E. coli.*	K. Murray
NRA/B7-4B	Rabbit antiserum raised against SDS- and DTT-denatured HBsAg.	P. Wingfield
Monoclonal antibody MA 18/7	Raised against HBsAg from serum. Reacts with the $preS_1$ region of P39 and GP42, native or denatured form, of all HBsAg subtypes.	W. H. Gerlich Heermann *et al.* (1984)
Monoclonal antibody Q19/10	Raised against HBsAg from serum. Reacts with the $preS_2$ region of GP33 and GP36 of all HBsAg subtypes, but does not react with unglycosylated P31.	W. H. Gerlich Heermann *et al.* (1987)
Monoclonal antibody RF-HBs-1	Raised against HBsAg from serum. Reacts with cyclic peptide (124-137) of HBsAg.	H. C. Thomas and J.A. Waters
Monoclonal antibody RF-HBs-2	Raised against HBsAg from serum. Reacts with cyclic peptide (124-137) of HBsAg.	H. C. Thomas and J.A. Waters

Monoclonal antibody RF-HBs-7	Raised against HBsAg from serum. Reacts with cyclic peptide (139-147) of HBsAg.	H. C. Thomas and J.A. Waters

2A.8 Synthetic peptides

$preS_{1(1-23)}$ (*adyw* subtype)

MGQNL STSNP LGFFP NHQLD PAF

$preS_{2(1-26)}$ (*adyw* subtype)

MQWNS TTFHQ TLQDP RVRGL YFPAG G

$HBs_{(111-156)}$ (*adyw* subtype)

PGSST TSTGP CRTCT TPAQG ISMYP SCCCT KPSDG NCTCI PIPSS W

Synthetic peptides were synthesized by R. Ramage of the Department of Chemistry, University of Edinburgh.

2B METHODS

2B.1 Bacterial cultures

2B.1.1 Growth of bacterial cultures

Bacterial culture broth, either LB or Terrific broth, was inoculated with *E. coli* from a single colony or 1/100 volume of an overnight culture in a test tube or a conical flask with a total capacity of 5 fold to that of the culture volume to ensure good aeration. Cultures were incubated at 37°C with shaking for 5-6 hr or overnight.

2B.1.2 Storage of bacterial cultures

For long-term storage, 900 μl of fresh overnight or 6-hr culture of bacteria grown in LB supplemented with antibiotics if necessary, was mixed with 100 μl of sterile glycerol, and stored in a sterile, plastic cryo tube (Nunc, Roskilde, Denmark) at -70°C. To recover the bacteria, the surface of the frozen culture was scraped with a sterile inoculating loop and directly streaked out onto a LB agar plate, with antibiotics if required. *E. coli* strain TG1 was streaked onto a minimal agar plate to maintain the F′ plasmid. After overnight incubation at 37°C, a single colony was picked to propagate a fresh bacterial culture. For short-term storage, an isolated colony was picked and stabbed into a vial containing LB agar. After tightening the caps, the vials were stored at room temperature in the dark.

2B.1.3 Transformation of *E. coli* with DNA

2B.1.3.1 Calcium chloride method

The method was based on the report from Mandel and Higa (1970) with the modifications of Dagert and Ehrlich (1979). To 50 ml of LB (supplemented with $MgCl_2$, 20 mM) was added 0.5-1 ml of an overnight culture of the *E. coli* strain to be

transformed. Growth was allowed at 37°C with vigourous shaking to 0.2 OD_{600nm}, the cells chilled on ice for 5 min, and pelleted by centrifugation at 3,000 rpm (1,250 x g; Denley BR401, Sussex, U.K.) at 4°C for 15 min. The cell pellet was resuspended in ice-cold transformation buffer ($CaCl_2$, 50 mM; Tris-HCl, 10 mM; pH 7.5; 20 ml), and kept in this buffer for 30 min on ice. The cells were repelleted by centrifugation for 15 min at 1,500 x g at 4° C, and resuspended in ice-cold transformation buffer (2 ml). Typically the cells were left overnight (12-16 hr) on ice (or at minimum for 2 hr) before use. Cells were transformed by adding 10 ng of DNA (typically a ligation mix), in a volume of 5-10 μl, to 100 μl of competent cells, mixing and holding on ice for 30 min. Cells were heat-shocked for 5 min at 37°C, warm LB (2 ml) added, and incubated for 1 hr to allow for expression of genes coded by the transformed plasmid DNA (i.e. antibiotic resistance). Cells were pelleted by centrifugation at 4°C and resuspended in LB (250 μl). Fifty μl aliquots were then plated to dryness on LB agar, with appropriate antibiotic selection and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)/isopropyl-β-D-thiogalactopyranoside (IPTG) if needed. The X-gal/IPTG allows recombinants to be identified; recombinant colonies appear colourless, while non-recombinant colonies appear blue. Cells were incubated overnight at 37°C for growth.

2B.1.3.2 DMSO method

This method for transformation of *E. coli* was initially described by Chung and Miller (1988) with minor modification. Cells were grown to 0.3 OD_{600nm} in LB supplemented with 20 mM of $MgCl_2$ (50 ml), kept for 10 min on ice, and pelleted from 10 ml of culture by centrifugation at 1,250 x g for 5 min at 4°C. The cell pellet was resuspended in transformation buffer [polyethylene glycol (PEG), molecular weight 3000, 10% (w/v); dimethylsulphoxide (DMSO), 5% (v/v); $MgCl_2$, 10 mM; $MgSO_4$, 10 mM; in LB; 1 ml], and incubated for 30 min on ice. DNA was added to cell suspension (100 μl), and the mixture was left for 30 min on ice. Transformation buffer containing 20 mM of glucose (900 μl) was added to the transformed cells, cells were shaken for 1 hr at 37°C to allow for expression of antibiotic resistant gene and

plated onto LB agar plates supplemented with suitable antibiotics immediately after incubation.

2B.2 Preparation of nucleic acids

2B.2.1 Phenol extraction of nucleic acid solution

Proteins present in DNA solution were removed by extraction with phenol or with phenol: chloroform: *iso*-amylalcohol (PCA). Distilled phenol was equilibrated with Tris-HCl (1 M; pH 8.0) prior to use. An equal volume of phenol or PCA was added to aqueous nucleic acid solution, and the two liquid phases were mixed by vigourous vortexing. The phases were separated by centrifugation at 17,000 x g for 5 min at room temperature. The upper, aqueous phase containing nucleic acid was recovered.

2B.2.2 Ethanol precipitation of DNA

Nucleic acids were precipitated from aqueous solutions by addition of 1/10 volume of sodium acetate (3 M; pH 5.2), and 2.5 volumes of cold absolute ethanol (-20°C). The mixture was inverted gently several times and left at -20°C or lower for 20 min. Precipitated DNA was recovered by centrifugation at 4°C at 17,000 x g for 15 min. The pellet was then washed twice with cold 70% ethanol (-20°C), dried under vacuum, and redissolved in distilled water or TE.

2B.2.3 Small-scale preparation of plasmid DNA (Minipreps)

Buffer P1: (kept at 4°C)

Tris-HCl, 50 mM; pH 8.0

EDTA, 10 mM

RNase A, 100 μg/ml

Buffer P2: (Kept in air-tight bottle)

NaOH, 0.2 M

SDS, 1% (w/v)

Buffer P3:

potassium acetate, 2.55 M; pH 4.8

The method used for small-scale preparation of plasmid DNA was a modification of that described by Ish-Horowicz and Burke (1981). Bacteria from a single colony were inoculated to LB or Terrific broth supplemented with antibiotics (5 ml) and incubated at 37°C ovemight with shaking. Cells (1.5 ml) were pelleted by centrifugation and resuspended in buffer P1 (300 μl). Cells were lysed by adding lysis buffer P2 (300 μl) and incubated at room temperature for 5 min. After the cell lysis, buffer P3 (300 μl) was added and mixed by gentle inversion. Precipitated complex consisting of chromosomal DNA, SDS and proteins was sedimented by centrifugation (17,000 x g) for 12 min. Contaminating proteins in the clarified supernatant were extracted with phenol. The upper, aqueous phase was recovered and nucleic acids were precipitated with isopropanol (600 μl). The DNA was washed twice with 70% ethanol, dried under vacuum, and resuspended in distilled water (50 μl). Plasmid "miniprep" DNA was then stored at -20°C.

2B.2.4 Large-scale plasmid preparation

2B.2.4.1 CsCl method

A 100-ml culture of bacteria carrying the desired plasmid was incubated overnight at 37°C with vigourous shaking in Terrific broth supplemented with suitable antibiotics. The cells were pelleted by centrifugation at 5,000 rpm (4080 x g; GSA rotor, Sorvall RC5B; Wilmington, U.S.A.) and resuspended in solution 1 (Tris-HCl, 25 mM, pH 8.0; EDTA, 10 mM; glucose, 50 mM; 10 ml). Cells were lysed on ice by adding lysis buffer [NaOH, 0.2 M; SDS, 1% (w/v); 10 ml], and left on ice for 20 min. Addition of potassium acetate (5 M, pH 5.0; 10 ml) precipitated complex of chromosomal

DNA, SDS and proteins, which was sedimented at 15,000 rpm (27,000 x g; SS34 rotor, Sorvall) for 30 min at 4°C. To the supernatant, isopropanol (11 ml) was added and left at room temperature for 30 min to precipitate plasmid DNA. The DNA was pelleted by centrifugation at 10,000 rpm (12,000 x g; SS34 rotor) for 20 min at 4°C. The pellet was washed with 70% ethanol, dried under vacuum, resuspended in TE containing 10 μg/ml of RNase (10 ml) and incubated at 37°C for 30 min. Plasmid DNA solution (9.4 ml) was transferred to a fresh tube to which ethidium bromide solution (10 μg/μl; 100 μl) and CsCl (9.02 g) were added, giving a density of 1.55 g/ml. The DNA was banded by centrifugation at 38,000 rpm (95,000 x g; 50Ti rotor, Sorvall OTD50B) for 40-48 hr at 20°C. DNA was visualised by side illumination with UV light. The lower band containing supercoiled plasmid DNA was removed by puncturing the tube with a 21-gauge needle and syringe. A second 21-gauge needle was inserted at the top of the tube to allow pressure release. The ethidium bromide was removed by extraction several times with butanol, and the CsCl was removed by dialysis against 3 to 4 changes of TE (2 l) for 8 to 15 hr at 4°C. The plasmid solution was then phenol extracted, and residual phenol removed by extraction with an equal volume of chloroform: *iso*-amylalcohol (CA) solution. Plasmid DNA was precipitated, washed and resuspended in distilled water and stored at -20°C.

2B.2.4.2 Column method (modified silica gel; Qiagen)

Buffer QBT:

NaCl, 750 mM; pH 7.0

3-(*N*-morpholino)propane-sulphonic acid (MOPS), 50 mM

ethanol, 15%

Triton X-100, 0.15%

Buffer QC:

NaCl, 1.0 M; pH 7.0

MOPS, 50 mM

ethanol, 15%

Buffer QF:

NaCl, 1.25 M; pH 8.2

MOPS, 50 mM

ethanol, 15%

The large-scale preparation of plasmid by this method followed the culture conditions of the CsCl method and the procedures of the small-scale plasmid preparation except using 10 ml of each solution (buffers P1, P2, and P3) instead of 300 μl. The complex containing chromosomal DNA, SDS and proteins was sedimented by centrifugation at 15,000 rpm (27,000 x g; SS34 rotor) for 30 min at 25°C. The supernatant was applied onto the Qiagen-tip 500 column (Diagen; Dusseldorf, Germany) which had been equilibrated by buffer QBT (10 ml). After washing three times with buffer QC (10 ml), the DNA was eluted with buffer QF (10 ml). DNA was then precipitated with 0.5 volume of isopropanol and washed twice with 70% ethanol. Dried DNA was resuspended for PCA and CA extraction, followed by ethanol precipitation and washing. DNA samples were redissolved in sterile distilled water and stored at -20°C.

2B.2.5 Preparation of M13 replicative form (RF), double-stranded DNA

A fresh, single plaque of M13mp18 or M13mp19 with or without inserts of heterologous DNA was picked with a sterile wooden toothpick and transferred into 2 x YT medium (3 ml) containing overnight culture of TG1 (50 μl), and the culture was grown for 4-5 hr with vigourous shaking at 37°C. For small-scale preparation of RF DNA, the cells were pelleted by centrifugation in a microcentrifuge and the procedure used was that for small-scale preparation of plasmid DNA.

2B.2.6 Preparation of M13 single-stranded template DNA

A fresh, single plaque of M13mp18 or M13mp19 with inserts of heterologous DNA was picked with a sterile wooden toothpick and transferred into 2 x YT medium (3 ml) containing overnight culture. of TG1 (50 μl), and the infected culture was

incubated for 4-5 hr with vigourous shaking. The suspension was then transferred to an Eppendorf tube and the bacteria pelleted. The supernatant, containing phages, was transferred to a fresh tube, the centrifugation repeated, and the supernatant transferred to a new tube. PEG/NaCl solution [PEG 6000, 20% (w/v); NaCl, 2.5 M; 200 μl] was added, mixed, and the tube left at room temperature for 15 min. The PEG/phage precipitate was pelleted by centrifugation in a microcentrifuge (17,000 x g) for 5 min, and the supernatant discarded. The centrifugation was repeated for a further 2 min and any residual supernatant removed by aspiration. At this stage a white pellet was visible. This pellet was suspended in TE (100 μl) and phenol extracted twice. The DNA was precipitated from aqueous solution, washed twice with 70% ethanol, dried, resuspended in sequencing TE (50 μl) and stored at -20°C.

2B.2.7 Quantification of DNA

The DNA sample was diluted in 1 ml of distilled water and the OD of absorbance at wavelengths 260nm and 280nm was measured by a spectrophotometer (Perkin-Elmer, Lambda 15, UV/VIS Spectrophotometer). An OD_{260nm} value of 1.0 represents a concentration of 50 μg/ml for double-stranded DNA, 40 μg/ml for single-stranded DNA, and 20 μg/ml for oligonucleotide. The ratio OD_{260nm}/OD_{280nm} provides an estimate for the purity of the nucleic acid. A value around 1.8 indicates pure preparations of DNA.

2B.3 DNA manipulation

2B.3.1 Digestion of DNA with restriction endonucleases

Most digestions of DNA with restriction enzymes were performed using Boehringer Mannheim enzymes and buffers. DNA was digested with approximately 5 U of restriction endonuclease per μg of DNA using buffer and temperature conditions recommended by the manufacturer. For double digests involving enzymes with different recommended buffers, the buffers were checked individually in double

digests to determine which gave most efficient digestion. If the optimal digestion conditions varied for buffers, the DNA was digested with enzyme at lower salt concentration, the enzyme reaction stopped by heat denaturation or phenol extraction, and the buffer concentration altered by addition of sufficient salt solution so that the final concentration was appropriate for digestion by the next enzyme. Digestion reactions were terminated by heating for 10 min at 65°C, by phenol extraction, or by addition of DNA sample buffer for agarose gel electrophoresis.

2B.3.2 Dephosphorylation

Calf intestinal alkaline phosphatase (CIP) catalyses the removal of 5′-phosphate residues from DNA, RNA, dNTP and rNTP. It can be used to remove 5′-phosphates from fragments of DNA to prevent self-ligation and from DNA or RNA prior to end-labelling with ^{32}P. The typical reaction consisted of 40 μl of DNA (~5 μg), 5 μl of 10 x CIP buffer (Tris-HCl, 0.5 M, pH 8.5; EDTA, 1 mM) and 5 μl of CIP (1 U/μl; Boehringer). The reaction mix was incubated at 37°C for 1 hr and the reaction stopped by addition of EDTA to a final concentration of 20 mM and heated at 65°C for 10 min followed by phenol extraction.

2B.3.3 Filling-in 3′ recessed termini of DNA

Recessed 3′ termini can be filled by the polymerase activity of the Klenow large fragment of *E. coli* DNA polymerase I in the presence of appropriate dNTP. The typical reaction consisted of 39 μl of DNA (~ 1 μg), 5 μl of 10 x Fill-in buffer (Tris-HCl, 0.5 M, pH 7.5; MgCl, 0.1 M; DTT, 10 mM; BSA, 0.5 mg/ml), 5 μl of 5 mM dNTP and 1 μl of Klenow enzyme (1 U/μl; Boehringer). The reaction mix was incubated for 1 hr at room temperature and the reaction stopped by addition of 2.5 μl of 0.5 M EDTA and 150 μl of TE followed by phenol extraction.

2B.3.4 Removing protruding 3′ termini of DNA by T4 DNA polymerase

T4 DNA polymerase carries a 3′→5′ exonuclease activity that can be used to remove protruding nucleotides from the 3′ termini of DNA. Its 3′ exonuclease activity is more than 200-fold more active than that of Klenow large fragment of *E. coli* DNA polymerase I. The typical reaction consisted of 38 μl of DNA (~ 5 μg), 5 μl of 10 x T4 DNA polymerase buffer (Tris-HCl, 0.5 M, pH 8.8; $MgCl_2$, 50 mM; DTT, 50 mM; BSA, 0.5 mg/ml), 2 μl of 5 mM dNTP and 5 μl of T4 DNA polymerase (1 U/μl; Boehringer). The reaction mix was incubated for 1 hr at room temperature and the reaction stopped by phenol extraction.

2B.3.5 Removing protruding 5′ termini of DNA by Mung bean exonuclease

Mung bean exonuclease degrades single-stranded DNA to mononucleotides or oligonucleotides with phosphate groups at their 5′ termini. Double-stranded DNA is relatively resistant to the enzyme. However, it digests double-stranded DNA completely if large amounts are used. The activity is less severe than nuclease S1. Mung bean exonuclease was used for converting protruding 5′ termini of DNA to blunt ends. The typical reaction consisted of 34.5 μl of DNA (~ 5 μg), 4 μl of 10 x buffer (sodium acetate, 0.3 M, pH 4.5; NaCl, 0.5 M; $ZnCl_2$ 10 mM; glycerol, 50%) and 1.5 μl of Mung bean exonuclease (13 U/μl; Pharmacia). The reaction mix was incubated for 30 min at room temperature and the reaction stopped by addition of NaCl to a final concentration of 0.2 M and kept on ice followed by PCA extraction.

2B.3.6 Ligation

The vector and insert DNA were cut to completion with appropriate restriction endonucleases. After restriction and removing the restriction endonucleases, DNA was ethanol precipitated. If needed, the digested DNA was separated in agarose gels and the desired fragments recovered. Typically, between 100-200 ng insert DNA were ligated in a reaction with vector to insert DNA concentration at a 1:3 molar ratio.

Ligations were carried out in 10 μl or 20 μl of reaction buffer (Tris-HCl, 50 mM, pH 7.6; $MgCl_2$, 10 mM; DTT, 1 mM; ATP, 1 mM; PEG 8000, 5%) and incubated overnight at 15°C (for cohesive ends), or room temperature (for blunt ends). One or 2 units of T4 DNA ligase (1U/μl; Boehringer) was used for blunt-end ligations, and 0.5-1 units for cohesive end-ligations. *E. coli* was then transformed with the ligation products.

2B.4 Electrophoresis of nucleic acids

2B.4.1 Electrophoresis of DNA in agarose gels

DNA fragments were separated in 0.8-1.5% (w/v) agarose (electrophoresis grade; Sigma) with ethidium bromide (0.5 μg/ml) in 1 x TBE buffer (for routine diagnosis gels) or 1 x TAE buffer (for band-recovery gels). Prior to loading, DNA samples were mixed with 1/10 volume of sample buffer (glycerol, 20%; EDTA, 100 mM; bromophenol blue, 0.1%). Electrophoresis was carried out horizontally across a potential difference of 1-10 v/cm. Bacteriophage λDNA cut with HindIII and $\phi\chi174$ RF DNA cut with HaeIII were used as size markers. DNA was visualised by UV illumination and photographed if necessary.

2B.4.2 Recovery of DNA from agarose gels

DNA was electrophoresed through 0.8% regular melting point agarose in 1 x TAE containing ethidium bromide (0.5 μg/ml). The desired fragment was visualised by UV illumination, cut out and extracted from the agarose using Geneclean™ kit (Bio101; La Jolla, U.S.A.). The agarose was weighed and 2-3 x volumes of 6 M NaI added. The agarose was dissolved by heating to 55°C for 5 min with occasional mixing and then cooled on ice for 5 min. The "glassmilk" (a silica matrix suspended in water; 5 μl) was added, and left on ice for 5 min with occasional mixing to allow DNA to bind to the silica matrix. The "glassmilk" was pelleted by centrifugation in a microcentrifuge, the supernatant discarded and the pellet washed three times with ice

cold NEW wash (NaCl/ethanol/water mix; 500 μl). After a final spin, all the NEW wash was discarded completely and the DNA eluted from the "glassmilk" in TE (5-10 μl) at 55°C. The mixture was centrifuged and the supernatant, containing the DNA, transferred to a new Eppendorf tube. The elution step was repeated once, the supernatant combined and stored at -20°C.

DNA fragments less than 300 bp long were recovered from the agarose using MERmaid™ kit (Bio101; La Jolla, U.S.A.). DNA was electrophoresed through 2% ultrapure agarose (MER™maid Biogel; Bio101) in 1 x TAE containing ethidium bromide (0.5 μg/ml). Agarose containing the DNA fragment was cut out, weighed and mixed with 3 x volumes of high salt binding solution (concentrated sodium perchlorate) in an Eppendorf tube. The "glassfog" (a silica based matrix in water; 8 μl) was added, the agarose melted and DNA bound to the "glassfog" by incubation at 55°C for 5 min. Adsorption was allowed to continue at room temperature for 5 min with occasional mixing to keep the "glassfog" in suspension. The "glassfog" was centrifuged, the supernatant discarded and the pellet washed three times with ethanol wash (300 μl). After the final wash, the tube was centrifuged again to ensure removal of all residual ethanol. The pellet of "glassfog" was resuspended in distilled water (10 μl) and the DNA eluted by incubation at room temperature for 5 min. The "glassfog" was centrifuged and the supernatant containing the DNA transferred to a fresh Eppendorf tube. The elution step was repeated once, the supernatant combined and stored at -20°C. Recovery of DNA using Geneclean or MERmaid kits was usually around 80%.

2B.5 Radiolabelling DNA

2B.5.1 Labelling DNA by random priming with hexadeoxyribonucleotide primers

DNA labelled to high specific radioactivities was obtained using the randomly primed DNA labelling method (Feinberg & Vogelstein, 1983). It is based on the hybridisation of a mixture of hexanucleotides to the DNA to be labelled. Many sequence

combinations are represented in the hexanucleotide primer mixture, which leads to binding of primer to template in a statistically random manner. The complementary strand is synthesized from the 3'OH termini of the random hexanucleotide primer using Klenow fragment of the *E. coli* DNA polymerase during which radiolabelled dNTP is incorporated into the newly synthesized DNA strand.

Oligo-labelling buffer (OLB) is made by mixing solution A (Tris-HCl, 1.25 M, pH 8.0; $MgCl_2$, 125 mM; 2-ME, 25 mM; dGTP, dTTP and dATP, 0.5 mM; 50 μl), solution B (HEPES, 2 M adjusted to pH 6.6 with NaOH; 125 μl) and solution C (random hexanucleotides, 1.8 μg/μl in TE; 75 μl).

The DNA fragment to be labelled was excised from the agarose gel and placed into an Eppendorf tube. Distilled water was added to the tube at a ratio of 3 ml H_2O per gram of gel and the tube incubated at 100°C for 10 min to melt the gel and denature the DNA followed by incubation at 37°C for 10 min. The labelling reaction was carried out at 37°C for 2 hr or at room temperature overnight.

DNA fragment in melted agarose (approx. 10-100 ng), 35 μl
OLB, 10 μl
BSA (10 mg/ml), 2 μl
[α-^{32}P]-dCTP (10 μCi/μl, Amersham), 5 μl
Klenow fragment of *E. coli* DNA polymerase I (5 U/μl), 1 μl

2B.5.2 Separation of unincorporated nucleotides from labelled DNA

A Sephadex G-50 (Pharmacia) column was used to remove unincorporated [α-^{32}P]-dCTP from labelled DNA fragment. Dry Sephadex G-50 beads were swollen in an excess of TE at 4°C overnight. The column was prepared by packing a 1 ml plastic syringe (without the plunger), in which the hole was plugged with glass wool, with the treated Sephadex G-50 followed by centrifugation (300 x g) for 3 min. The column was equilibrated with STE (0.5 ml) followed by centrifugation (300 x g) for 3 min. The equilibration step was repeated once. After completion of the labelling

reaction, STE (50 μl) was added to the reaction mixture and loaded to the Sephadex G-50 column. Unincorporated [α-^{32}P]-dCTP was trapped in the column and labelled DNA was collected by centrifugation (300 x g) for 5 min.

2B.6 Colony and plaque hybridisation

2B.6.1 Transfer of DNA from *E. coli* colonies or bacteriophage M13 plaques to membranes

The method used for transfer of DNA from colonies was that described by Grunstein and Hogness (1975). Colonies harbouring putative plasmids of interest were replica plated by spotting onto two LB agar plates containing the appropriate antibiotics and the plates were incubated at 37°C overnight. Colonies were lifted, from one plate onto Hybond™-N nylon membrane (Amersham) by placing the filter onto the plate to contact colonies, and peeling off the filter as soon as it had become moistened. Cells were lysed and DNA denatured by placing the filters, colonies face-up, onto Whatman No. 1 blotting paper (Maidstone, U.K.) saturated with denaturation solution (NaOH, 0.5 M; NaCl, 1.5 M) for 2 min at room temperature. They were neutralised by placing the colony side up on blotting paper saturated with neutralisation solution (Tris-HCl, 1 M, pH 8.0; NaCl, 1.5 M) for 5 min at room temperature. The neutralisation step was repeated, the filters were dried on blotting paper and baked in a vacuum oven at 80°C for 2 hr.

The method used for plaque lifts was that described by Benton and Davis (1977). Colourless plaques harbouring the putative insert of interest were picked with a sterile wooden toothpick and replica plated onto two plates containing cell lawns. A lawn of TG1 cells was prepared by adding an overnight culture in LB (100 μl) to molten top-layer agar (3-3.5 ml) containing X-gal (20 mg/ml in dimethylformamide; 30 μl) and IPTG (200 mg/ml in H_2O; 20 μl) and plating on minimal agar plates. Bacteriophages were allowed to grow overnight and the plates were cooled at 4°C. The plaques were lifted onto nitrocellulose membrane as described previously. The

denaturation and neutralisation steps were similar to those of colony lifts.

2B.6.2 Hybridisation

By adjusting the stringency of hybridisation, it is possible to distinguish between closely- and distantly-related members of a sequence family. In practice, to distinguish between the distantly-related members of a family of sequences, hybridisation should take place under conditions of low stringency followed by washing under progressively more stringent conditions. To identify closely-related members, a stringent hybridisation followed by a stringent wash is better. The stringency of hybridisation depends on a number of factors, such as *T*m (melting temperature) which is dependent on base composition and denaturing agents, temperature of reaction, and ionic strength of the solution.

100 x Denhardt's solution:

BSA (fraction V; BDH), 2% (w/v)
Ficoll (type 400; Pharmacia), 2% (w/v)
polyvinyl pyrrolidone, 2% (w/v)

Prehybridisation solution:

6 x SSC
SDS, 0.2% (w/v)
sonicated salmon sperm DNA, 200 μg/ml
5 x Denhardt's solution
formamide, 40% (v/v)

Hybridisation solution:

6 x SSC
SDS, 0.2% (w/v)
sonicated salmon sperm DNA, 200 μg/ml
1 x Denhardt's solution

formamide, 40% (v/v)

The filter containing DNA transferred from colonies or plaques was incubated in prehybridisation solution for at least 60 min with gentle shaking. The prehybridisation solution was removed and replaced with hybridisation solution. The radiolabelled probe which had been denatured by incubating at 100°C for 5 min and cooled by placing immediately in ice was added and left to hybridise at 37°C overnight. The filter was recovered and washed twice in 2 x SSC containing SDS (0.1%) at room temperature for 15 min followed by further wash twice in 0.5 x SSC containing SDS (0.1%) at room temperature for 20 min. The filter was sealed wet in a plastic bag and autoradiographed.

2B.6.3 Autoradiography

Autoradiography was used to visualise and quantitate, on film, radioactive molecules hybridised to membranes. X-ray film (X-OMAT AR, Kodak, Rochester, U.S.A.) was hypersensitised by exposure to a flash of light provided by a photographic flash unit and exposed to the sealed filter in a light-proof cassette with intensifying screens (Cronex Lightning Plus; Du Pont). The cassette was stored at -70°C for various times depending on the intensity of the signal obtained.

2B.7 DNA sequencing

The method for DNA sequencing was the dideoxynucleotide chain termination method based on that described by Sanger *et al.* (1977 and 1980).

2B.7.1 M13 sequencing using *E. coli* DNA polymerase I (Klenow fragment)

10 x TM:

Tris-HCl, 100 mM pH 8.5

$MgCl_2$, 50 mM

Chain-termination mix (T°, C°, G° and A°):

	Chain-termination mix (numbers are volumes in μl)			
Nucleotide	T°	C°	G°	A°
dTTP, 0.5 mM	12.5	250	250	250
dCTP, 0.5 mM	250	12.5	250	250
dGTP, 0.5 mM	250	250	12.5	250
ddTTP, 10 mM	25			
ddCTP, 10 mM		4		
ddGTP, 10 mM			8	
ddATP, 10 mM				0.5
sequencing TE	500	500	500	250

dNTP chase mix:

dTTP, 0.25 mM

dCTP, 0.25 mM

dGTP, 0.25 mM

dATP, 0.25 mM

Klenow reaction mix (per template, 4 reactions):

[α-^{35}S]-dATP (8 μCi/μl, Amersham), 4 μCi

E. coli DNA polymerase I (Klenow fragment) (5 U/μl), 1.5 U

DTT, 10 mM, 8 μl

Tris-HCl, 10 mM (pH 8.5), 6.5 μl

Formamide dye:

xylene cyanol, 0.1% (w/v)

bromophenol blue, 0.1% (w/v)

EDTA, 10 mM

in formamide

The sequencing primer (1 pmole/μl; 1 μl) was annealed to the template (100 ng/μl; 8 μl) in the presence of 10 x TM (1 μl) at 80°C for 5 min and slowly cooled to room temperature over 30 min. The reactions were carried out in 96-well U-shaped microtitre plates or HLA plates. The annealed template/primer mix (2 μl) was dispensed into each of 4 wells followed by addition of one of the chain-termination mixes (G°, A°, T° or C°; 2 μl) and the Klenow reaction mix. The solutions were mixed thoroughly by tapping the plate and incubated at room temperature for 25 min. dNTP chase mix (2 μl) was then added to each well, mixed thoroughly and the plate incubated for a further 20 min at room temperature. Sequencing reactions were stopped by addition of formamide dye (2 μl) to each well and the double-stranded reaction products were denatured by incubating the solutions at 80°C for 15 min and leaving on ice before separation by electrophoresis through urea-polyacrylamide gels.

2B.7.2 Plasmid sequencing using T7 DNA polymerase

The plasmid sequencing was performed as outlined for Sequenase™ version 2.0 (United Stated Biochemical Co.) using T7 DNA polymerase or Sequenase ™ version 2.0 DNA polymerase (a modified T7 DNA polymerase). Purified, RNA-free plasmid DNA (3-5 μg) in TE (18 μl) was denatured by adding NaOH (2 M; 2 μl) at room temperature for 5 min, the mixture was neutralised by adding ammonium acetate (5 M, pH 5.4; 8 μl), and the DNA precipitated with ethanol. The DNA pellet was redissolved in distilled water (7 μl) and ready for use as template for sequencing.

Sequencing reagents:

5 x sequencing buffer	Tris-HCl, 200 mM, pH 7.5; $MgCl_2$, 100 mM; NaCl, 250 mM
Enzyme dilution buffer	Tris-HCl, 10 mM, pH 7.5; DTT, 5 mM; BSA, 0.5 mg/ml
Labelling mix (5 x)	dGTP, 7.5 μM; dCTP, 7.5 μM; dTTP, 7.5 μM
Termination mixes	Each mixture contains dGTP, 80 μM; dATP, 80 μM; dCTP, 80 μM; dTTP, 80 μM and NaCl, 50 mM. In addition, the G° mix contains ddGTP, 8 μM; the A° mix ddATP, 8 μM; the T° mix ddTTP, 8 μM and the C° mix ddCTP, 8 μM
Stop solution	formamide, 95%; EDTA, 20 mM; bromophenol blue, 0.05% (w/v); xylene cyanol FF, 0.05% (w/v)
Labelled ATP	[α-^{35}S]-dATP, 1000 Ci/mmole
Sequenase™ (USB)	13 U/μl

The sequencing primer (1 pmole/μl; 1 μl) was annealed with DNA template (3-5 μg; 7 μl) in the presence of 5 x sequencing buffer (2 μl). The mixture was incubated at 65°C for 2 min and cooled slowly to room temperature. The annealed primer was then extended with Sequenase™ and a nucleotide mixture, in a reaction consisting of:

template-primer	10 μl
DTT (0.1M)	1 μl
1 x labelling mix	2 μl
[α-^{35}S]-dATP	0.5 μl
Sequenase (1:8 diluted)	2 μl

This reaction was incubated at room temperature for 2-5 min. When the labelling reaction was complete, the reaction mixture (3.5 μl) was dispensed to each of four Eppendorf tubes containing prewarmed ddNTP (termination mix; G°, C°, A° or T°; 2.5

μl) and the solution was mixed thoroughly. The termination reaction was carried out at 37°C for 5 min and stopped by addition of stop solution (4 μl). The samples were heated to 80°C for 5 min and loaded (2-3 μl/lane) on the sequencing gel.

2B.7.3 Resolution of sequencing reaction products by electrophoresis in urea-polyacrylamide gels

6% urea-polyacrylamide solution:

urea	17 g
40% (w/v) acrylamide	6 ml
[38% (w/v) acrylamide,	
2% (w/v) *N,N'-bis*-methylene acrylamide]	
10 x TBE	2 ml
Add H_2O made up to 40 ml	

To the solution, ammonium persulphate (10%; 240 μl) and *N,N,N',N'*-tetramethylethylenediamine (TEMED; 35 μl) were added. The solution was poured immediately between 2 glass plates with gel dimensions of 380 mm x 200 mm x 0.5 mm with a shark-tooth comb inserted and allowed to polymerise. The nested set of extension products in the sequencing reactions were loaded onto the gel and were size fractionated by electrophoresis using 1 x TBE buffer at constant power of 25-30 watts. The gels were run until the required fragment separation was achieved (bromophenol blue migrates with DNA fragments of about 25 nucleotides in length, xylene cyanol migrates with DNA fragments of about 75 nucleotides in length). After electrophoresis, the gel was retrieved and fixed by soaking in a solution containing methanol [5% (v/v)] and glacial acetic acid [5% (v/v)] for 15 min, transferred to blotting paper, covered with thin plastic film (Saran Wrap), dried under vacuum at 80°C, and autoradiographed.

2B.8 Site-directed mutagenesis

2B.8.1 Phosphorylation of 5′ end of oligodeoxynucleotide with T4 polynucleotide kinase

Phosphorylation of the 5′ end of the mutant oligonucleotide, 412G, 411G or 359G, was carried out at 37°C for 90 min in the following reaction mixture:

oligonucleotide (40 pmoles/μl) 6 μl
10 x polynucleotide kinase buffer (Tris-HCl, 0.5 M, pH 7.5; $MgCl_2$, 0.1 M; DTT, 50 mM; ATP, 10 mM; BSA, 0.5 mg/ml) 1 μl
T4 polynucleotide kinase (0.5 U/μl, prepared by S. Bruce) 2 μl
H_2O 1 μl

After completion of the reaction, the mixture was heat-treated (70°C for 10 min) following the addition of distilled water (40 μl) and PCA extracted. The DNA was precipitated with ethanol and resuspended in distilled H_2O (8 μl) which was used for the following annealing reaction.

2B.8.2 Synthesis of mutant strand DNA and recovery of mutant clones

The bacteriophage M13 single-stranded DNA template used for mutagenesis was the vector M13mp19 with a 594-bp EcoRI-SalI fragment carrying the $HBc_{(3-144)}$ and $HBs_{(111-156)}$ sequences. The phosphorylated, mutant primer (30 pmoles/μl; 8 μl) and the M13 -20 universal primer (1 pmole/μl; 3 μl) were annealed to the template DNA (1 μg/μl; 7 μl) in the presence of 10 x TM (2 μl) at 80°C for 5 min and cooled slowly to room temperature.

Synthesis of the mutant strand DNA was carried out at room temperature for 90 min in the following reaction mixture:

Annealed mixture from the above reaction 20 μl
dNTP chase mix 20 μl

ATP (50 mM) 1 μl

E. coli DNA polymerase I (Klenow fragment) (5 U/μl) 1.5 μl

T4 DNA ligase (1 U/μl) 5 μl

sequencing TE 2.5 μl

After completion of the reaction, the mixture was PCA extracted and the DNA was precipitated with ethanol and suspended in TE (10 μl). The double-stranded DNA insert was excised from the bacteriophage M13 by digestion with EcoRI and SalI and the resulting 594-bp fragment was ligated to M13 mp19 RF DNA that had been linearised by digestion with EcoRI and SalI. *E. coli* strain BMH71-18 *mut*L was transformed with the ligation mixture and plated onto minimal agar plates containing X-gal and IPTG in the presence of TG1 as target cells. The colourless plaques were isolated and single-stranded DNA prepared. M13 clones containing a mutated HBV DNA insert were identified by T-track sequencing. The candidate mutants were then fully sequenced for the entire insert. The mutated insert was excised from the M13 vector by digestion with EcoRI and SalI, and the resulting 594-bp fragment was ligated to pHBcX linearised by digestion with EcoRI and SalI (see Section 3.4.2), thus $pHBcS_{145R}$, $pHBcS_{145E}$ and $pHBcS_{145K}$ were constructed.

2B.9 Amplification of DNA using the polymerase chain reaction (PCR)

$HBs_{(111\text{-}156)}$ fragment carrying wild type, gly_{145}→arg or gly_{145}→glu mutation at amino acid residue 145 of HBsAg was amplified by using 010H and 011H primers which share 17-nucleotide homology to the 5′ and 3′ ends of $HBs_{(111\text{-}156)}$ sequence, respectively, but contain additional nucleotides for an EcoRI cleavage site at the 5′ end in each primer. pHinG2, $pHinG2_{145R}$ and $pHBcS_{145E}$ were the templates for amplification of $HBs_{(111\text{-}156)}$ carrying wild type, gly_{145}→arg and gly_{145}→glu mutation at amino acid residue 145 of HBsAg, respectively. All reactions were carried out using Tri-thermoblock TB1 (Biometra, Gottingen, Germany) in the following reaction mixture (50 μl):

10 x PCR buffer (Tris-HCl, 0.1 M; KCl, 0.5 M; $MgCl_2$, 15 mM;

gelatine, 1 mg/ml; pH 8.3) 5 μl

dNTP (10 mM) 0.5 μl

010H (52.5 pmoles/μl) 0.5 μl

011H (26 pmoles/μl) 0.8 μl

Taq polymerase (5 U/μl; Boehringer) 0.5 μl

DNA template (30 ng in Tris-HCl, 10 mM, pH 8.0; EDTA, 0.1 mM) 0.5 μl

H_2O 42 μl

Submersion under 1 drop of mineral oil (Sigma)

The reactions also include mixtures in the absence of DNA template, one or two primers to serve as negative controls. Cycle conditions were 3 min at 93°C, 1 min at 50°C and 0.5 min at 70°C for 3 cycles followed by 1 min at 93°C, 1 min at 65°C and 0.5 min at 70°C for 27 cycles. After completion of the reactions, DNA (1 μl) from each reaction mixture was run in an agarose gel to assess the DNA concentration. The reaction mixtures were then PCA extracted twice, ethanol precipitated, and suspended in distilled water for further manipulation.

2B.10 Protein methods

2B.10.1 Protein assay (Lowry method)

Lowry reagent:

A 2% Na_2CO_3 (anhydrous) in 0.1 N NaOH

B 0.5% $CuSO_4.5H_2O$ in 1% sodium citrate

C 50 ml A + 1 ml B (freshly prepared)

D Folin-Ciocalteau reagent (Sigma) diluted 1:1 with water

Protein sample (0.4 ml) was added to reagent C (2 ml) and the solution was mixed and left at room temperature for 10 min. Reagent D (200 μl) was added, mixed well, and the mixture was stood for 30 min at room temperature. OD_{550nm} was recorded for each sample. The assay was calibrated using BSA at a concentration of 10 to 200 μg/ml.

2B.10.2 Electrophoresis of proteins in SDS-polyacrylamide gels (SDS-PAGE)

Acrylamide stock solution (30%):

acrylamide	29.2% (w/v)
N,N'-bis-methylene acrylamide	0.8% (w/v)

in H_2O, filter and store in the dark at 4°C (1 month maximum).

Separation gel solution: (numbers are volumes in ml)

Gel	30% Acrylamide	1.5 M Tris-HCl pH 8.8	H_2O
5%	1.66	4.18	4
7.5%	2.5	3.34	4
10%	3.34	2.5	4
12.5%	4.16	1.68	4
15%	5	0.84	4

Add SDS (10% w/v; 100 μl), ammonium persulphate (10% w/v; 50 μl) and TEMED (5 μl).

Stacking gel solution:

Tris-HCl (0.5 M, pH 6.8)	2.5 ml
acrylamide stock 30%	1.3 ml
distilled H_2O	6.1 ml
SDS [10% (w/v)]	100 μl
ammonium persulphate [10% (w/v)]	50 μl
TEMED	10 μl

10 x runner buffer (pH 8.3):

Tris-HCl	0.25 M
glycine	1.92 M
SDS	1% (w/v)

2 x sample buffer:

Tris-HCl, pH 6.8	125 mM
SDS	4% (w/v)
glycerol	20% (v/v)
2-ME	10% (v/v)
(or DTT	100 mM)
bromophenol blue	0.02% (w/v)

Proteins were separated by SDS-PAGE as described by Laemmli (1970). Separation gel solution (12.5% or 15%) was prepared and poured into the Mini-protein II dual slab cell (Bio-Rad, Richmond, U.S.A.) or between two sealed 16 cm x 16 cm glass plates separated at their edges by 1.5 mm spacers leaving 3 cm from the top for a stacking gel. The poured separation solution was overlaid with butanol (water saturated) to a depth of about 1 cm and the assembly left in an upright position at room temperature to allow polymerisation. The butanol was poured off the surface and the top rinsed with water. The stacking gel solution was prepared and poured onto the separating gel, and a Teflon slot-forming comb (10 or 15 wells x 0.75 mm for mini-gels; 10 or 15 wells x 1.5 mm for big gels) was inserted. The gel was allowed to polymerise and the comb was then removed. The gel assembly was transferred to an electrophoresis tank which was filled with 1 x runner buffer and the wells of the gel flushed out with running buffer. Samples were prepared by mixing with an equal volume of 2 x sample buffer and boiling at 100°C for 5 min. The samples were then short spun in a microcentrifuge to collect condensation and pellet any cell debris before being loaded on the gel. Low molecular weight markers (Pharmacia), including phosphorylase b (94 kD), albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20 kD), and α-lactalbumin (14.4 kD), were also loaded into one well, for molecule weight determination. Rainbow™ coloured protein molecular weight markers (2.35, 3.4, 6.5, 14.3, 21.5, 30 and 46 kD; Pharmacia) were included in the gels for immunoblot analysis. Electrophoresis was performed at 25-30 mA.

2B.10.3 Staining of proteins with Coomassie blue in gels following electrophoresis

After electrophoresis, polyacrylamide gels were removed and stained in staining solution consisting of methanol [40% (v/v)], acetic acid [10% (v/v] and Coomassie brilliant blue R-250 [0.2% (w/v); Sigma] with gentle shaking at room temperature. The time required for staining depends on the thickness of the gel and its polyacrylamide concentration. For mini-gel, 20 min of staining was sufficient. Gels were destained in destaining solution containing methanol [40% (v/v)] and acetic acid [10% (v/v)] with shaking at room temperature.

2B.10.4 Immunoblotting (Western blot)

Immunoblotting consists of separation of the proteins by electrophoresis, transfer and immobilisation of the proteins onto nitrocellulose membranes, and detection of specific proteins or antigens using antibodies as the probe. The method is a modification of the procedures described by Towbin *et al.* (1979).

Transfer buffer (pH 8.3):

Tris-HCl	25 mM
glycine	192 mM
methanol	20% (v/v)

10 x Tris-saline (TS) (pH 8.1):

Tris-HCl	10 mM
NaCl	150 mM

Blocking solution:

nonfat dry milk (Marvel)	5% (w/v)
[or BSA (IgG free, fraction V; BDH)	3% (w/v)]
sodium azide	0.02% (w/v)
in 1 x TS	

Alkaline phosphatase buffer:

Tris-HCl (pH 9.5)	100 mM
NaCl	100 mM
$MgCl_2$	50 mM

2B.10.4.1 Transfer of proteins from gels to nitrocellulose membranes

After separation of the proteins by SDS-PAGE, the stacking gel was removed and the separation gel equilibrated in transfer buffer prior to blotting. The proteins were electroblotted using the Trans-Blot™ cell (Bio-Rad). The gel was overlaid with a nitrocellulose membrane (BioBlot™-NC; Costar, Cambridge, Mass., U.S.A.) and this was sandwiched between 3MM filter paper and foam sponge. All the components were soaked in transfer buffer before assembly. The electroblot apparatus was assembled and filled with transfer buffer, with the nitrocellulose membrane facing the anode, and the proteins transferred to the nitrocellulose at 60V (350 mA) for 3-4 hr or 30V (120 mA) overnight.

After transfer and prior to probing blots for the presence of a specific antigen, the total composition of the proteins transferred to the membrane can be stained reversibly with Ponceau S [0.5% (w/v) Ponceau S (Sigma) in 3% TCA (trichloroacetic acid)] for 30-60 seconds at room temperature with agitation and destained with distilled water. In some experiments, Rainbow™ coloured protein molecular weight markers (Pharmacia) were run in parallel on the gels to serve as internal markers for transfer and molecular weight calibration.

2B.10.4.2 Immunostaining of specific proteins using antibodies

Before the blot could be processed for antigen detection, it was incubated in blocking solution at room temperature for at least 1 hr with agitation to prevent nonspecific adsorption of the immunological reagents. The blocking solution was poured off, and the blot incubated with the appropriate dilution of primary antibody in blocking

solution at room temperature for 2 hr or longer with agitation. The primary antibody was removed, and the blot was washed with 5 changes of 1 x TS for 5 min each. Depending on the animal source of the primary antibody, the secondary antibody was either goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma) or sheep anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (Sigma). The blot was incubated with a 1:7500 dilution of secondary antibody in blocking solution for 2 hr at room temperature with agitation. The blot was washed with 5 changes of 1 x TS for 5 min each.

Just prior to developing the blot, a fresh substrate solution was prepared by adding nitroblue tetrazolium (NBT, 5% in 70% dimethylformamide; 66 μl) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, 5% in 100% dimethylformamide; 33 μl) to alkaline phosphatase buffer (10 ml). Substrate solution was added to the blot with agitation to develop a colour reaction usually within 10 min. When the bands were suitably dark, the reaction was stopped by washing the blot in several changes of distilled water.

2B.10.5 Purification of HBcAg fusion proteins produced in *E. coli*

Cultures of *E. coli* strain RB791 harbouring recombinant plasmids directing the expression of various HBcAg fusion proteins were grown overnight at 37°C with shaking in LB supplemented with ampicillin (100 μg/ml) (L-amp medium) to high cell density and diluted with fresh L-amp medium (1:50) the following day. When the culture reached OD_{600} =1.0, expression was induced by the addition of IPTG to 0.5 mM. After 16-18 hr of shaking at 37°C, the bacteria were harvested by centrifugation (12,000 x g) for 10 min at 4°C. The cell pellet was suspended in 1/200 volume of the culture medium of lysis buffer [Tris-HCl, 50 mM, pH 8.0; Triton-X100, 1% (v/v); phenylmethyl-sulphonyl fluoride (PMSF), 0.2 mM] and sonicated for 8-10 x 30 sec (Soniprobe, Dawe instruments type 7530A). The cellular debris in the lysate was sedimented by centrifugation (12,000 x g) for 10 min at 4°C and the supernatant, ie. crude extract, was recovered. If *E. coli* strain TG1 was used for the production of

HBcAg fusion proteins in Terrific broth, instead of *E. coli* strain RB791 in LB, approximately double the cell density was obtained.

Proteins in the crude extract were precipitated by adding ammonium sulphate to 30% saturation. The precipitate was centrifuged (27,000 x g) for 15 min at 4°C and the pellet resuspended in 50 mM of Tris-HCl (pH 8.0) in the same volume as the lysis buffer. The ammonium sulphate fraction was dialysed against three changes of 50 mM of Tris-HCl (pH 8.0) at 4°C overnight. The fusion protein particles were collected by ultracentrifugation at 100,000 x g (TH640 rotor; Combi, Sorvall) for 1-1.5 hr at 4°C, resuspended in 50 mM of Tris-HCl (pH 8.0; 1 ml), and centrifuged at 12,000 x g for 3-5 min to remove few precipitated pellets. The supernatant containing HBcAg fusion proteins was chromatographed on Sepharose 4B-Cl in the same solvent containing PMSF (0.2 mM) and NaN_3 (5 μM). Fractions containing the fusion proteins identified by SDS-PAGE were pooled, and the product was collected by ultracentrifugation (100,000 x g) for 1 hr. The final products were suspended in PBS and dialysed against the same buffer to remove trace amount of NaN_3.

2B.11 Immunological methods

2B.11.1 Preparation of immunogens for immunisation

The antigens, eg. HBcAg, HBsAg and HBcAg fusion proteins, diluted in PBS were added to an equal volume of complete Freund's adjuvant (CFA) for immunisation or incomplete Freund's adjuvant (IFA) for boost, and two phases were mixed by using a tissue homogeniser (Omni-mixer; Sorvall, Newtown, Conn., U.S.A.) or by passing the mixtures from a syringe into a glass universal bottle and back vigourously, and repeatedly until a thick emulsion developed.

2B.11.2 Immunisation and bleeding of animals

Outbred Dutch male rabbits (~6 months of age; ~1.5 kg) were immunised

intramuscularly (I.M.) with 2 ml of immunogens containing various concentration of HBcAg fusion proteins (ranging from 0.2 mg to 1 mg) or other antigens (see Table 5.1) emulsified in CFA and were subsequently boosted for a number of times (see Table 5.1) with the same antigen emulsified in IFA (2 ml).

Inbred BALB/c mice were obtained from the Department of Medical Microbiology, University of Edinburgh, while inbred B10.S/Ola/Hsd ($H\text{-}2^s$) mice were purchased from Harlan Olac Ltd., Oxon, U.K. Mice were immunised intraperitoneally (I.P.) with various concentrations of immunogens emulsified in CFA (0.2 ml) and were subsequently boosted for a number of times with the same immunogen emulsified in IFA (0.2 ml).

The injection and bleeding of the animals were carried out by the staff of the Medical Microbiology Transgenic Unit, University of Edinburgh. Rabbits were bled from the rear marginal ear vein. A very small amount of xylene was applied to the tip of the ear to encourage vein dilation and the vein was cut across by the tip of a pointed scalpel blade. The blood was collected into a glass universal tube containing heparin (15 U for 1 ml of blood) for isolation of PBLs or without heparin for separation of serum. Mice were bled from retroorbital plexus after anaesthesia. For terminal bleeding, cardiac puncture was carried out.

2B.11.3 Separation of sera from blood

Rabbit blood collected in a glass universal tube was allowed to clot for 30-60 min at 37°C. The clot was then separated from the walls of the container using a Pasteur pipette and left at 4°C overnight for the clot to contract. The serum was withdrawn from the clot and was centrifuged at 10,000 x g for 10 min at 4°C to remove red blood cells. The rabbit sera were heat-inactivated at 56°C for 30 min, aliquoted and stored at -20°C.

Mouse blood pooled from each group and collected in an Eppendorf tube was allowed

to clot for 30-60 min at 37°C and centrifuged for 5 min to remove red blood cells. The sera, aliquoted or not, were stored at -20°C.

2B.11.4 Serum preparation from plasma

Plasma obtained from blood collected in vessels containing heparin was centrifuged at 500 x g for 20 min to remove the cell fraction which was used for isolation of PBLs. To prepare serum from plasma, 1/100 volume of thrombin solution (100 iu/ml in 1M $CaCl_2$) was added to the plasma which had been warmed to 37°C and mixed vigourously to promote clot formation. The treated plasma was left at room temperature for 60 min and the formed clot was compressed to withdraw the maximum serum. If clot did not form, protamine sulphate was added to enhance its formation. The supernatant was withdrawn, centrifuged at 17,000 x g at 4°C for 20 min, and the serum collected.

2B.11.5 Solid-phase RIAs

2B.11.5.1 Preparation of a protein- or synthetic peptide-coated solid phase

Polystyrene beads (polystyrene balls, 6.4 mm diameter, specular finish, NBL) were placed in a 50-ml centrifuge tube (Falcon) and coated with HBcAg (0.54 or 0.72 μg/ml) or HBsAg (0.15 or 0.5 μg/ml) in coating buffer (carbonate-bicarbonate buffer, 0.05 M, pH 9.6) for 6-7 hr or overnight at room temperature. For preparation of solid phase coated with synthetic peptides, polystyrene beads were coated with synthetic peptide (20 or 50 μg/ml in coating buffer), ie. $preS_{1(1-23)}$, $preS_{2(1-26)}$ or $HBs_{(111-156)}$, overnight at room temperature. For preparation of solid phase coated with monoclonal anti-HBs or polyclonal anti-HBc or anti-HBs antibodies, polystyrene beads were coated with mouse ascites fluids (1:150 or 1:200 in coating buffer) containing monoclonal anti-HBs antibodies (approx. 2.5-10 μg/ml of IgG), RFHBs-1, -2 or -7, or with polyclonal anti-HBc IgG or anti-HBs IgG (60 μg/ml in coating buffer) overnight at room temperature.

The protein- or peptide-coated beads were washed three times with PBS and the remaining sites for protein binding were blocked with PBS containing 10% FCS, donkey or sheep serum, depending on the animal source of the second antibody used in the RIA, for at least 2 hr or overnight at room temperature. The beads were washed three times with PBS and blotted dry on tissue paper. The beads were then ready for use and were stored at 4°C.

2B.11.5.2 Assay of HBc antigenicity by the sandwich RIA

The HBcAg reactivity was determined by the sandwich RIA as illustrated in Figure 2.1 (Stahl *et al.*, 1982). The protein samples were diluted in RIP buffer (0.5% BSA in PBS) to adjust the concentration and 200 μl of each was added in duplicate to appropriate wells containing anti-HBc-coated beads. The reaction was carried out overnight at room temperature. On the following day, the samples were removed by aspiration and the beads were washed 10 times by filling the well each time with distilled water and removing it by aspiration. ^{125}I-labelled human anti-HBc IgG (300,000 cpm/ml in RIP buffer; 200 μl), which was kindly provided by P. McCulloch and G. Leadbetter of the Department of Medical Microbiology, University of Edinburgh, was added to each well. The plate was then sealed and incubated at 45°C for 2 hr. After extensive washings with distilled water, the beads were transferred to counting tubes and counted in a γ counter (1275 Minigamma; LKB, Turku, Finland).

2B.11.5.3 Assay of HBs antigenicity by the sandwich RIA

A commercial solid-phase sandwich RIA (AUSRIA II-125, Abbott Laboratories) as illustrated in Figure 2.1 was used for detection of HBsAg and the protocol was according to the procedure B recommended by the manufacturer. The protein samples were diluted in PBS and 200 μg of each was added to appropriate wells containing beads coated with guinea pig antibody to HBsAg. The reaction was carried out overnight at room temperature. The samples were removed by aspiration and the beads were washed extensively with distilled water. ^{125}I-labelled human anti-HBs

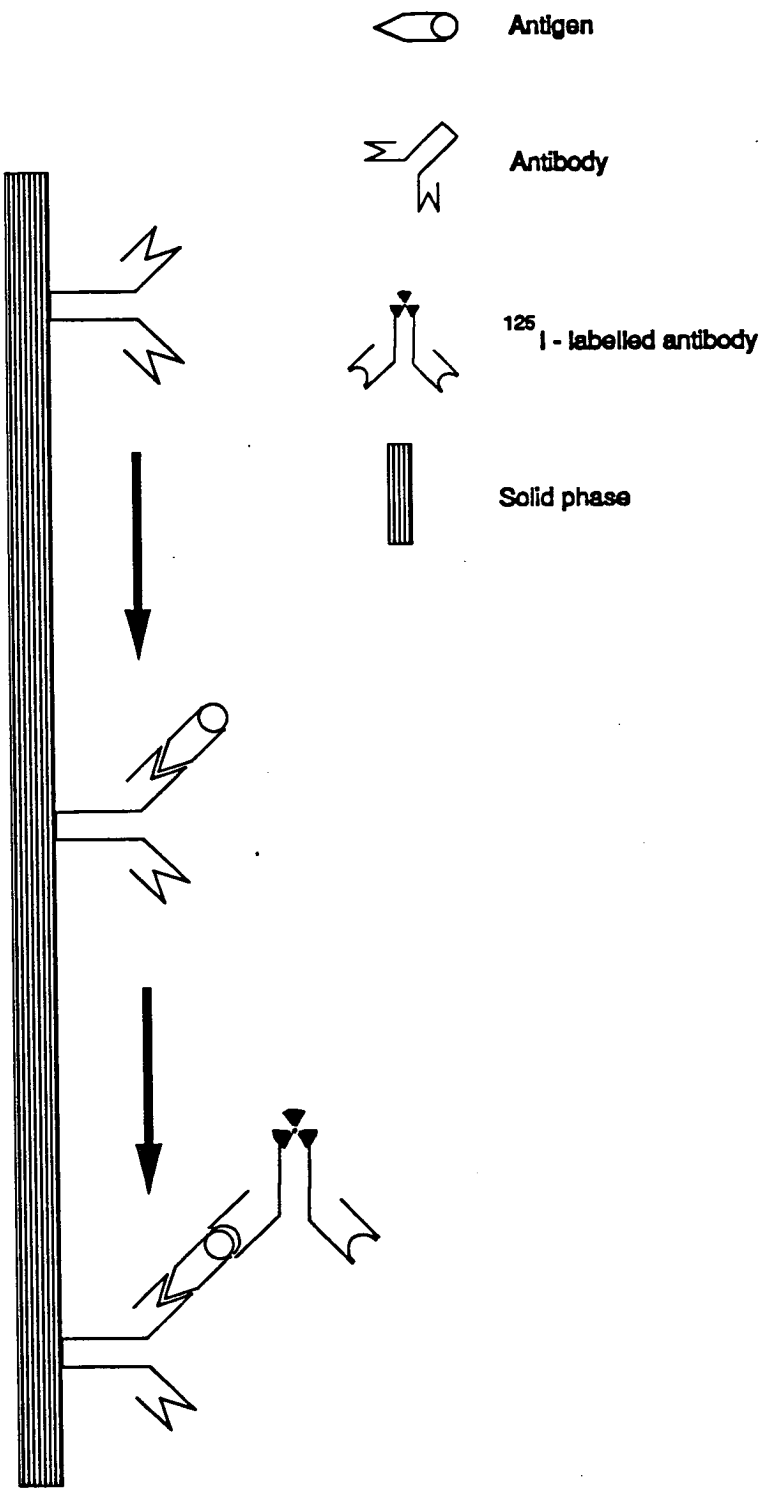


Figure 2.1 Antigen-capture (sandwich) RIA.

(0.74 μCi/ml; 200 μl) was added to each well and incubated with the beads for 1 hr at 45°C. After extensive washings with distilled water, the beads were transferred to counting tubes and counted in a γ counter (1275 Minigamma, LKB). The titre is expressed as the P/N ratio which represents cpm ratio of samples measured (P) and negative control (N). The P/N ratio equal to or greater than 2.1 is to be considered positive for HBsAg.

2B.11.5.4 Assay of HBc/HBs antigenicity in the HBcAg fusion protein by the sandwich RIA

The HBc/HBs antigenicity was determined using a modification of the sandwich RIA (Figure 2.1) in which the HBcAg fusion proteins were captured on the solid phase coated with human anti-HBc antibody and were then detected by rabbit anti-HBs antibody directed against an HBsAg epitope on the tested proteins. The HBcAg fusion proteins were diluted in blocking buffer (PBS containing 10% donkey serum) and 200 μl was added in duplicate to appropriate wells containing anti-HBc-coated beads. After incubation for 2 hr at 37°C, the samples were removed by aspiration and the beads washed with washing buffer (PBS containing 0.05% Tween 20) for 5-6 times and anti-HBs antibody (R82 1:1000 dilution in blocking buffer; 200 μl) was added to each well. After an hour incubation at 37°C, the beads were washed and ^{125}I-labelled donkey anti-rabbit immunoglobulin (250,000 cpm/ml in blocking buffer; ~1,800 Ci/mmole, Amersham; 200 μl) was added to each well. After a further hour at 37°C, the beads were washed with washing buffer extensively, transferred to counting tubes and counted in a γ counter (1275 Minigamma, LKB).

2B.11.5.5 Anti-HBs monoclonal antibody (RFHBs-1, -2 , and -7) binding assay

The method used to determine binding of the HBcAg fusion proteins to anti-HBs MAbs was the sandwich RIA (Figure 2.1) similar to that described for HBc/HBs antigenicity (Section 2B.11.5.4). The test samples were diluted in blocking buffer (PBS containing 10% donkey serum) and 200 μl of each was added in duplicate to

appropriate wells containing polystyrene beads coated with RFHBs-1, RFHBs-2 or RFHBs-7. After incubation for 2 hr at 37°C, the samples were removed by aspiration and the beads washed with washing buffer for 5-6 times and anti-HBs antibody (R82 1:2000 dilution in blocking buffer; 200 μl) was added to each well. After an hour incubation at 37°C, the beads were washed and ^{125}I-labelled donkey anti-rabbit immunoglobulin (250,000 cpm/ml in blocking buffer; ~1,800 Ci/mmole, Amersham; 200 μl) was added to each well. After a further hour at 37°C, the beads were washed with washing buffer extensively, transferred to counting tubes and counted in a γ counter (1275 Minigamma, LKB). The binding is expressed as the P/N ratio which represents cpm ratio of samples measured (P) and the background control (N) containing the blocking buffer only.

2B.11.5.6 Assay of anti-HBc antibody by the competitive RIA

The competitive RIA (Figure 2.2) was used to detect anti-HBc as described by Murray *et al.* (1984). Sera to be tested, including positive and negative sera, for anti-HBc were serially 2-fold diluted in RIP buffer and 100 μl of each was added in duplicate to appropriate wells of the reaction tray followed by the addition of ^{125}I-labelled human anti-HBc IgG (480,000 cpm/ml in RIP buffer; 100 μl), which was kindly provided by P. McCulloch and G. Leadbetter of the Department of Medical Microbiology, University of Edinburgh. After adding one HBcAg-coated bead to each well, the reaction tray was incubated overnight at room temperature. On the following day, the beads were washed with PBS or distilled water for 6-10 times and their radioactivity counted by a γ counter (1275 Minigamma, LKB). The anti-HBc titre is expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled anti-HBc binding to the solid-phase HBcAg. The titres were calibrated using positive anti-HBc antiserum (R87) as the standard.

2B.11.5.7 Assay of anti-HBs antibody by the sandwich RIA

A commercial solid-phase sandwich RIA (AUSAB, Abbott Laboratories) as illustrated

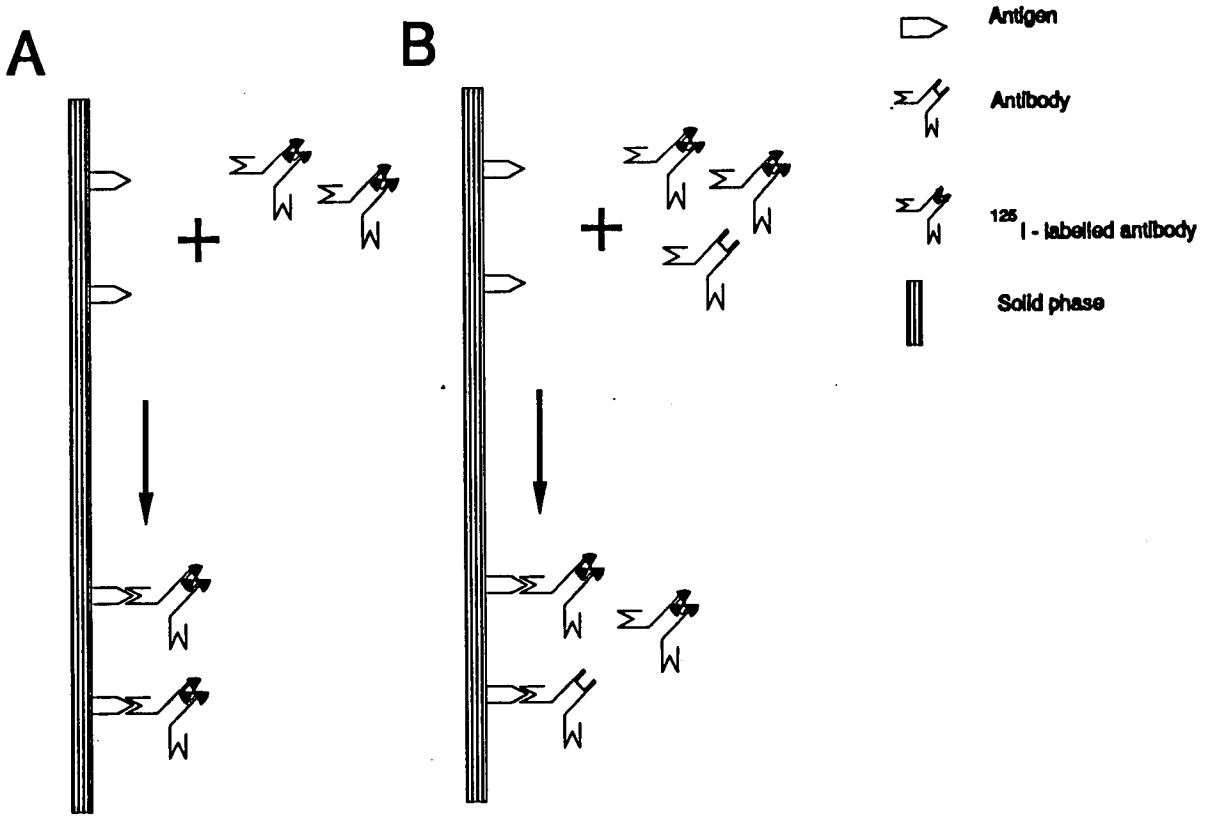


Figure 2.2 Competitive RIA. (A) ^{125}I-labelled antibody binds to antigen on the solid phase. (B) Unlabelled antibody inhibits the binding of labelled antibody.

in Figure 2.3 for detection of anti-HBs antibody was used according to the manufacturer's instructions. Test serum (200 μl), including positive and negative controls supplied by the kit, was added to one well containing an HBsAg-coated bead and the reaction tray was covered and incubated for 16-20 hr at room temperature. The samples were removed by aspiration and the beads were washed with distilled water extensively. ^{125}I-labelled HBsAg (0.74 μCi/ml; 200 μl) was added to each well and incubated with the beads at room temperature for 4 hr. After extensive washings with distilled water, the beads were transferred to counting tubes and counted in a γ counter (1275 Minigamma, LKB). The estimated RIA unit value is determined by calculating the ratio of the reactivity of the test serum to the positive control and selecting the estimated RIA unit value for this ratio according to the data supplied by the manufacturer. The RIA unit shown in the data is the mean of the value obtained from two or three independent experiments.

2B.11.5.8 Assay of anti-peptide antibodies by the indirect antibody RIA

The serum samples were assayed for anti-$preS_{1(1-23)}$, anti-$preS_{2(1-26)}$ and anti-$HBs_{(111-156)}$ antibodies by the indirect antibody RIA (Catty and Murphy, 1989) as illustrated in Figure 2.4. The tested rabbit or mouse sera were diluted 1:100 and 1:1000 with blocking buffer (PBS containing 10% donkey serum for rabbit serum samples or sheep serum for mouse serum samples) and 200 μl of each was added in duplicate to appropriate wells containing synthetic peptide [$preS_{1(1-23)}$, $preS_{2(1-26)}$ or $HBs_{(111-156)}$]-coated beads. The reaction tray was incubated at 37°C for 2 to 2.5 hr. The sera were removed by aspiration and the beads were washed extensively with washing buffer. ^{125}I-labelled donkey anti-rabbit immunoglobulin (250,000 cpm/ml; ~1,800 Ci/mmole, Amersham; 200 μl) or ^{125}I-labelled sheep anti-mouse immunoglobulin (250,000 cpm/ml; ~2,400 Ci/mmole, Amersham; 200 μl) was added to each well and incubated with the beads at 37°C for 1 to 1.5 hr. After extensive washings with washing buffer, the beads were transferred to counting tubes and counted in a γ counter (1275 Minigamma, LKB). The titre is expressed as the P/N ratio which represents cpm ratio of samples measured (P) and negative control (N). The P/N ratio equal to or greater

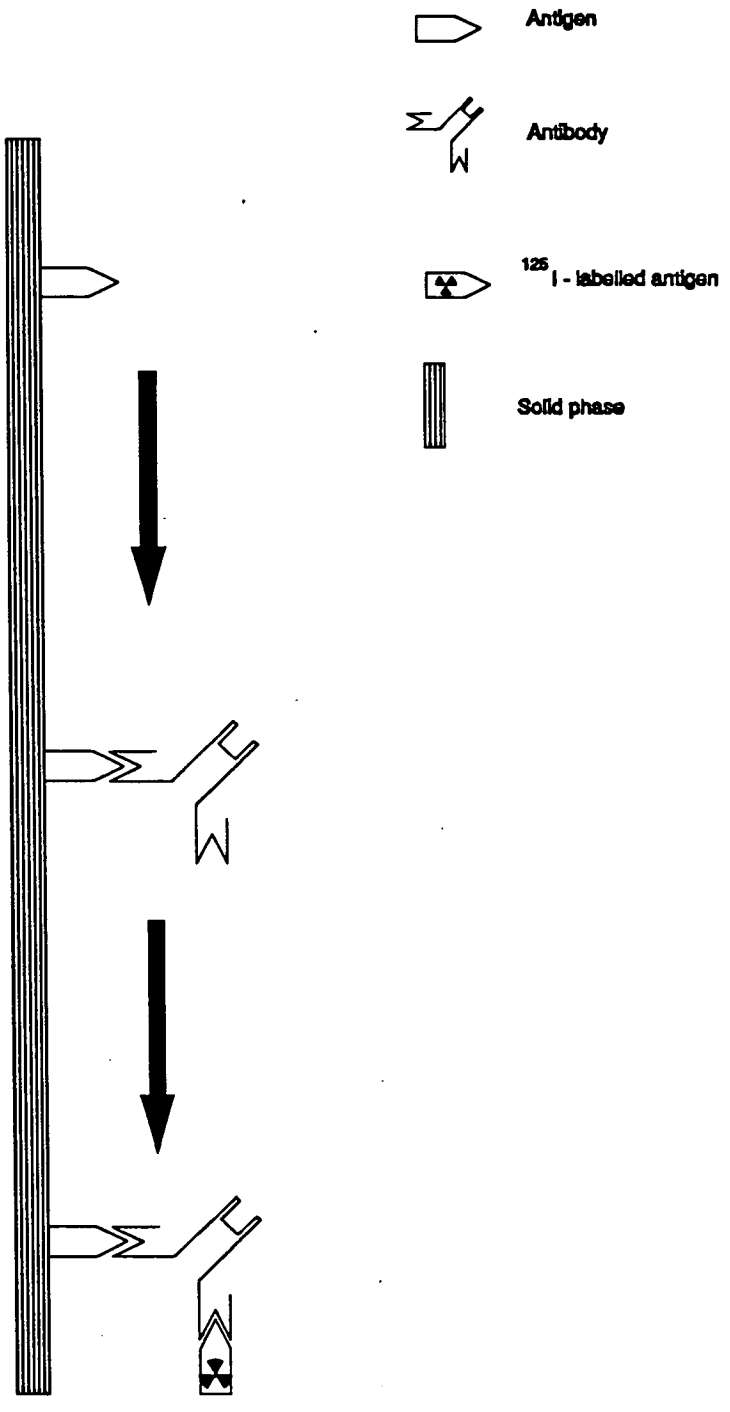


Figure 2.3 Antibody-capture (sandwich) RIA.

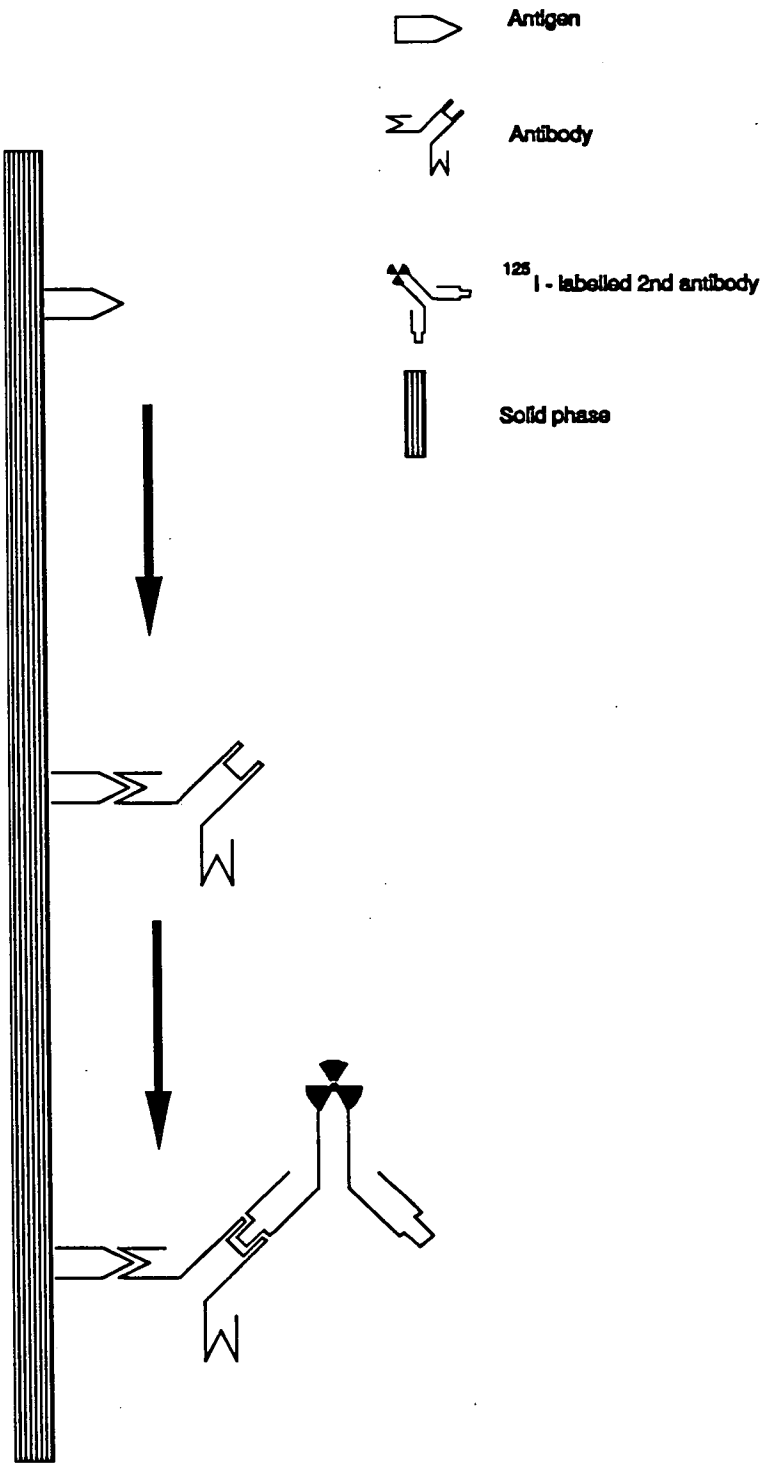


Figure 2.4 Indirect antibody RIA.

than 2.1 is to be considered positive for anti-peptide antibody.

2B.11.6 Liquid-phase RIAs

2B.11.6.1 Assay of anti-HBs antibody by the double antibody radio-immunoprecipitation (DARIP) test

The method for the detection of anti-HBs antibody in the liquid-phase DARIP assay is based on that described by Burrell *et al.* (1978) as illustrated in Figure 2.5. In the original method, the test human serum is incubated with ^{125}I-labelled HBsAg followed by precipitation of the human globulin (with or without complexed ^{125}I-labelled HBsAg) by the addition of goat anti-human IgG.

The test rabbit sera and negative rabbit control serum were diluted 1:10 in RIP buffer, while positive rabbit control serum (R82) was 10-fold diluted from 10^{-3} to 10^{-6} in 1:100 normal rabbit serum (in RIP buffer) as carrier proteins. The diluted test serum (50 μl) was added in duplicate to ^{125}I-labelled HBsAg (*adw* or *ayw* subtype, 3,000 cpm, corresponding to approx. 0.1-0.5 ng HBsAg; 50 μl) which was kindly provided by P. McCulloch and G. Leadbetter of the Department of Medical Microbiology, University of Edinburgh. More RIP buffer (100 μl) was added, mixed thoroughly, and the reaction mixture incubated for 24 hr at 4°C. The second antibody (100 μl), donkey anti-rabbit IgG serum with appropriate dilution in RIP buffer was added to each reaction mixture, mixed thoroughly, and incubated overnight at 4°C to precipitate IgG. Each tube containing the reaction mixture was measured for radioactivity as the "total count". Finally, RIP buffer containing starch [0.1% (w/v); 1 ml] was added to each test and centrifuged at 2,500 x g for 15 min at room temperature. The supernatant was decanted and the amount of radioactivity in the pellet determined. The amount of anti-HBs antibody in the test serum is expressed as the mean of the percentage of ^{125}I-HBsAg co-precipitated, ie. cpm in the pellet divided by cpm added (total count), from duplicate tests.

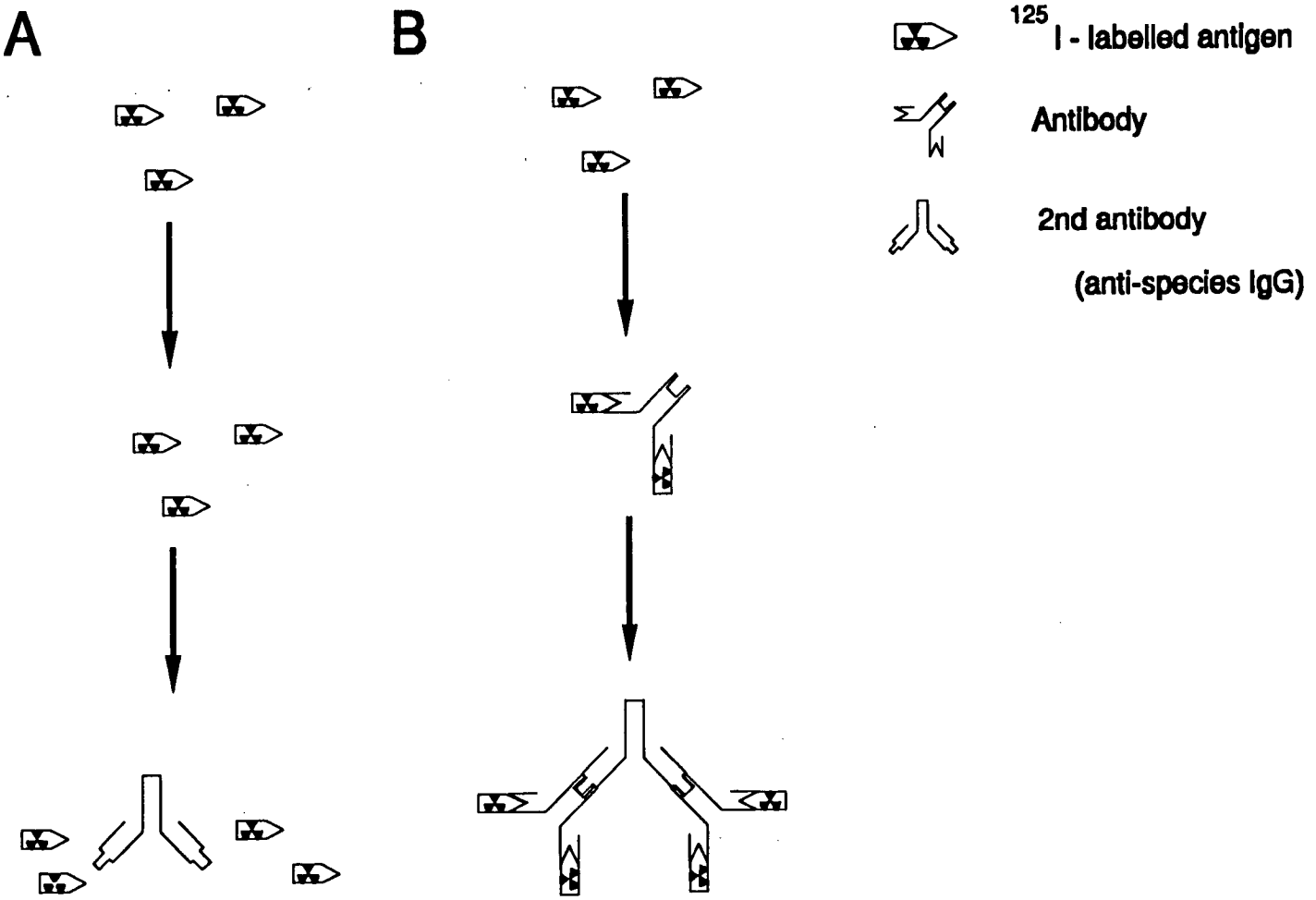


Figure 2.5 DARIP test. (A) In the absence of antibody, precipitate does not form. (B) ^{125}I-labelled antigen is co-precipitated with the complex of antibody and anti-species (depending on the origin of antibody) IgG.

2B.11.6.2 Assay of HBs antigenicity by the DARIP inhibition test

The method for the detection of HBsAg in the liquid-phase DARIP inhibition test is also based on that described by Burrell *et al.* (1978). The assay uses the test samples to inhibit anti-HBs antibody from binding to the same antigen radiolabelled. Therefore, the amount of HBsAg in the sample can be expressed as percent inhibition of a positive DARIP test.

The dilutions of first antibody (rabbit anti-HBs antibody), anti-rabbit IgG and carrier normal rabbit serum used in the DARIP inhibition test were determined by pilot titration experiments in this work. The test antigen (100 μl, diluted in RIP buffer) was incubated with anti-HBs antibody, R82 antiserum diluted 10^{-4} in 1:200 normal rabbit serum (diluted in RIP buffer), at 37°C for 3 hr. ^{125}I-labelled HBsAg (*adw* or *ayw* subtype, 3000 cpm; 50 μl) provided by P. McCulloch and G. Leadbetter of the Department of Medical Microbiology, University of Edinburgh, was added to the reaction, mixed thoroughly, and the mixture incubated for 24 hr at 4°C. Each tube containing the reaction mixture was measured for radioactivity as the "total count". Finally, RIP buffer containing starch [0.1% (w/v); 1 ml] was added to each test and centrifuged at 2,500 x g for 15 min at room temperature. The supernatant was decanted and the amount of radioactivity in the pellet determined. The amount of HBsAg in the test sample is expressed as the mean of the percent inhibition of ^{125}I-HBsAg co-precipitated from duplicate tests.

2B.11.7 Lymphocyte cultures

2B.11.7.1 Isolation of rabbit PBLs

Heparin-treated rabbit blood (10 ml) was centrifuged at 500 x g for 20 min and the plasma fraction was kept at 4°C for later use (see Section 2B.10.5). The cellular fraction (~5 ml) was diluted 1:4 with PBS and carefully layered on Ficoll-Paque solution (Pharmacia; 15 ml) and centrifuged at 1,490 rpm (400 x g; 43121-302 rotor,

Mistral 1000, MSE Scientific Instruments, Rawley, U.K.) for 40 min at room temperature. After centrifugation, erythrocytes and granulocytes were at the bottom layer, while the mononuclear cells, mostly lymphocytes, were found at the interface between the upper layer (PBS) and the Ficoll-Paque solution. The upper layer was then carefully drawn off by aspiration, leaving the white lymphocyte layer undisturbed. The lymphocyte layer was transferred to a sterile centrifuge tube by using a Pasteur pipette, mixed with at least 3 volumes of PBS, and centrifuged at 1,300 rpm (304 x g) for 5-10 min. The cell pellet was washed by resuspending in RPMI 1640 medium (10 ml) and centrifuging at 304 x g for 5-10 min for 3 times. The cells were resuspended in culture medium (RPMI 1640 medium containing L-glutamine 2 mM, HEPES 10 mM, 2-ME 5 x 10^{-5}M, $NaHCO_3$ 10 mM, and FCS 5%; 5 ml), from which 50 μl was removed and diluted 1:5 with trypan blue (0.4%) for counting cell numbers on a haemocytometer (Counter chamber; Housser Scientific, Horsham, PA., U.S.A). The cell suspension was adjusted to an appropriate density, eg. 5 x 10^6 cells/ml with culture medium. The cell yield was 5-15 x 10^6/ml of blood with a 90-99% cellular viability.

2B.11.7.2 Isolation of mouse splenocytes

Mice were sacrificed by cervical dislocation and their spleens removed aseptically and placed in a 100-mm petri dish containing 10 ml of RPMI 1640 medium supplemented with penicillin (200 U/ml) and streptomycin (200 μg/ml). After trimming off any contaminating tissue, the spleen was transferred to another petri dish containing the same medium (10 ml) and macerated with the bottom of a 5ml-syringe plunger or with a cell dissociation sieve (Grinder kit, Sigma). To ensure a single cell suspension formed, the cell suspension was passed through a 23-gauge needle twice. Cells were then washed three times with RPMI 1640 medium and adjusted to a density to 5 x 10^6 cells/ml with culture medium.

2B.11.8 Lymphocyte-proliferative assay

Rabbit PBLs or mouse splenocytes were cultured at a cell concentration of 2 x 10^5 or 5 x 10^5 cells/well in the culture medium with a total volume of 0.2 ml in flat-bottomed 96-well microtitre plates (Falcon) in the medium alone or in the presence of Concanavalin A (Con A, Pharmacia; 2 μg/ml), poly(Na-glutamate-alanine-tyrosine) (GAT; 6:3:1, M.W. 20,000-50,000, Sigma; 5 μg/ml), or various concentrations of antigens. Con A, a potent T-cell mitogen, was used to monitor the proliferative potential of lymphocytes, while GAT was used to monitor non-specific T-cell activation. The antigens used in the experiments include recombinant HBcAg (Biogen; Cambridge, Mass., U.S.A.), recombinant HBsAg (Biogen; or Green Cross; Osaka, Japan), and HBcAg fusion proteins purified in this work as well as $preS_{1(1-23)}$ and $preS_{2(1-26)}$ synthetic peptides. Each concentration of stimulants was assayed in triplicate or quadruplicate.

Following 4 days of culture with or without stimulants at 37°C (humidified 6.5% CO_2, 93.5% air atmosphere), 1 μCi of ^{3}H-thymidine (5 Ci/mmole, Amersham) was added to each well for an additional 18 hr of culture. The cells were harvested onto glass fibre filter strips (Flow Laboratories) using the Titertek™ cell harvester (Skatron; Lierbyen, Norway). The filter disks obtained from the strips were placed in plastic vials (Ponyl vial, Packard) containing scintillation fluid (2 ml), and the amount of ^{3}H-thymidine incorporated into DNA was counted with a liquid scintillation spectrometer (Tri-CARB™, 2000CA, Packard). Cell proliferation is expressed as the mean of the cpm of a triplicate or quadruplicate culture ± standard deviation (S.D.), while stimulation index (S.I.) is calculated by dividing the cpm of ^{3}H-thymidine incorporated into stimulated cells by the cpm incorporated into non-stimulated cells.

CHAPTER 3: CONSTRUCTION OF GENE FUSIONS TO HBcAg AND PURIFICATION OF HBcAg FUSION PROTEINS

3.1 Introduction

The epitopes from the envelope proteins of HBV, $HBs_{(111-156)}$, $preS_{1(1-20)}$ and $preS_{2(1-26)}$, were selected for fusion to HBcAg. $PreS_{1(1-20)}$ was chosen because it carries T-cell and B-cell epitopes (Milich *et al.*, 1987a) and the sequence used in this study was derived from the *adyw* subtype which lacks 11 N-terminal amino acid residues compared to the adw_2 or *adr* subtype and corresponds to $preS_{1(12-31)}$ of adw_2 subtype with substitution of 3 amino acid residues (Neurath, 1989). $PreS_{2(1-26)}$, the N-terminal half of the $preS_2$ region, which may elicit the production of neutralising antibody when conjugated to carrier proteins or not, was selected for the presence of both B- and T-cell epitopes (Neurath *et al.*, 1984; Thornton *et al.*, 1987 and 1989). $HBs_{(111-156)}$ which encompasses the *a* determinant of HBsAg was also chosen to provide B-cell epitopes of the S region. Several gene fusions were constructed in which coding sequences of inserted epitopes were linked to those for N-, C-, or both termini of truncated HBcAg. All plasmids expressing fusion proteins were based on ptacHpaII (Stahl and Murray, 1989), which contains the coding sequence for amino acid residues 3-144 of HBcAg fused to β-galactosidase (amino acids 1-8) with 3 extra amino acids from a linker sequence, with gene expression being controlled by the tac promoter (de Boer *et al.*, 1983).

The HBcAg gene fusions constructed here could be divided into 3 categories. First, the fusions carrying a monomer or a dimer of $HBs_{(111-156)}$ as well as $preS_1$ and $preS_2$ epitopes. Secondly, the fusions carrying the mutant sequences at gly_{145} of the immunodominant region of HBsAg, $HBs_{(111-156)}$; these include $HBcS_{145R}$, $HBcS_{145E}$ and $HBcS_{145K}$, which carry $HBs_{(111-156)}$ sequence with gly_{145} change to arginine, glutamic acid and lysine, respectively. Thirdly, the fusions carrying similar insertions as the first category, but containing one mutant sequence at gly_{145} of $HBs_{(111-156)}$. The principal HBcAg fusion proteins are presented schematically in Figure 3.1. The fusion proteins carrying wild-type and/or mutants at amino acid residue 145 of $HBs_{(111-156)}$ are shown in Figures 3.2 and 3.3.

Figure 3.1 Schematic representation of HBcAg fusion proteins encoded by the corresponding plasmids. Names and total number of amino acids (aa) of the proteins are at the left. Numbers in the boxes indicate the positions of the amino acids in the native proteins. β-gal, β-galactosidase. The first three recombinant plasmids were constructed by Stahl and Murray (1989).

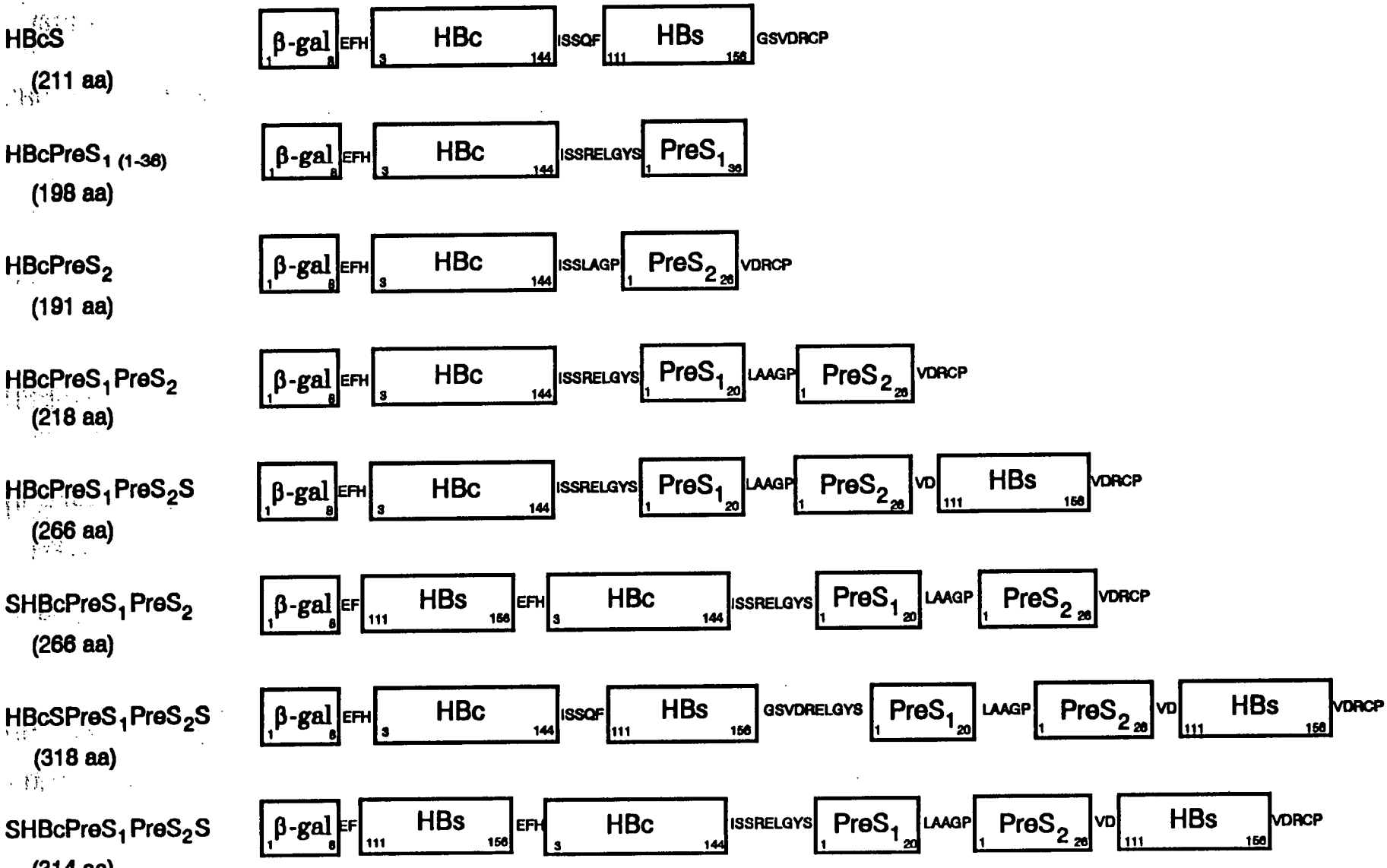

HBcS
(211 aa)
β-gal 1 8 EFH HBc 3 144 ISSQF HBs 111 156 GSVDRCP
HBcPreS1 (1-36)
(198 aa)
β-gal 1 8 EFH HBc 3 144 ISSRELGYS PreS1 1 36
HBcPreS2
(191 aa)
β-gal 1 8 EFH HBc 3 144 ISSLAGP PreS2 1 26 VDRCP
HBcPreS1PreS2
(218 aa)
β-gal 1 8 EFH HBc 3 144 ISSRELGYS PreS1 1 20 LAAGP PreS2 1 26 VDRCP
HBcPreS1PreS2S
(266 aa)
β-gal 1 8 EFH HBc 3 144 ISSRELGYS PreS1 1 20 LAAGP PreS2 1 26 VD HBs 111 156 VDRCP
SHBcPreS1PreS2
(266 aa)
β-gal 1 8 EF HBs 111 156 EFH HBc 3 144 ISSRELGYS PreS1 1 20 LAAGP PreS2 1 26 VDRCP
HBcSPreS1PreS2S
(318 aa)
β-gal 1 8 EFH HBc 3 144 ISSQF HBs 111 156 GSVDRELGYS PreS1 1 20 LAAGP PreS2 1 26 VD HBs 111 156 VDRCP
SHBcPreS1PreS2S
(314 aa)
β-gal 1 8 EF HBs 111 156 EFH HBc 3 144 ISSRELGYS PreS1 1 20 LAAGP PreS2 1 26 VD HBs 111 156 VDRCP

Figure 3.2 Schematic representation of HBcAg fusion proteins, HBcS and $HBcPreS_1PreS_2S$, with wild-type or mutant sequence in gly_{145} of $HBs_{(111\text{-}156)}$ encoded by the corresponding plasmids. Names of the proteins are at the left. Numbers at the right indicate amino acid positions on the native proteins.

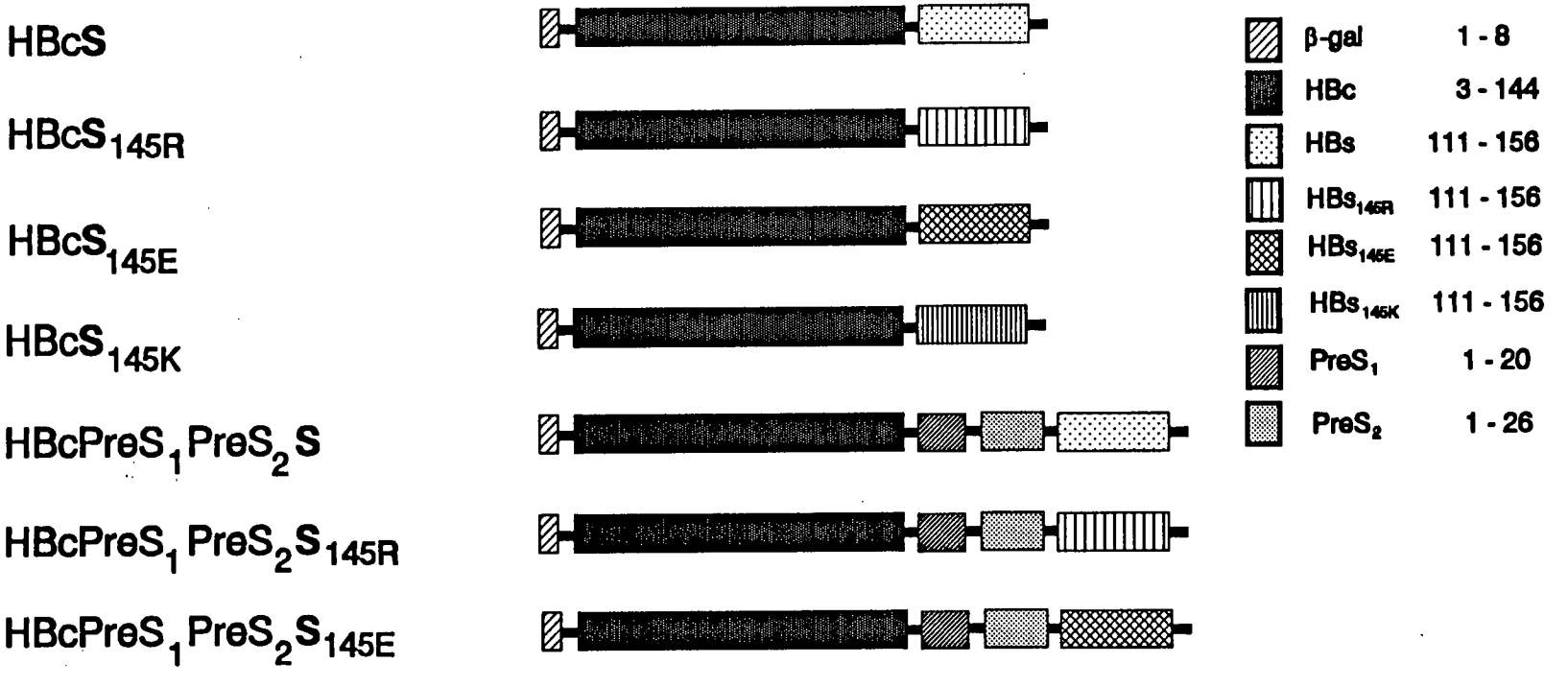
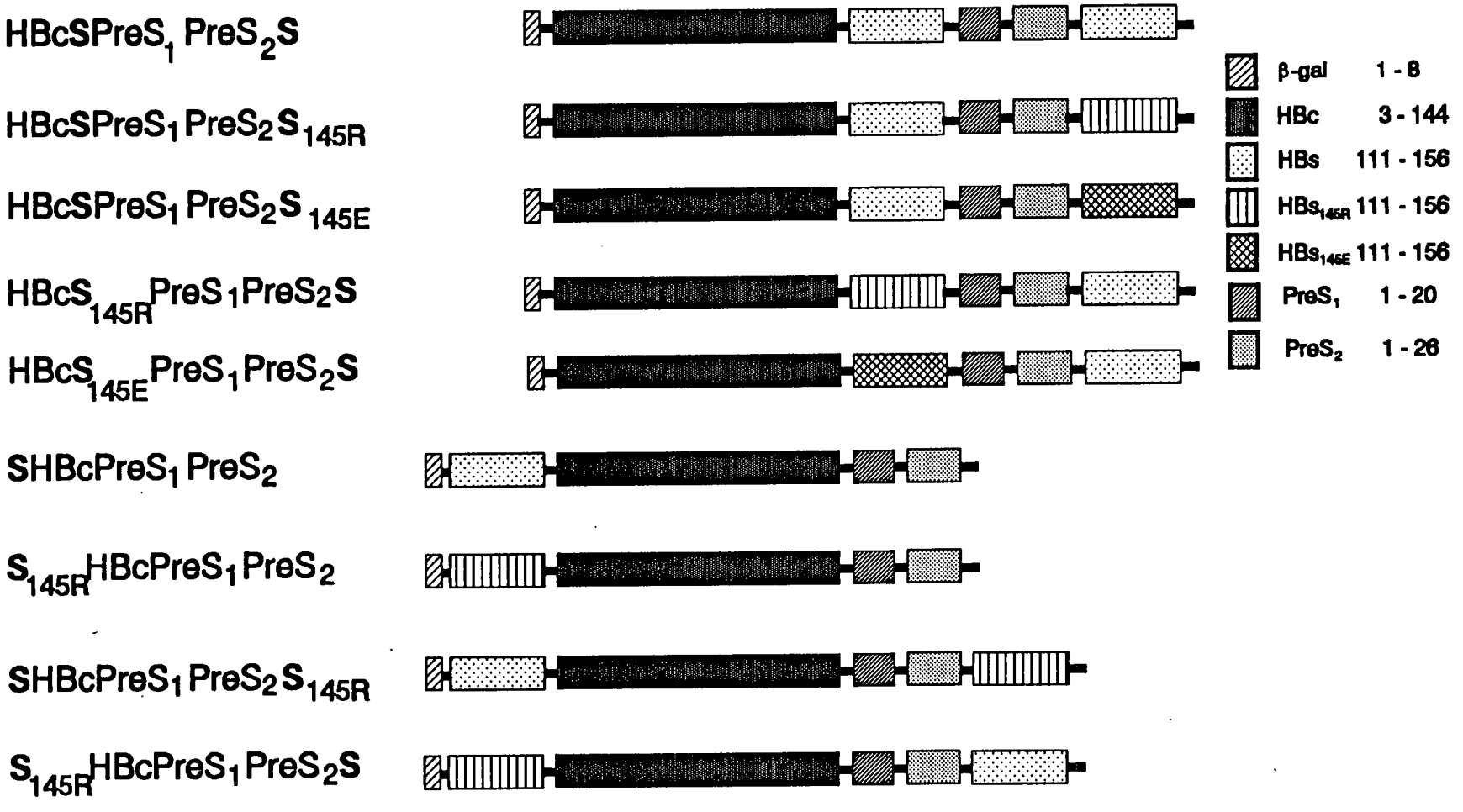

HBcS
HBcS145R
HBcS145E
HBcS145K
HBcPreS1PreS2S
HBcPreS1PreS2S145R
HBcPreS1PreS2S145E
β-gal 1 - 8
HBc 3 - 144
HBs 111 - 156
HBs145R 111 - 156
HBs145E 111 - 156
HBs145K 111 - 156
PreS1 1 - 20
PreS2 1 - 26

Figure 3.3 Schematic representation of HBcAg fusion proteins, HBcSPreS$_1$PreS$_2$S, SHBcPreS$_1$PreS$_2$ and SHBcPreS$_1$PreS$_2$S, with wild-type or mutant sequence in gly$_{145}$ of HBs$_{(111\text{-}156)}$ encoded by the corresponding plasmids. Names of the proteins are at the left. Numbers at the right indicate amino acid positions on the native proteins.


HBcSPreS1 PreS2S
HBcSPreS1 PreS2S145R
HBcSPreS1 PreS2S 145E
HBcS145RPreS1PreS2S
HBcS145EPreS1PreS2S
SHBcPreS1 PreS2
S145RHBcPreS1 PreS2
SHBcPreS1 PreS2S145R
S145RHBcPreS1 PreS2S
β-gal 1 - 8
HBc 3 - 144
HBs 111 - 156
HBs145R 111 - 156
HBs145E 111 - 156
PreS1 1 - 20
PreS2 1 - 26

3.2 Sequences fused to the C-terminal region of HBcAg

Plasmid pHBcPreS$_1$PreS$_2$ directs the expression of fusion protein HBcPreS$_1$PreS$_2$ which contains preS$_{1(1-20)}$ followed by preS$_{2(1-26)}$ fused to the C-terminus of HBcAg. The strategy for plasmid construction is shown in Figure 3.4, which was to replace the coding sequence for HBcAg from pHBcPreS$_2$ (Stahl and Murray, 1989) by coding sequences for HBcAg and preS$_{1(1-20)}$ from pHBcPreS$_{1(1-36)}$ (Stahl and Murray, 1989). Plasmid pHBcPreS$_2$ was digested with HindIII and the linearised molecule was rendered blunt-ended by digestion with Mung bean exonuclease. The large vector-containing fragment (4.25 kb) carrying preS$_{2(1-26)}$ was isolated after digestion of the blunt-end molecule with EcoRI. The DNA fragment carrying the coding sequence for HBc$_{(3-144)}$ and preS$_{1(1-20)}$ was isolated from pHBcPreS$_{1(1-36)}$ by digestion with BamHI, filling in cohesive ends of the linearised molecule with DNA polymerase (Klenow fragment) and digestion with EcoRI. The resulting 528-bp fragment was ligated to the 4.25-kb fragment from pHBcPreS$_2$.

Plasmid pHBcPreS$_1$PreS$_2$S directs the expression of fusion protein HBcPreS$_1$PreS$_2$S which contains preS$_{1(1-20)}$, preS$_{2(1-26)}$ and HBs$_{(111-156)}$ fused to the C-terminus of HBcAg and was constructed as follows (Figure 3.5). pHBcPreS$_1$PreS$_2$ was linearised by digestion with SalI and the cohesive ends filled in with DNA polymerase (Klenow fragment). The coding sequence for HBs$_{(111-156)}$ was isolated from amplified pHinG2, which contains the whole coding sequence for HBsAg in a *S. cerevisiae/E. coli* shuttle vector (Murray *et al.*, 1984), by using a PCR protocol. The synthetic oligonucleotides used as primers for PCR reactions were 010H (5'-GGA ATT CCC AGG AAC ATC AAC CAC-3') and 011H(5'-GGA ATT CCC AGG ATG ATG GGA TG-3') which carry an EcoRI site at their 5' end. The PCR product containing HBs$_{(111-156)}$ was digested with EcoRI and the cohesive ends rendered blunt by digestion with Mung bean exonuclease. The PCR fragment was then fused to linearised pHBcPreS$_1$PreS$_2$.

Plasmid pHBcSPreS$_1$PreS$_2$S directs the expression of fusion protein HBcSPreS$_1$PreS$_2$S and was constructed as follows (Figure 3.6). pHBcS$_{(111-156)}$ (Stahl and Murray, 1989)

Figure 3.4 Construction of pHBcPreS$_1$PreS$_2$ for generating HBcAg fusion protein with the preS$_{1(1-20)}$ and preS$_{2(1-26)}$ fragments at the C-terminus. Selected restriction sites are shown.

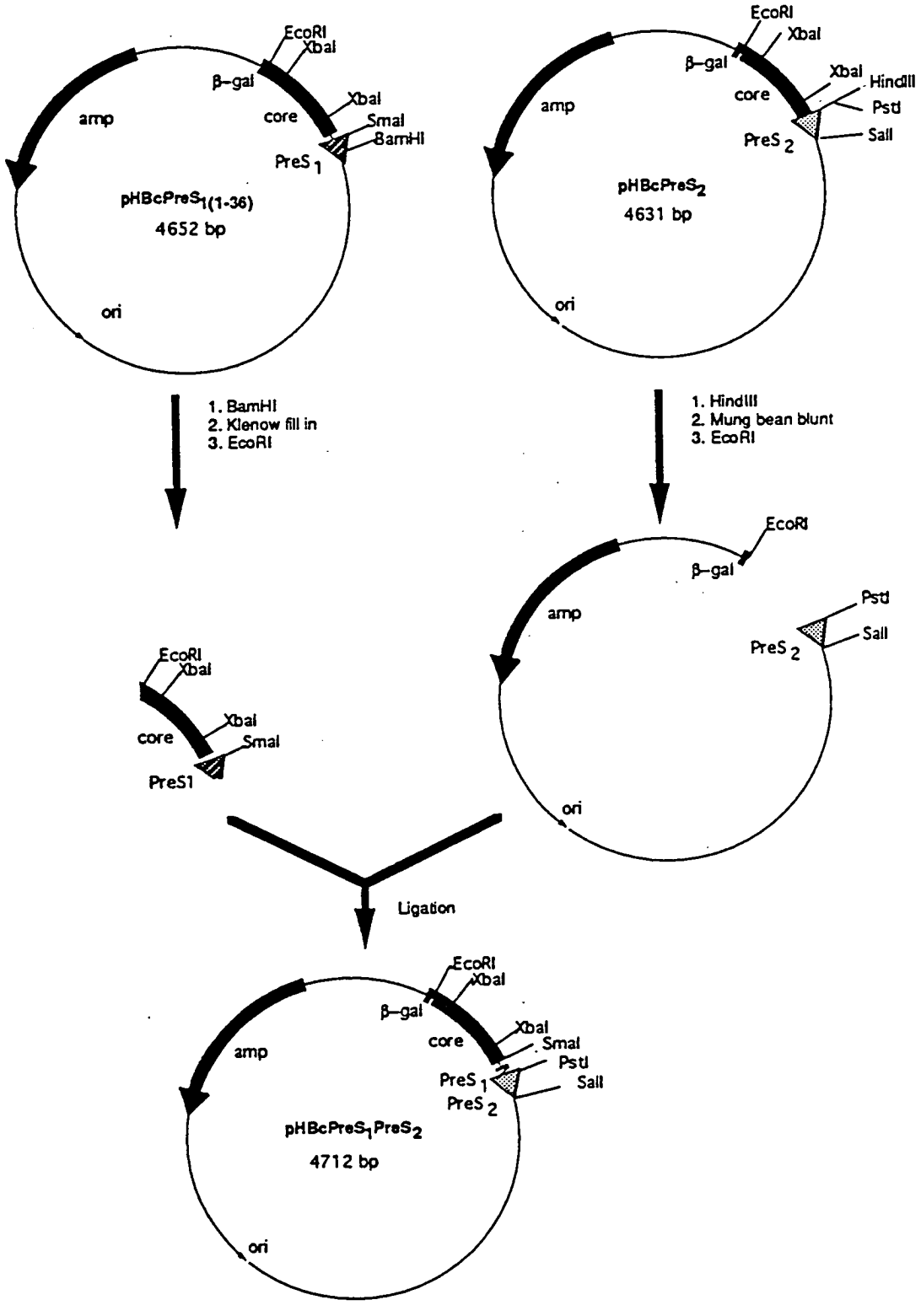

EcoRI
XbaI
β-gal
amp
core
XbaI
SmaI
BamHI
PreS 1
pHBcPreS1(1-36)
4652 bp
ori
1. BamHI
2. Klenow fill in
3. EcoRI
EcoRI
XbaI
XbaI
HindIII
β-gal
core
PstI
amp
PreS 2
SalI
pHBcPreS2
4631 bp
ori
1. HindIII
2. Mung bean blunt
3. EcoRI
EcoRI
β-gal
amp
PstI
SalI
PreS 2
ori
EcoRI
XbaI
XbaI
core
SmaI
PreS1
Ligation
EcoRI
XbaI
β-gal
XbaI
core
SmaI
amp
PstI
PreS 1
SalI
PreS 2
pHBcPreS1PreS2
4712 bp
ori

Figure 3.5 Construction of pHBcPreS$_1$PreS$_2$S for generating HBcAg fusion protein with the preS$_{1(1-20)}$, preS$_{2(1-26)}$ and HBs$_{(111-156)}$ fragments at the C-terminus. Selected restriction sites are shown.

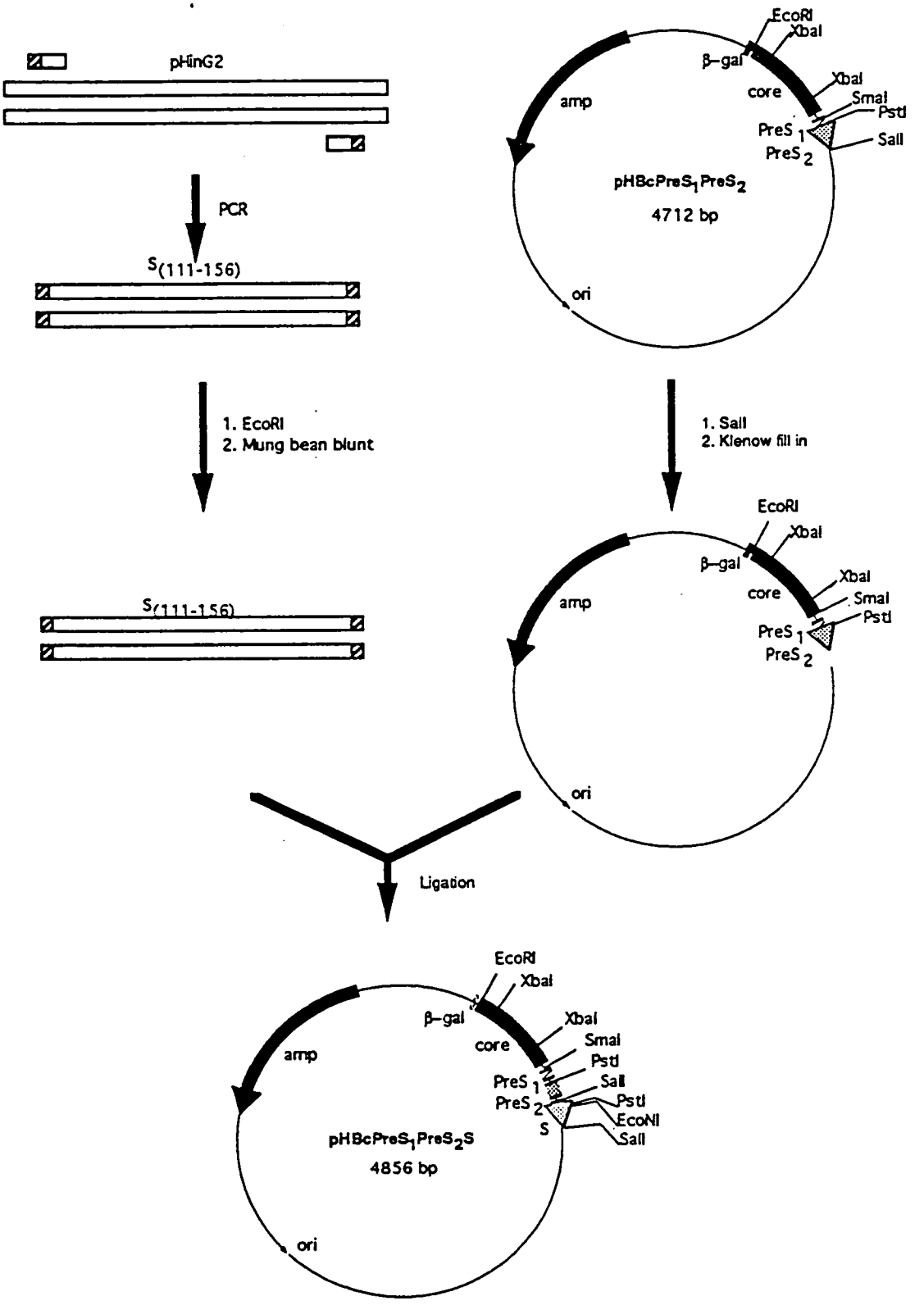
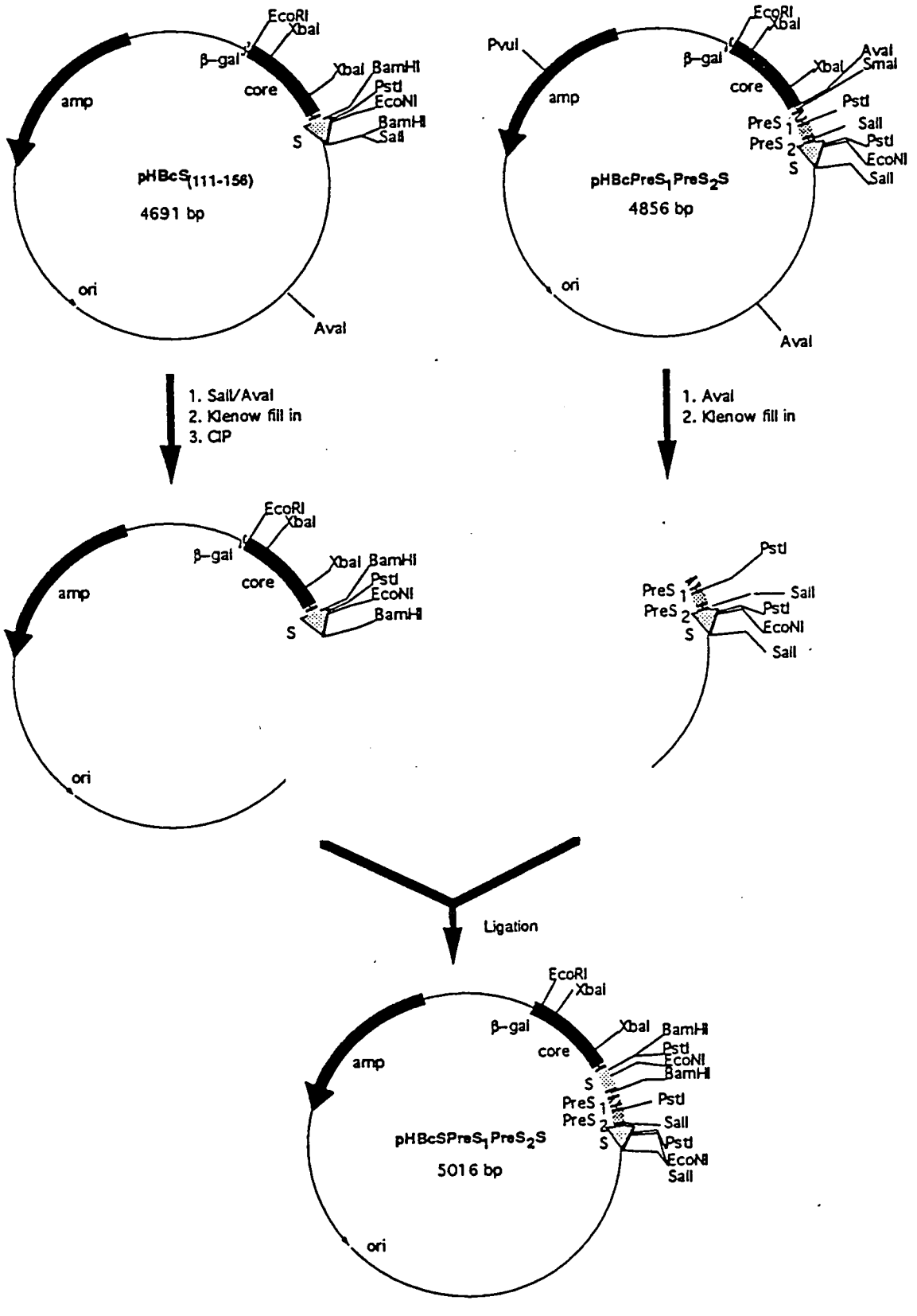

pHinG2
PCR
S(111-156)
1. EcoRI
2. Mung bean blunt
S(111-156)
EcoRI
Xbal
β-gal
amp
core
Xbal
Smal
Pstl
PreS 1
PreS 2
Sall
pHBcPreS1PreS2
4712 bp
ori
1. Sall
2. Klenow fill in
EcoRI
Xbal
β-gal
amp
core
Xbal
Smal
Pstl
PreS 1
PreS 2
ori
Ligation
EcoRI
Xbal
β-gal
amp
core
Xbal
Smal
Pstl
PreS 1
Sall
PreS 2
Pstl
S
EcoNI
Sall
pHBcPreS1PreS2S
4856 bp
ori

Figure 3.6 Construction of pHBcSPreS$_1$PreS$_2$S for generating HBcAg fusion protein with the preS$_{1(1-20)}$ and preS$_{2(1-26)}$ fragments sandwiched by two HBs$_{(111-156)}$ fragments at the C-terminus. Selected restriction sites are shown.


EcoRI
XbaI
β-gal
XbaI BamHI
PstI
EcoNI
BamHI
SalI
core
amp
S
pHBcS(111-156)
4691 bp
ori
AvaI
PvuI
AvaI
SmaI
PreS1
PreS2
pHBcPreS1PreS2S
4856 bp
1. SalI/AvaI
2. Klenow fill in
3. CIP
1. AvaI
2. Klenow fill in
Ligation
pHBcSPreS1PreS2S
5016 bp

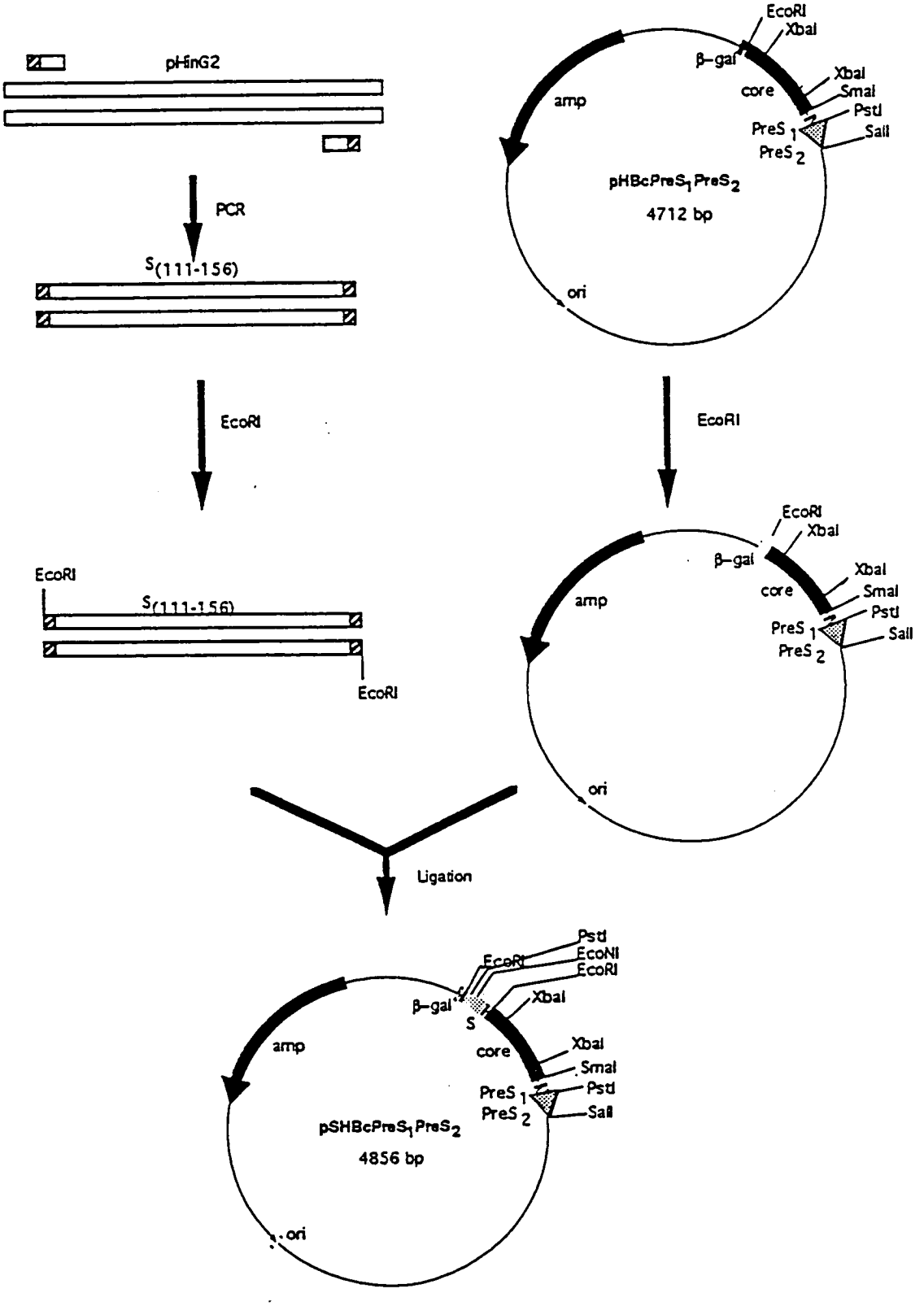
was digested with SalI and AvaI and the cohesive ends of the large fragment were filled in with DNA polymerase (Klenow fragment). The resulting 3.9-kb fragment carrying the coding sequences for $HBc_{(3-144)}$ and $HBs_{(111-156)}$ was ligated to a 1.1 kb fragment containing $preS_{1(1-20)}$, $preS_{2(1-26)}$ and $HBs_{(111-156)}$ which had been generated from $pHBcPreS_1PreS_2S$ by digestion with AvaI and filling in the cohesive ends of the small fragment with DNA polymerase (Klenow fragment). The coding sequence for HBsAg in $pHBcS_{(111-156)}$ was subcloned from a variant of plasmid pHBV130 (Gough and Murray, 1982) which contains a missense transition (HBV nucleotide 1794, C changed to T) resulting in a substitution from proline to serine at amino acid residue 120 of HBsAg. The plasmid pHinG2 used for amplification of the $HBs_{(111-156)}$ sequence was, in fact, a mutant plasmid with a substitution from serine to threonine at amino acid residue 113 of HBsAg. Therefore, in $HBcSPreS_1PreS_2S$, there are two amino acids different between the two $HBs_{(111-156)}$ fragments.

The nucleotide sequence in the junction region was determined by DNA sequencing. In addition, the coding sequence for $HBs_{(111-156)}$ from the PCR product in $pHBcPreS_1PreS_2S$ was confirmed by DNA sequencing.

3.3 Sequences fused to the N-terminal region of $HBcPreS_1PreS_2$

Plasmid $pSHBcPreS_1PreS_2$ directs the expression of fusion protein $SHBcPreS_1PreS_2$ and was constructed as follows (Figure 3.7). The coding sequence for $HBs_{(111-156)}$ was amplified from pHinG2 by PCR as described previously (Section 3.2). The amplified DNA fragment carrying the coding sequence for $HBs_{(111-156)}$ with EcoRI sites at both ends was digested with EcoRI and fused to $pHBcPreS_1PreS_2$ that had been linearlised and digested with EcoRI. The fusion protein $SHBcPreS_1PreS_2S$, therefore, carries foreign epitopes at both N- and C-termini of HBcAg. The coding region of $HBs_{(111-156)}$ within the vector was verified by DNA sequencing.

Figure 3.7 Construction of pSHBcPreS$_1$PreS$_2$ for generating HBcAg fusion protein with the HBs$_{(111\text{-}156)}$ fragment at the N-terminus as well as with the preS$_{1(1\text{-}20)}$ and preS$_{2(1\text{-}26)}$ fragments at the C-terminus. Selected restriction sites are shown.


pHinG2
PCR
S(111-156)
EcoRI
EcoRI
S(111-156)
EcoRI
EcoRI
Xbal
β-gal
core
amp
Xbal
Smal
Pstl
Sall
PreS 1
PreS 2
pHBcPreS1PreS2
4712 bp
ori
EcoRI
Xbal
β-gal
core
amp
Xbal
Smal
Pstl
Sall
PreS 1
PreS 2
ori
Ligation
Pstl
EcoNI
EcoRI
EcoRI
β-gal
S
Xbal
amp
core
Xbal
Smal
Pstl
Sall
PreS 1
PreS 2
pSHBcPreS1PreS2
4856 bp
ori

3.4 Mutant sequences of $HBs_{(111-156)}$ fused to the C-terminal and/or N-terminal regions of HBcAg

The impetus for creating mutant sequences of $HBs_{(111-156)}$ fused to HBcAg is the finding of the vaccine-induced escape mutant with an amino acid substitution from glycine to arginine at amino acid residue 145 of HBsAg, which cannot be neutralised by vaccine-induced anti-HBs antibody (Carman *et al.*, 1990). By using site-directed mutagenesis, three mutant HBcAg fusion proteins were constructed which carry the $HBs_{(111-156)}$ sequence with gly_{145} change to arginine, mimicking the escape mutant described previously, or glutamic acid or lysine. A study by Ashton-Rickardt and Murray (1989b) indicated that site-directed mutagenesis can be used to change the immunological specificity of an antigen. Therefore, an attempt has been made to alter the immunological specificity of HBcS carrying wild-type or gly_{145} mutant $HBs_{(111-156)}$. In addition, on the basis of these mutant sequences, fusions were also constructed carrying a dimer of $HBs_{(111-156)}$, one from wild-type and the other from a mutant sequence, accompanied by $preS_{1(1-20)}$ and $preS_{2(1-26)}$ sequences. The goal of this part of the study is an attempt to create multivalent immunogens based on HBcAg that may induce antibody with a wider specificity to confer immunity against HBV infection, including antigenic variants.

3.4.1 Site-directed mutagenesis of $pHBcS_{(111-156)}$

The target for mutagenesis, a 594-bp EcoRI-SalI fragment, bearing the $HBc_{(3-144)}$ and $HBs_{(111-156)}$ sequences, was excised from $pHBcS_{(111-156)}$, and subcloned into the M13mp19 vector (Norrander *et al.*, 1983), which resulted in the construction of M13HBcS. The following three 23- or 26-mer oligonucleotides were used for site-directed mutagenesis. 412G: 5′-ACC TTC GGA TAG AAA CTG CAC CT-3′ (nucleotide 1869 in HBV G→A, amino acid residue 145 in HBsAg gly→arg). 411G: 5′-ACC TTC GGA TGA AAA CTG CAC CT-3′ (nucleotide 1870 in HBV G→A, amino acid residue 145 in HBsAg gly→glu). 359G: 5′-AAA CCT TCG GAT AAA AAC TGC ACC TG-3′ (nucleotides 1869 and 1870 in HBV GG→AA, amino acid

residue 145 in HBsAg gly→lys). Site-directed mutagenesis was performed to convert amino acid residue 145 in HBsAg from glycine to arginine, glutamic acid, or lysine. The M13mp19 clones containing a mutated insert were identified by T-track sequencing. The candidate mutants were then fully sequenced for the entire insert. No undesired mutations occurred during the reactions.

3.4.2 Construction of mutant pHBcS$_{(111\text{-}156)}$ recombinants

Three mutants to pHBcS$_{(111\text{-}156)}$ at amino acid residue 145 of HBsAg were constructed as follows (Figure 3.8). Plasmid pHBcX (Rossner, 1991) which contains the coding regions for HBc$_{(3\text{-}144)}$, HBs$_{(111\text{-}120)}$ and HBx$_{(1\text{-}154)}$ was digested with EcoRI and SalI and the large fragment from this digest was then ligated to EcoRI-SalI fragment excised from M13 RF DNA carrying mutant sequence (M13HBcS$_{145R}$, M13HBcS$_{145E}$ and M13HBcS$_{145K}$). This resulted in recombinant plasmids pHBcS$_{145R}$, pHBcS$_{145E}$ and pHBcS$_{145K}$, which directed the expression of fusion protein HBcS$_{145R}$, HBcS$_{145E}$, and HBcS$_{145K}$, respectively. The entire coding regions for HBc$_{(3\text{-}144)}$ and HBs$_{(111\text{-}156)}$ in three mutant recombinant plasmids were verified by M13 sequencing after subcloning 594-bp EcoRI-SalI fragments bearing the coding sequence of HBc$_{(3\text{-}144)}$ and HBs$_{(111\text{-}156)}$ into M13mp18 (Norrander *et al.*, 1983). The sequences of the mutated sites in the three mutants are presented in Figure 3.9.

3.4.3 Mutant sequence of HBs$_{(111\text{-}156)}$ with preS$_{1(1\text{-}20)}$ and preS$_{2(1\text{-}26)}$ fused to the C-terminal region of HBcAg

Plasmids pHBcPreS$_1$PreS$_2$S$_{145R}$ and pHBcPreS$_1$PreS$_2$S$_{145E}$ were constructed using the same strategy as that for pHBcPreS$_1$PreS$_2$S (Figure 3.5). The coding sequences for HBs$_{(111\text{-}156)}$ with gly$_{145}$ changed to arg and glu were derived from pHinG2$_{145R}$ carrying the mutant sequence for arg$_{145}$ in HBsAg, and pHBcS$_{145E}$ carrying the mutant sequence for glu$_{145}$ in HBsAg, respectively. The two mutant recombinant plasmids thus directed the expression of fusions proteins HBcPreS$_1$PreS$_2$S$_{145R}$ and HBcPreS$_1$PreS$_2$S$_{145E}$.

Figure 3.8 Construction of $pHBcS_{145R}$ for generating HBcAg fusion protein with $HBs_{(111-156)}$ fragment carrying arg substitution at amino acid residue 145. Selected restriction sites are shown. $pHBcS_{145E}$ and $pHBcS_{145K}$ were constructed using the same strategy.

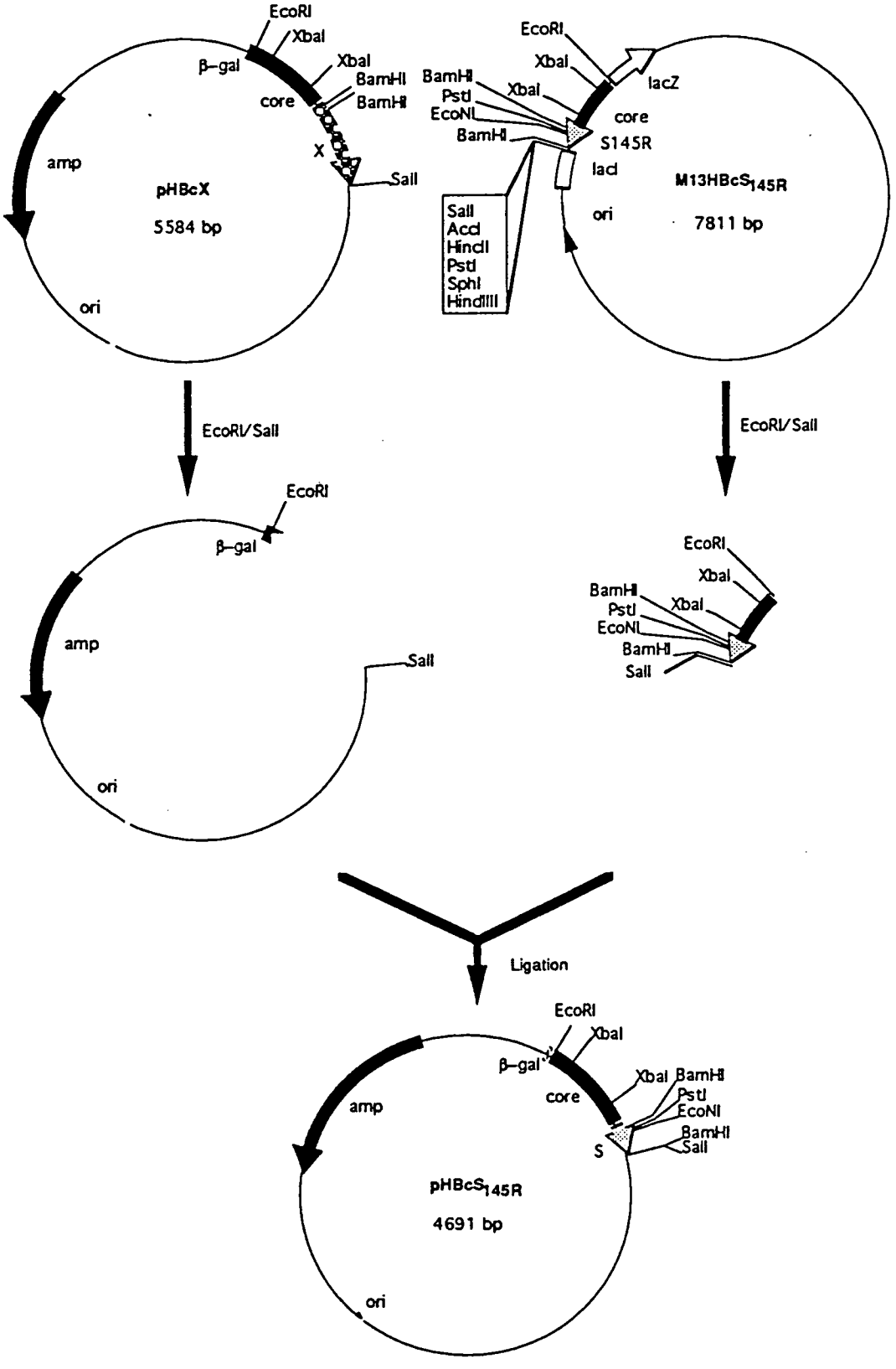
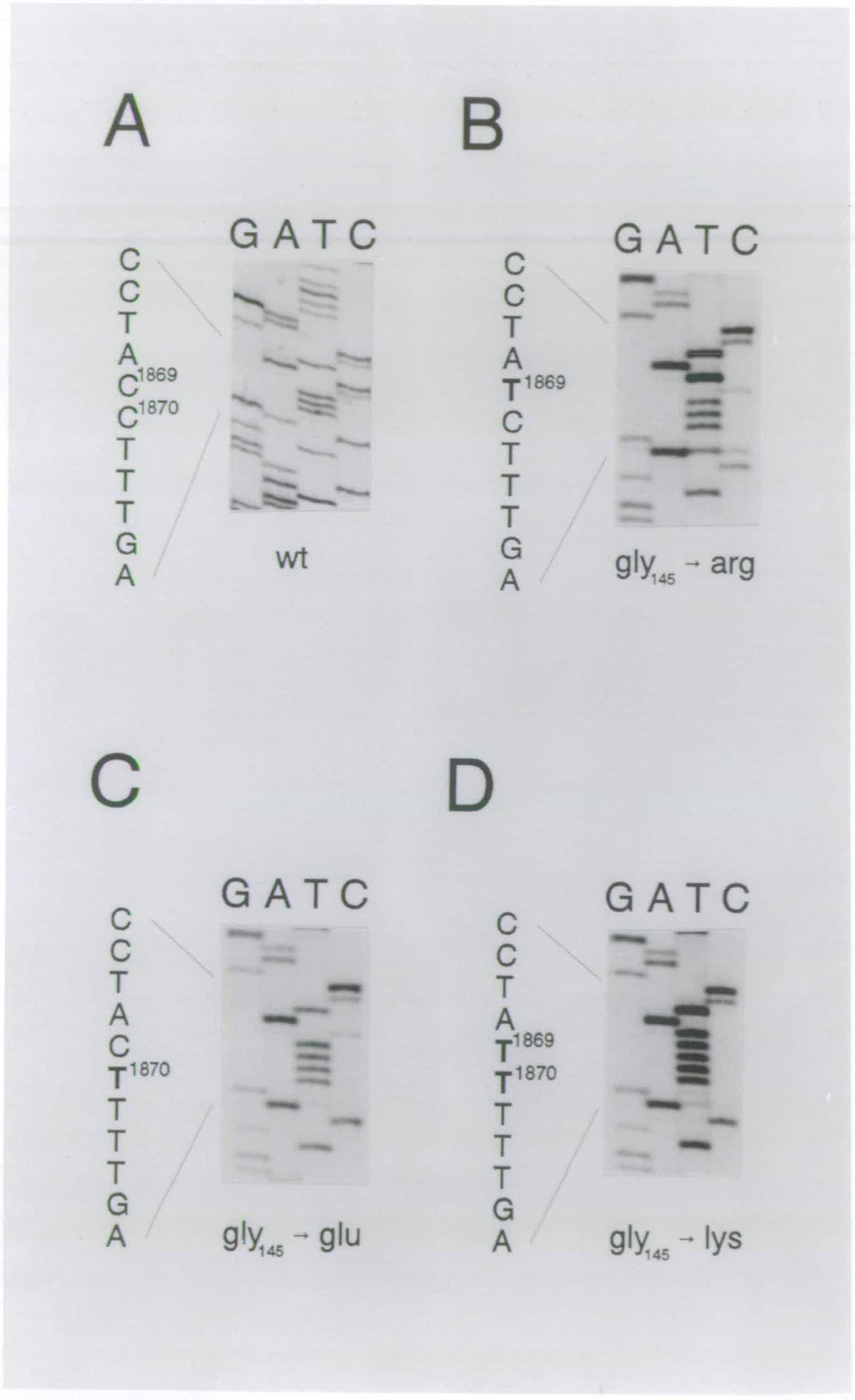

EcoRI
XbaI
β–gal
XbaI
BamHI
core
BamHI
X
SalI
amp
pHBcX
5584 bp
ori
EcoRI
XbaI
BamHI
PstI
EcoNI
BamHI
lacZ
core
S145R
lacI
ori
M13HBcS145R
7811 bp
SalI
AccI
HincII
PstI
SphI
HindIII
EcoRI/SalI
EcoRI/SalI
EcoRI
β–gal
amp
SalI
ori
EcoRI
XbaI
BamHI
PstI
EcoNI
XbaI
BamHI
SalI
Ligation
EcoRI
XbaI
β–gal
XbaI
BamHI
core
PstI
EcoNI
S
BamHI
SalI
amp
pHBcS145R
4691 bp
ori

Figure 3.9 Nucleotide (non-coding strand) sequences of HBsAg gene mutated at amino acid residue 145 of HBsAg. Plasmids $pHBcS_{145R}$, $pHBcS_{145E}$ and $pHBcS_{145K}$ were digested with EcoRI and SalI and the resulting 594-bp fragment bearing coding strand of $HBc_{(3-144)}$ and $HBs_{(111-156)}$ sequences were subcloned into M13mp18 for sequencing. For comparison, the corresponding wild-type sequence obtained from plasmid sequencing of $pHBcS_{(111-156)}$ is also shown.


A
G A T C
C C T A C 1869 C 1870 T T T G A
wt
B
G A T C
C C T A T 1869 T C T T T G A
gly145 → arg
C
G A T C
C C T A C T 1870 T T T G A
gly145 → glu
D
G A T C
C C T A T 1869 T 1870 T T T G A
gly145 → lys

Four mutant recombinant plasmids, pHBcSPreS$_1$PreS$_2$S$_{145R}$, pHBcSPreS$_1$PreS$_2$S$_{145E}$, pHBcS$_{145R}$PreS$_1$PreS$_2$S, and pHBcS$_{145E}$PreS$_1$PreS$_2$S, were constructed using the same strategy as that for pHBcSPreS$_1$PreS$_2$S (Figure 3.6). The coding sequence for HBcAg and its adjacent HBs$_{(111-156)}$ was derived from wild-type or mutant pHBcS$_{(111-156)}$, while the coding sequence for preS$_{1(1-20)}$, preS$_{2(1-26)}$ and HBs$_{(111-156)}$ at the C-terminus of the fusion protein was derived from pHBcPreS$_1$PreS$_2$S with the wild-type or mutant sequence at amino acid residue 145 of HBsAg. The four mutant recombinant plasmids direct the expression of the corresponding HBcAg fusion proteins. The nucleotide sequences in the junction region of the recombinant plasmids were confirmed by DNA sequencing.

3.4.4 Construction of plasmids pS$_{145R}$HBcPreS$_1$PreS$_2$, pS$_{145R}$HBcPreS$_1$PreS$_2$S and pSHBcPreS$_1$PreS$_2$S$_{145R}$

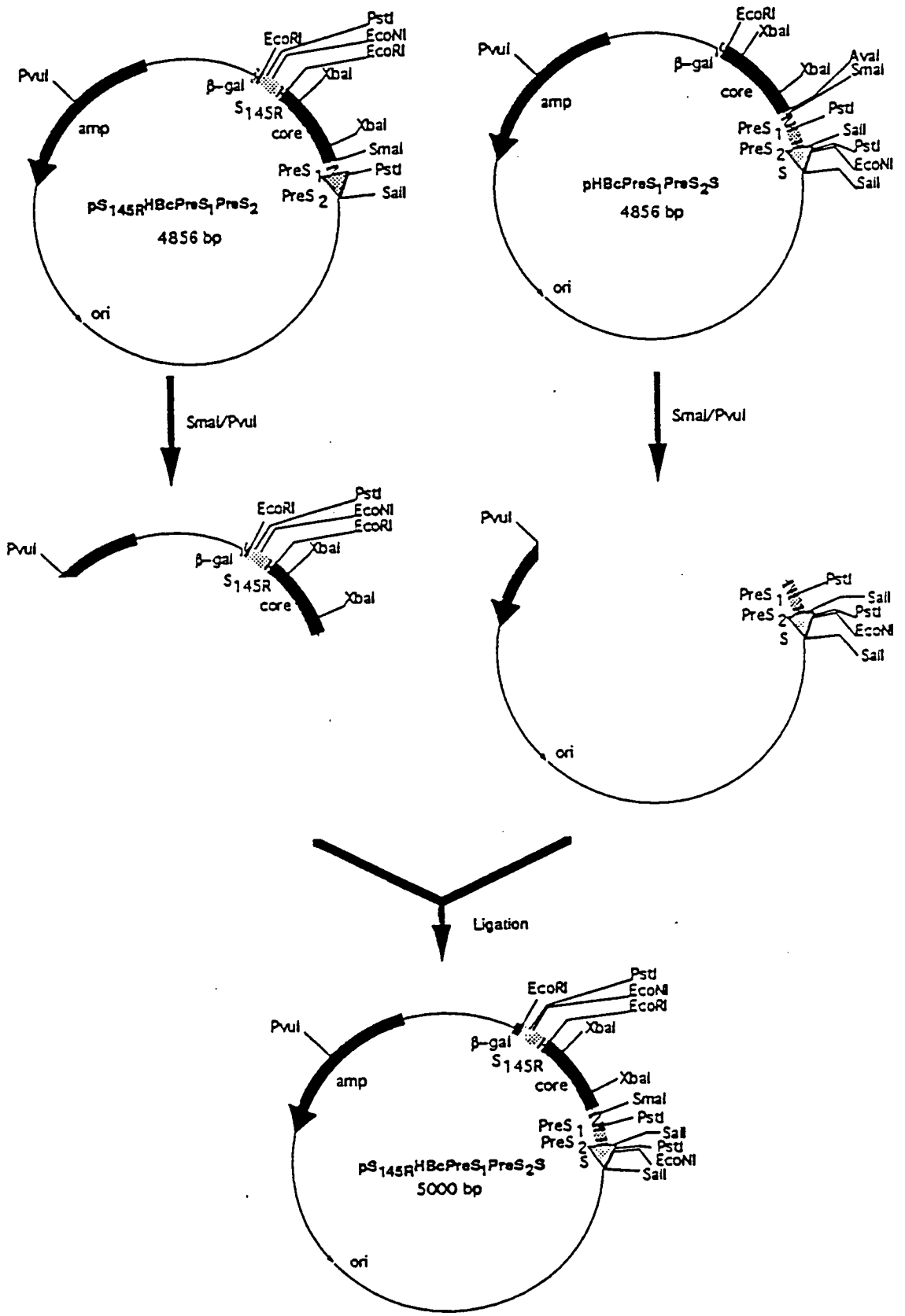
Plasmid pS$_{145R}$HBcPreS$_1$PreS$_2$ directs the expression of fusion protein S$_{145R}$HBcPreS$_1$PreS$_2$ and was constructed similarly to pSHBcPreS$_1$PreS$_2$ except that the coding sequence for HBs$_{(111-156)}$ with gly$_{145}$ changed to arg was derived from pHinG2$_{145R}$ carrying the mutant sequence for arg$_{145}$ in HBsAg.

Plasmid pS$_{145R}$HBcPreS$_1$PreS$_2$S directs the expression of fusion protein S$_{145R}$HBcPreS$_1$PreS$_2$S and was constructed as follows (Figure 3.10). Plasmid pS$_{145R}$HBcPreS$_1$PreS$_2$ was digested with SmaI and PVUI and the small fragment (1.6 kb) from this digest was fused to a large SmaI-PVUI fragment (3.4 kb) from pHBcPreS$_1$PreS$_2$S. Plasmid pSHBcPreS$_1$PreS$_2$S$_{145R}$ directs the expression of fusion protein SHBcPreS$_1$PreS$_2$S$_{145R}$ and was constructed similarly to pS$_{145R}$HBcPreS$_1$PreS$_2$S, but using pSHBcPreS$_1$PreS$_2$ and pHBcPreS$_1$PreS$_2$S$_{145R}$ instead. The nucleotide sequences in the junction region were also verified by DNA sequencing.

3.5 Production and purification of HBcAg fusion proteins in *E. coli*

Cultures of the *lac*I^q strains of *E. coli* RB791 or TG1 harbouring various recombinant

Figure 3.10 Construction of $pS_{145R}HBcPreS_1PreS_2S$ for generating HBcAg fusion protein with the $HBs_{(111-156)}$ fragment carrying gly_{145}→arg mutation at the N-terminus as well as with $preS_{1(1-20)}$, $preS_{2(1-26)}$ and $HBs_{(111-156)}$ fragments at the C-terminus. Selected restriction sites are shown.


PvuI
amp
β-gal
S145R
core
EcoRI
PstI
EcoNI
EcoRI
XbaI
XbaI
SmaI
PreS 1
PreS 2
PstI
SalI
pS145RHBcPreS1PreS2
4856 bp
ori
SmaI/PvuI
EcoRI
XbaI
AvaI
SmaI
XbaI
PstI
PreS 1
PreS 2
S
SalI
PstI
EcoNI
SalI
pHBcPreS1PreS2S
4856 bp
ori
SmaI/PvuI
Ligation
pS145RHBcPreS1PreS2S
5000 bp

plasmids were grown in LB supplemented with ampicillin, and the *tac* promoter was induced with IPTG. After shaking at 37°C overnight, the bacteria were harvested and lysed by sonication and the cellular debris was removed by centrification (12,000 x g, 10 min). The soluble fractions were initially analysed on 12.5% or 15% SDS-PAGE. At this stage, it was usually possible to visualise expressed HBcAg fusion proteins by Coomassie blue staining, but the natural host proteins of similar molecular weight sometimes obscured the gel profile. The crude extract was then precipitated by ammonium sulphate at 30% saturation. The pellets from ammonium sulphate precipitation were dissolved in 0.1 M of Tris-HCl (pH 7.0) buffer and dialysed against the same buffer. At this stage, the induced HBcAg fusion proteins were easily visualised by Coomassie blue staining after SDS-PAGE compared to a counterpart from uninduced cell extract. Figure 3.11 shows SDS-PAGE profiles of HBcS wild-type and the three gly_{145} mutants as well as $HBcS_{(111-165)}$ (Stahl and Murray 1989) with 7 amino acid residues more than HBcS. The mutant protein $HBcS_{145E}$, containing a glutamic acid substitution at gly_{145} of HBsAg, runs slower than expected, whereas no change is observed for $HBcS_{145R}$ and $HBcS_{145K}$ containing arginine and lysine substitutions, respectively. It has been reported that alteration of a single amino acid by mutagenesis may alter the mobility of some proteins in SDS-PAGE (Fasano *et al.*, 1984). However, the reason for the decrease in electrophoretic mobility in $HBcS_{145E}$ is not clear.

The HBcAg fusion proteins were collected by ultracentrifugation at 100,000 x g for 1 hr and resuspended in 0.1 M of Tris-HCl (pH 7.0). The HBcAg fusion proteins carrying inserts only at the C-terminus were soluble and readily chromatographed on Sepharose 4B-Cl, whereas those carrying both N- and C-terminal inserts, e.g. $SHBcPreS_1PreS_2$ and $SHBcPreS_1PreS_2S$, were aggregated as insoluble fractions, which impede them from being purified by the method used for HBcAg and its C-terminal fusion derivatives. In addition, the production of HBcAg fusion proteins containing both N- and C-terminal inserts appeared to be less than that obtained with those containing only C-terminal inserts when estimated in SDS-PAGE (results not shown). The purification of HBcAg fusion proteins containing both N- and C-terminal inserts

Figure 3.11 SDS-PAGE of expressed HBcAg fusion proteins. Protein samples from soluble fractions of crude extracts after 30% ammonium sulphate precipitation from IPTG-induced or uninduced cultures harbouring recombinant plasmids were fractionated in a (A)15% or (B)12.5% acrylamide gel and stained with Coomassie blue.

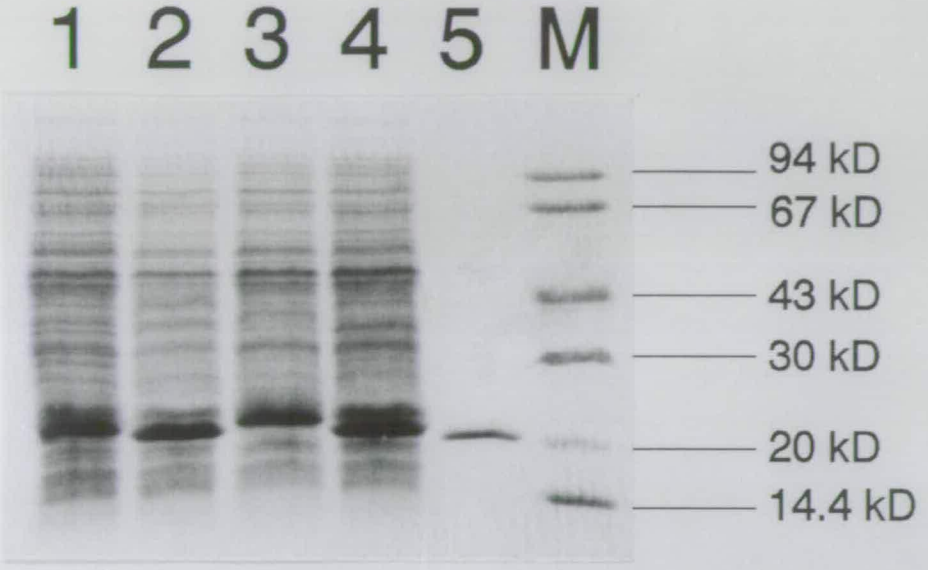
(A) Lanes:

M	-	Molecular weight size standards
1	-	HBcS from IPTG-induced cultures
2	-	$HBcS_{145K}$ from IPTG-induced cultures
3	-	$HBcS_{145E}$ from IPTG-induced cultures
4	-	$HBcS_{145R}$ from IPTG-induced cultures
5	-	3 μg purified HBcAg

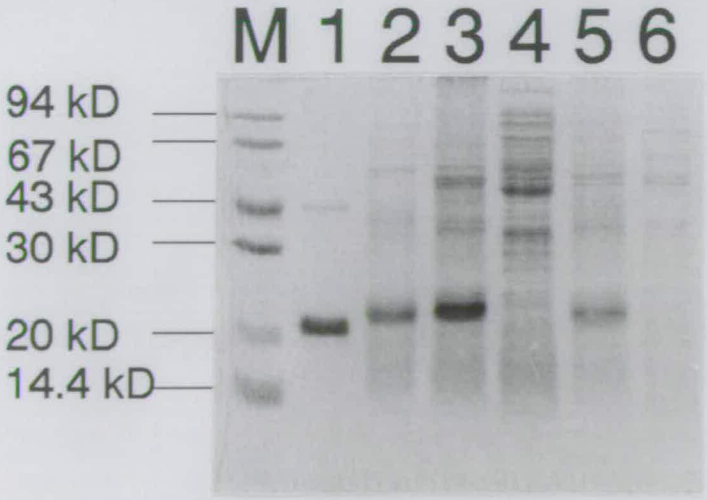
(B) Lanes:

M	-	Molecular weight size standards
1	-	3 μg purified HBcAg
2	-	HBcS from IPTG-induced cultures
3	-	$HBcS_{(111-165)}$ from IPTG-induced cultures
4	-	$HBcS_{(111-165)}$ from uninduced cultures
5	-	$HBcS_{145K}$ from IPTG-induced cultures
6	-	$HBcS_{145K}$ from uninduced cultures

A


1 2 3 4 5 M
94 kD
67 kD
43 kD
30 kD
20 kD
14.4 kD

B


M 1 2 3 4 5 6
94 kD
67 kD
43 kD
30 kD
20 kD
14.4 kD

was not further explored. Other approaches, such as SDS, urea, or guanidinium chloride treatments, might be useful for solubilising the proteins; however, they should not cause protein denaturation, for this would complicate the immunogenicity experiments.

After fractionation by chromatography, fractions containing HBcAg fusion proteins visualised by SDS-PAGE were pooled and collected by ultracentrifugation. Purified proteins were analysed by SDS-PAGE on 15% gels followed by Coomassie blue staining. The differences among the apparent molecular sizes of the various HBcAg fusion proteins lacking or carrying insertions are in good agreement with the lengths of the amino acid sequences encoded by the inserts. Yields of the HBcAg C-terminal fusion proteins range from 5 to 34 mg per litre of culture. The purified proteins reacted with rabbit anti-HBc antibody (R87 rabbit serum) as shown in immunoblots (Figure 3.12).

Since HBcAg fusion proteins can be pelleted by ultracentrifugation as in the method employed for the purification of HBcAg, it is likely that they all form particles. Direct visualisation by using electron microscopy, which was performed by D. Notman of the Department of Medical Microbiology, University of Edinburgh, shows that the HBcAg fusion proteins carrying inserts only at the C-terminus were assembled into particles similar to recombinant HBcAg produced from *E. coli* (Figure 3.13). In addition, electron microscopy of the ammonium sulphate fraction containing $SHBcPreS_1PreS_2S_{145R}$ which carries both N- and C-terminal inserts, also revealed core-like particles (result not shown). Therefore, the assembly of core-like particle was unimpeded by the addition of peptide sequences to both the N- and C-termini of the truncated HBcAg.

3.6 Discussion

Apart from the higher immunogenicity of HBcAg in terms of T-cell activation and antibody production compared to HBsAg, the wide range of hosts for its expression

Figure 3.12 Immunoblot analysis of HBcAg fusion proteins probed by anti-HBc serum. Purified protein samples were electrophoresed through a 15% SDS-PAGE and transferred to nitrocellulose membrane and HBcAg was detected by incubation with (A) Rabbit 87 anti-HBc serum diluted 1:250 or (B) mouse anti-HBc serum diluted 1:500 followed by incubation with appropriate anti-species IgG conjugated with alkaline phosphatase and subsequent incubation with NBT and BCIP.

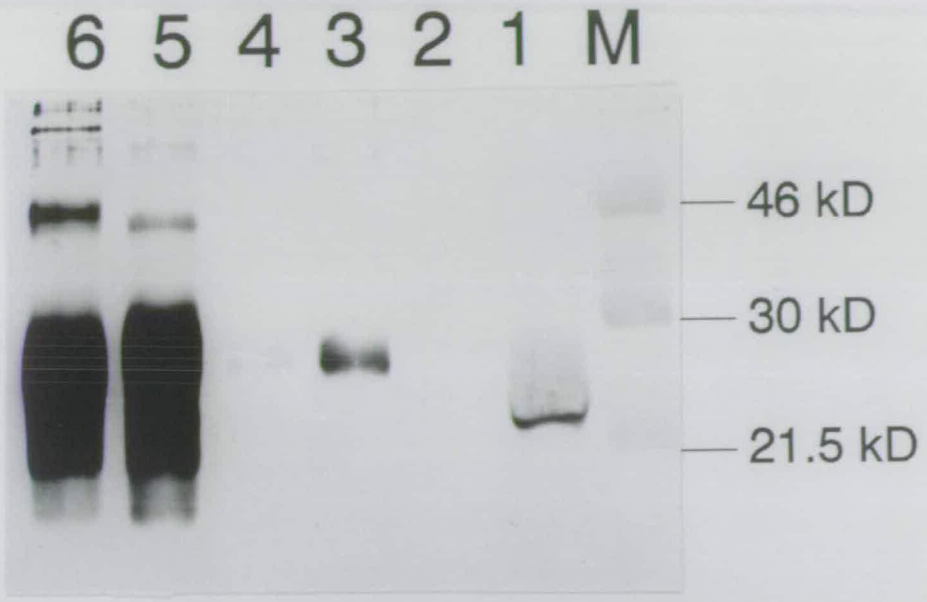
(A) Lanes:

M	-	Coloured molecular weight size standards
1	-	HBcAg
2	-	HBsAg
3	-	HBcS
4	-	$HBcS_{145R}$
5	-	$HBcS_{145E}$
6	-	$HBcS_{145K}$

(B) Lanes:

M	-	Coloured molecular weight size standards
1	-	HBcAg
2	-	HBsAg
3	-	$HBcPreS_1PreS_2S$
4	-	$HBcPreS_1PreS_2S_{145R}$
5	-	$HBcPreS_1PreS_2S_{145E}$
6	-	$HBcSPreS_1PreS_2S_{145R}$

A



B

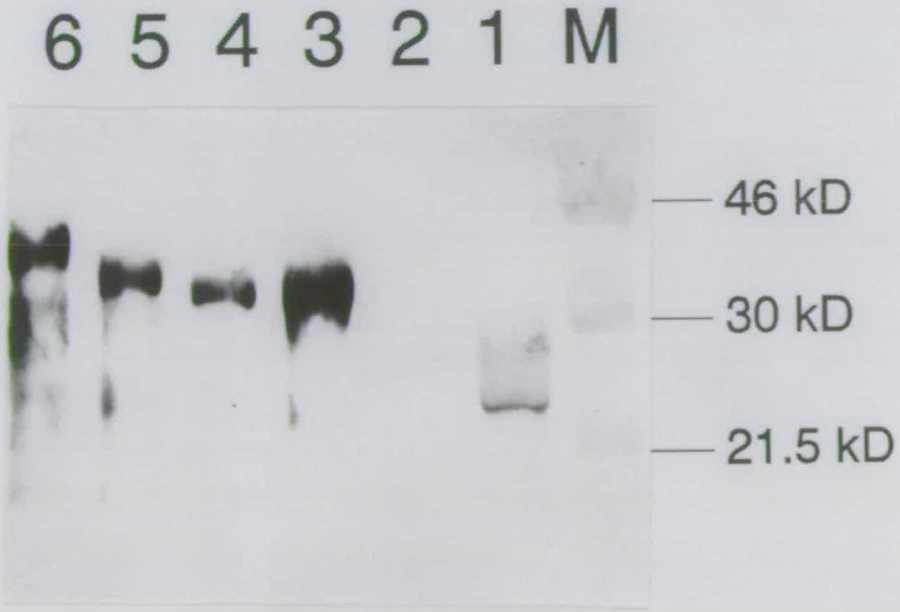
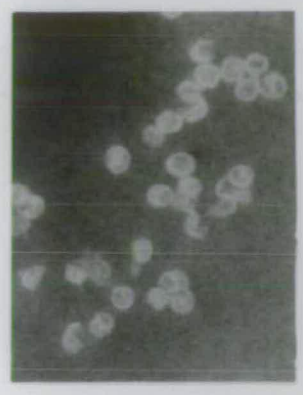
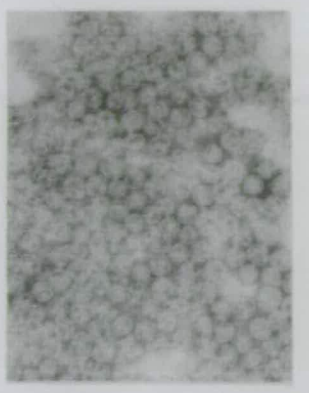


Figure 3.13 Electron micrographs of HBcAg fusion proteins (75,000 x magnification).

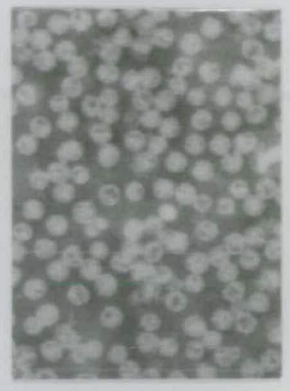
HBcAg



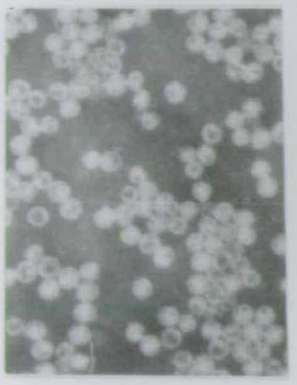
HBcS



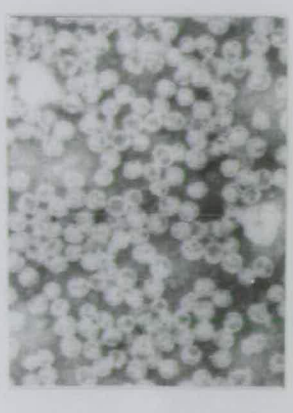
$HBcS_{145R}$



$HBcS_{145E}$

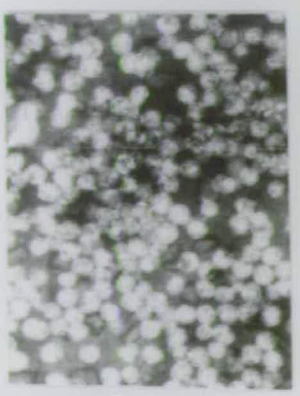


$HBcS_{145K}$

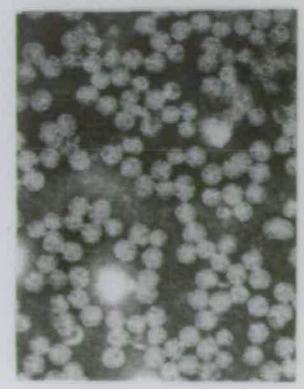


$HBcPreS_1PreS_2$

$HBcPreS_1PreS_2$ S



$HBcSPreS_1PreS_2$ S



$HBcSPreS_1PreS_2$ S_{145R}

makes it very attractive as a carrier for presentation of foreign epitopes. HBcAg can be expressed in a wide range of hosts, including *E. coli* (Pasek *et al.*, 1979), *B. subtilis* (Hardy *et al.*, 1981), *Salmonellae* (Schodel *et al.*, 1990), yeast (Kniskern *et al.*, 1986) and mammalian cells (Rossinck *et al.*, 1986; Clarke *et al.*, 1987). The HBcAg fusion proteins carrying foreign epitopes at the C-terminus of truncated HBcAg expressed in *E. coli* were produced in high yield and could be purified following the method described previously (Stahl *et al.*, 1982; Stahl and Murray, 1989) with some modifications. They assembled into core-like particles, which allow easy purification of the fusion proteins.

The largest HBcAg fusion protein, HBcSPreS$_1$PreS$_2$S, has 165 amino acid residues attached at the C-terminus of residue 144 of HBcAg via 5 residues from the linker sequence and has 318 residues in all, compared with 183 of the normal, full-length HBcAg. A similar size of extension has been reported in the HBcX fusion protein carrying HBs$_{(111\text{-}120)}$ and the complete X antigen (1-154) fused to the C-terminus of truncated HBcAg (Rossner, 1991). However, unlike the C-terminal fusion particles made in this study and those described by Stahl and Murray (1989), HBcX could not be dissociated by treatment with 1% Triton-X 100 and harsher treatments were needed to dissociate the aggregated proteins.

The HBcAg fusion proteins carrying inserts at both N- and C-termini of truncated HBcAg displayed different physical and/or chemical characteristics from those carrying only C-terminal inserts. They were insoluble when pelleted by ultracentrifugation after 30% ammonium sulphate precipitation of the soluble fractions from crude extracts. For this reason, these HBcAg fusion proteins with inserts at both termini were not purified, and their immunogenicity was not studied. The aggregation of SHBcPreS$_1$PreS$_2$ and SHBcPreS$_1$PreS$_2$S carrying the HBs$_{(111\text{-}156)}$ fragment at both N- and C-terminal region of truncated HBcAg may be due to a peculiarity of the HBs$_{(111\text{-}156)}$ sequence used for the N-terminal fusion. The HBs$_{(111\text{-}156)}$ sequence consists of 46 amino acid residues, among which 7 are cysteine residues. If some of the cysteine residues are exposed on the particle surface, they may be available to form

interparticle disulphide bonds. There is evidence that the synthetic $HBs_{(124-147)}$ peptide carrying 5 cysteine residues spontaneously self-assembles to yield a heterogenous mixture of multiple forms via disulphide bonds (Manivel *et al.*, 1992b). Recently, a synthetic 323-bp gene with the ORF of a multiple-epitope polypeptide consisting of epitopes from $preS_1$, $preS_2$ and S regions separated by pairs of glycine residues has been assembled, cloned and expressed in *E. coli* (Kumar *et al.*, 1992). The expressed polypeptide appeared in the cell pellets and only urea could solubilise it from the pellet. Nevertheless, HBcS, $HBcPreS_1PreS_2S$ and $HBcSPreS_1PreS_2S$ carrying a monomer or a dimer of the $HBs_{(111-156)}$ sequence fused to the C-terminal region of truncated HBcAg are soluble. How the position within HBcAg of the inserted epitopes influences aggregation of the formation of disulphide bonding of the HBcAg fusion proteins is not known.

Clarke *et al.* (1987) first reported the presentation of the major immunogenic sequence from FMDV comprising amino acids 141-160 from the viral protein VP1 on the surface of HBcAg fused to the N-terminal part of the HBV pre-core sequence. Because of toxicity problems, the fusion protein could not be produced in *E. coli*, but was produced by expression in a mammalian cell system. Later, the problem of expression in *E. coli* was overcome and a bacterial expression system developed for production of HBcAg fusion proteins with FMDV or rhinovirus epitopes joined through a short linker sequence to 6 residues of the HBV pre-core sequence followed by the full HBcAg sequence (Clarke *et al.*, 1990; Francis *et al.*, 1990). Recently, similar constructs containing epitopes from the preS region of HBV (Schodel *et al.*, 1992) or simian immunodeficiency virus (Yon *et al.*, 1992) were made and expressed in *E. coli* without difficulty.

The strategy for N-terminal fusion in this study differs from that used by other groups. The $HBs_{(111-156)}$ sequence was fused upstream of $HBc_{(3-144)}$ using a unique EcoRI site in $pHBcPreS_1PreS_2$ and $pHBcPreS_1PreS_2S$ for insertion of the S sequence, so that the resulting gene fusions contained the β-galactosidase$_{(1-8)}$ sequence followed by 2 amino acid residues from the linker sequence, the inserted epitope $HBs_{(111-156)}$, 3 amino acid

residues from another linker sequence, and $HBc_{(3-144)}$ further followed by the insertions at the C-terminus. Recently, it has been reported that a $preS_1$ epitope linked to the N-terminus of HBcAg was not surface accessible and not immunogenic, although the hybrid HBcAg proteins were particulate; while that linked to the amino terminus through the C-terminal 4 amino acid residues of the pre-core sequence, became surface accessible (Schodel *et al.*, 1992). The authors claimed that the N-terminus of native HBcAg might not be exposed on the particle surface, and heterologous epitopes were exposed at the exterior of HBcAg particles by virtue of the pre-core and/or linker amino acids.

Gene fusions to the C-terminus of HBcAg have been made at amino acid residue 144 via a short linker sequence (Stahl and Murray, 1989) or directly (Borisova *et al.*, 1989) and at amino acid residue 156 (Schodel *et al.*, 1990 and 1992) so that the arginine-rich region at the C-terminus of HBcAg was not present. The fusion proteins produced in bacteria assembled into core-like particles, indicating that the arginine-rich C-terminal region is dispensable for core particle assembly.

Insertion of foreign epitopes at an internal site in HBcAg has also been explored. PreS epitopes and non-HBV viral epitopes have been inserted into a polylinker sequence positioned between amino acid residues 144 and 145 of HBcAg and the fusion proteins formed core-like particles (Borisova *et al.*, 1989). Moreover, the introduction of foreign epitopes between amino acid residues 80 and 81 (Yon *et al.*, 1992) as well as between 75 and 83, thereby deleting residues 76 to 82, (Schodel *et al.*, 1992) did not prevent particle formation.

Based on the concept that presentation of foreign epitopes on the surface of particulate structures enhanced immunogenicity, the use of self-assembling particles using HBsAg as carrier moieties has also been explored. However, the HBsAg fusion proteins were expressed in yeast or mammalian cells (Delpeyroux *et al.*, 1986; Michel *et al.*, 1988; Schlienger *et al.*, 1992) and were unstable in *E. coli.* In contrast, the HBcAg fusion proteins are produced in high yields in *E. coli*, which facilitates their purification.

Schodel *et al.* (1990) expressed HBcAg fusion proteins in strains of various *Salmonella* species for the development of oral vaccines.

In conclusion, in the work described here, the $preS_{1(1-20)}$, $preS_{2(1-26)}$ and $HBs_{(111-156)}$, wild-type or gly_{145} mutant, sequences were fused to truncated HBcAg at the N- and C-termini and the HBcAg fusion proteins were expressed in *E. coli.* Fusions of the epitopes at the C-terminus of HBcAg were soluble and could be purified based on their particulate nature. However, insertion of the epitopes at both N- and C-termini of HBcAg appeared to alter the physical and/or chemical properties of the proteins, and rendered them aggregated. Nevertheless, they still formed particles. The purified HBcAg fusion proteins were then evaluated for antigenicity and immunogenicity.

CHAPTER 4: ANTIGENICITY OF HBcAg FUSION PROTEINS

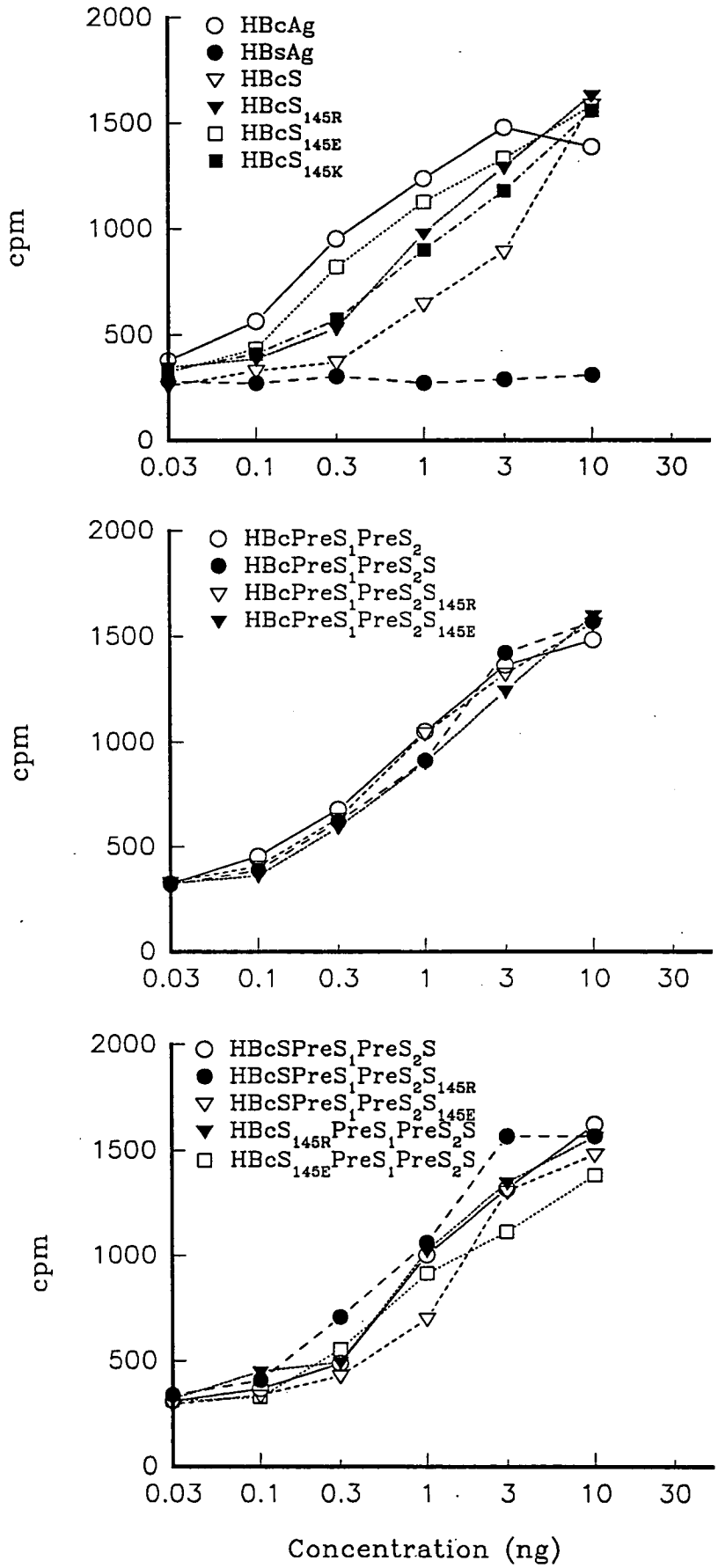
4.1 Introduction

The purified HBcAg fusion proteins including HBcS, HBcPreS$_1$PreS$_2$, HBcPreS$_1$PreS$_2$S and HBcSPreS$_1$PreS$_2$S in which the inserted HBs sequence possesses either wild-type or mutant amino acid residue at gly$_{145}$ were tested for HBc and HBs antigenicity. The reactivities of HBcAg fusion proteins with anti-HBc and anti-HBs antibodies were tested by immunoblot analyses and RIAs. Because suitable antibodies against preS$_{1(1-20)}$ and preS$_{2(1-26)}$ epitopes were not available in this laboratory, the preS$_1$ and preS$_2$ antigenicity of the HBcAg fusion proteins was not examined. However, immunogenicity in terms of the production of anti-preS$_1$ and anti-preS$_2$ antibodies were assessed (see Chapter 5).

4.2 HBc antigenicity

The result of the immunoblot experiment is shown in Figure 3.12. Polyclonal anti-HBc antiserum recognised HBcAg and all the HBcAg fusion proteins. The HBcAg fusion proteins with both N- and C-terminal inserts, e.g. SHBcPreS$_1$PreS$_2$ and SHBcPreS$_1$PreS$_2$S, although not purified due to aggregation during the purification process, also reacted with rabbit anti-HBc serum in immunoblot analysis when the reduced, boiled, insoluble samples were applied to SDS-PAGE (result not shown). The antigenic reactivity of HBcAg was further analysed by a sandwich RIA in which HBcAg fusion proteins were trapped on a solid phase with human anti-HBc IgG. When ^{125}I-labelled human anti-HBc is added, it binds to antigen-antibody complex on the solid phase, creating a radioactive antibody-antigen-antibody "sandwich". Figure 4.1 shows that all HBcAg fusion proteins display similar HBcAg reactivity. The assay is specific to HBcAg reactivity because HBsAg in the same concentrations failed to bind to the beads coated with anti-HBc IgG and all the HBcAg fusion proteins showed dose-response binding to anti-HBc antibody similar to that of full-length HBcAg. In this antigen-capture sandwich assay, the signal generated is not high, hence the detection range is between 1 to 10 ng of HBcAg and the assay reaches a plateau when more than 10 ng of antigen is present. However, the signal in the assay could be

Figure 4.1 HBc antigenicity of HBcAg fusion proteins. The HBc antigenicity was measured by the sandwich RIA in which HBcAg fusion proteins were trapped with anti-HBc IgG and the bound antigens were measured using ^{125}I-labelled anti-HBc serum. All samples were assayed concomitantly.


2000
1500
1000
500
0
cpm
HBcAg
HBsAg
HBcS
HBcS145R
HBcS145E
HBcS145K
0.03
0.1
0.3
1
3
10
30
HBcPreS1PreS2
HBcPreS1PreS2S
HBcPreS1PreS2S145R
HBcPreS1PreS2S145E
HBcSPreS1PreS2S
HBcSPreS1PreS2S145R
HBcSPreS1PreS2S145E
HBcS145RPreS1PreS2S
HBcS145EPreS1PreS2S
Concentration (ng)

amplified to some extent by using an unlabelled polyclonal anti-HBc antibody from mice, while the coating anti-HBc antibody was from human, and ^{125}I-labelled sheep anti-mouse immunoglobulin (data not shown).

These results indicate that all HBcAg fusion proteins bearing amino acid 3 to 144 residues of HBcAg and containing up to 318 amino acid residues compared with 183 of the full-length HBcAg or 157 of its HBcAg counterpart used for the construction of these gene fusions, retain HBc antigenicity.

4.3 HBs antigenicity

The reactivity of HBcAg fusion proteins carrying the $HBs_{(111\text{-}156)}$ sequence with anti-HBs antibody was tested in the DARIP inhibition assay and also by the AUSRIA II RIA detection kit (Abbott laboratories). In the DARIP inhibition test, an ^{125}I-labelled HBsAg and anti-HBs antibody reaction can be demonstrated by concentration-dependent competitive inhibition in the presence of increasing amounts of HBsAg. In Table 4.1, HBsAg, serving as a positive control, demonstrated a dose-response inhibition of the binding of ^{125}I-labelled HBs to anti-HBs antibody. However, no inhibition was observed with the HBcAg fusion proteins at the 200 ng level. The result indicates that HBcS, not only wild-type but also the three gly_{145} mutant proteins, display little or no HBsAg antigenicity. Since HBcS showed no reactivity, whether the antigenic determinants in the three gly_{145} mutant proteins have changed could not be assessed. Besides, the anti-HBs used for the assay was a polyclonal serum against several B-cell epitopes within HBsAg, thus it is unlikely that the possible change in one single epitope would abolish the binding of anti-HBs with HBcAg fusion proteins carrying the $HBs_{(111\text{-}156)}$ sequence. Nevertheless, it is tempting to suggest that the inserted $HBs_{(111\text{-}156)}$ sequence may be surface inaccessible or may exhibit conformational changes that alter the accessibility of HBs epitopes on HBcAg fusion proteins and hinder them from reacting to anti-HBs antibody.

The HBsAg antigenicity of HBcS carrying unmutated or mutated gly_{145} was also

Table 4.1 HBs antigenicity of HBcAg fusion proteins examined in the DARIP inhibition assay

Sample[a]	Concentration (ng)	%^{125}I-HBsAg precipitated[b]	% Inhibition
HBsAg	200	7.2	91.6
	100	6.8	92.1
	30	6.3	92.7
	10	5.5	93.6
	3	6.2	92.8
	1	7.3	91.5
	0.3	30.4	64.6
	0.1	53.1	38.2
	0.03	74.1	13.7
	0.01	80.3	6.5
	0.003	83.9	2.3
HBcAg	200	89.1	-3.7
HBcS	200	83.5	2.8
$HBcS_{145R}$	200	82.0	4.5
$HBcS_{145E}$	200	83.0	3.4
$HBcS_{145K}$	200	86.3	-0.5
$HBcPreS_{1(1-36)}$	200	79.5	7.5
Buffer[c]	-	85.9	0

[a] The tested samples were used as the inhibitor in the DARIP inhibition assay.

[b] A percentage of ^{125}I-HBsAg (*ayw*) bound to the precipitate compared to total ^{125}I added in the reaction.

[c] RIP buffer (0.5% BSA in PBS) used as the diluent in the assay.

examined with the Abbott AUSRIA II RIA. In this "sandwich" RIA, beads coated with guinea pig anti-HBs antibody are incubated with test samples. Any HBsAg present in the samples is bound to the solid phase antibody. Human ^{125}I-labelled anti-HBs is allowed to react with antibody-antigen complex on the bead. The reactivity of HBsAg in these HBcAg fusion proteins, either wild-type or mutants, was poor, since high concentrations of the samples (100 μg), containing approximately 20 μg of $HBs_{(111-156)}$ fragment produced only weak binding (Table 4.2), equivalent to the binding of approximately 1 ng of native HBsAg. $HBcPreS_{1(1-36)}$ served as the negative control also produced weak binding (P/N ratio = 2.4 for 10 μg). In addition, when 500 μg of the HBcAg fusion proteins was used in the AUSRIA test, the P/N ratio remained low, ranging from 3.0 to 3.6 (data not shown).

In immunoblot analysis, however, HBcS wild-type and the three mutant proteins reacted not only with anti-SDS-denatured HBs antibody (result not shown), but also with anti-native HBs antibody (Figure 4.2). These results confirm that the HBs sequence was presented in HBcAg fusion proteins containing $HBs_{(111-156)}$ sequence. The immunoblotting method involves antigen presentation on a solid support, thus allowing antibody to bind in a multivalent manner. However, it is likely to favour sequence determinants, rather than conformational determinants.

In the AUSRIA test, the antigen to be tested must present at least one target epitope on each side of the molecule. In other words, the two antibody components are required to bind spatially distinct antigen epitopes. Although the two anti-HBs antibodies, one as capture and the other as labelled reagents, used in the assay are polyclonal, the binding of HBs epitopes in HBcAg fusion proteins to the solid phase antibody might result in steric hindrance that prevents the HBs epitopes from reacting efficiently with ^{125}I-labelled anti-HBs antibody. Therefore, another antigen-capture sandwich assay was set up in which HBcAg fusion proteins were trapped on polystyrene beads coated with human anti-HBc IgG and then tested for the presence of anti-HBs reactive epitopes on the surface of the fusion proteins by using rabbit anti-HBs serum and appropriate ^{125}I-labelled second antibody (^{125}I-labelled donkey anti-

Table 4.2 HBs antigenicity of HBcAg fusion proteins assessed by the AUSRIA II RIA

Sample	Concentration	P/N ratio[a]
HBsAg	10.0 ng	14.0
	3.0 ng	7.1
	1.0 ng	3.4
	0.3 ng	2.4
	0.1 ng	1.7
	0.03 ng	1.7
HBcS	100.0 μg	2.5
	10.0 μg	2.0
$HBcS_{145R}$	100.0 μg	2.8
	10.0 μg	2.3
$HBcS_{145E}$	100.0 μg	3.2
	10.0 μg	2.0
$HBcS_{145K}$	100.0 μg	3.1
	10.0 μg	2.7
$HBcPreS_{1(1-36)}$	100.0 μg	1.8
	10.0 μg	2.4

[a] The reactivity of HBsAg in the samples was assessed by the AUSRIA II RIA and the results are expressed as P/N ratio which represents cpm ratio of samples measured (P) and negative control (N).

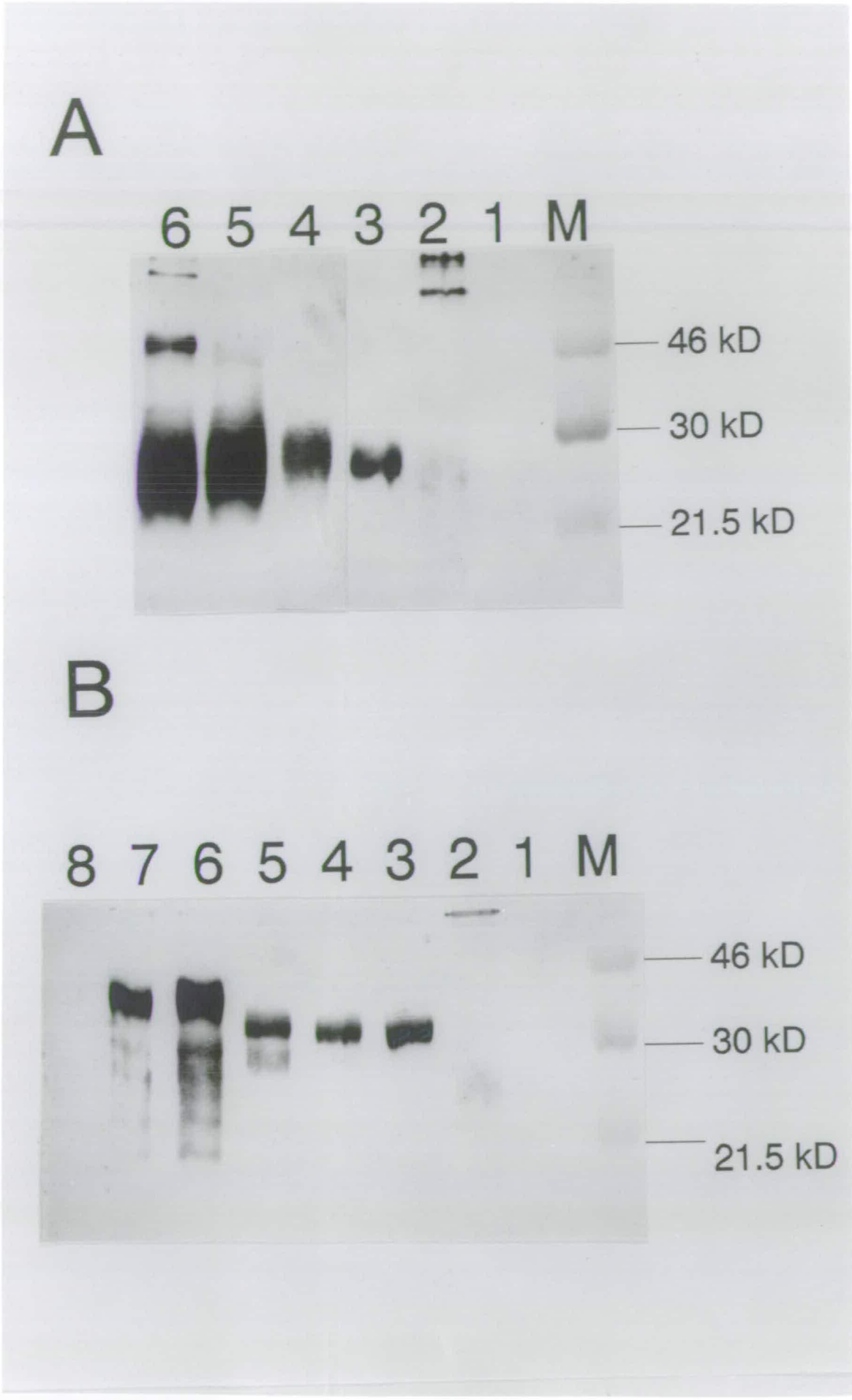
Figure 4.2 Immunoblot analysis of HBcAg fusion proteins probed by anti-HBs serum. Purified protein samples were electrophoresed through a 15% SDS-PAGE and transferred to nitrocellulose membrane and HBsAg was detected by incubation with Rabbit 82 anti-HBs serum diluted 1:200 followed by incubation with appropriate anti-species IgG conjugated with alkaline phosphatase and subsequent incubation with NBT and BCIP.

(A) Lanes:

M	-	Coloured molecular weight size standards
1	-	HBcAg
2	-	HBsAg
3	-	HBcS
4	-	$HBcS_{145R}$
5	-	$HBcS_{145E}$
6	-	$HBcS_{145K}$

(B) Lanes:

M	-	Coloured molecular weight size standards
1	-	HBcAg
2	-	HBsAg
3	-	$HBcPreS_1PreS_2S$
4	-	$HBcPreS_1PreS_2S_{145R}$
5	-	$HBcPreS_1PreS_2S_{145E}$
6	-	$HBcSPreS_1PreS_2S$
7	-	$HBcSPreS_1PreS_2S_{145R}$
8	-	$HBcPreS_1PreS_2$

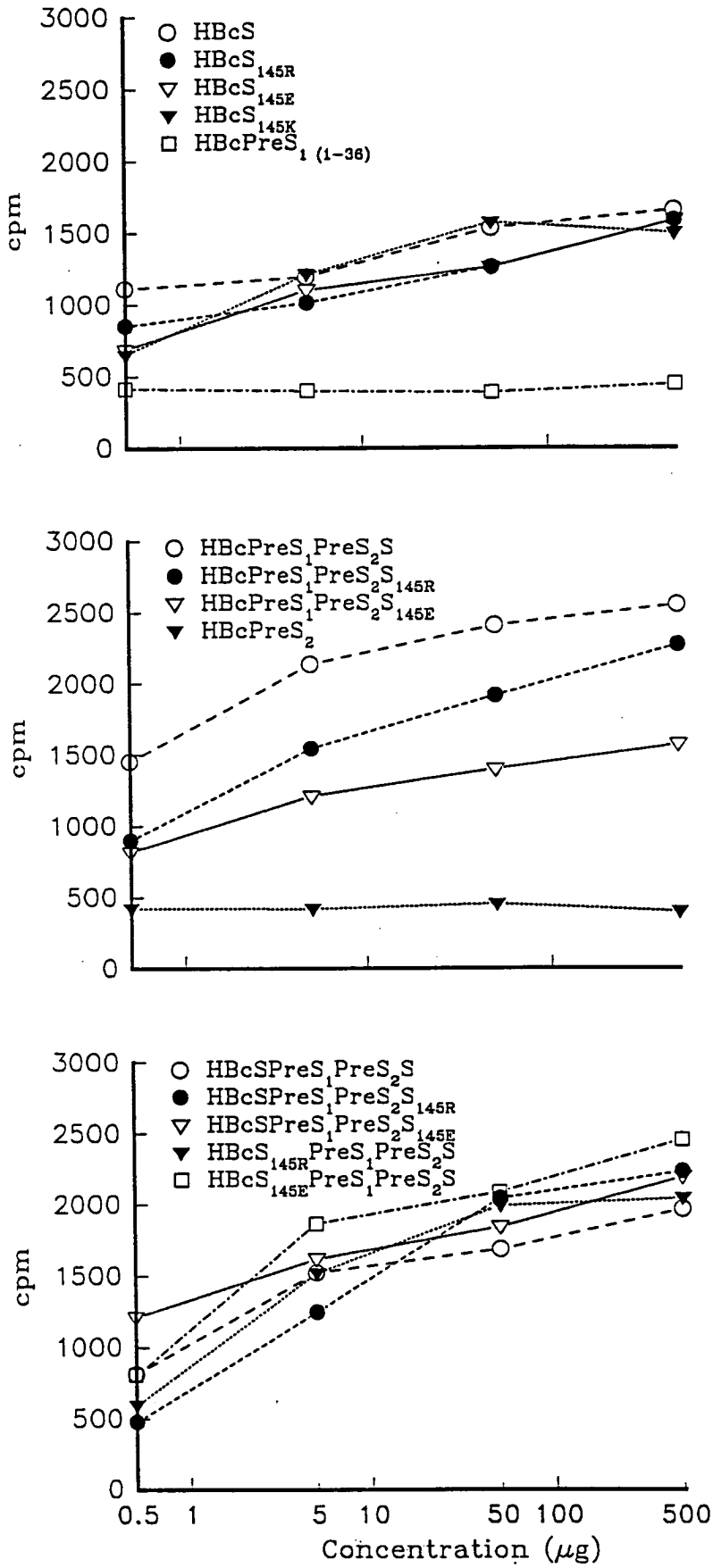

A
6 5 4 3 2 1 M
46 kD
30 kD
21.5 kD
B
8 7 6 5 4 3 2 1 M
46 kD
30 kD
21.5 kD

rabbit immunoglobulin). As shown in Figure 4.3, HBs antigenicity was detected in all the HBcAg fusion proteins carrying wild-type or mutant $HBs_{(111-156)}$ sequences, whereas HBcAg, $HBcPreS_{1(1-36)}$ and $HBcPreS_2$ showed no HBs antigenicity, demonstrating that the assay is specific for the detection of HBs antigenicity. Comparing the assays for HBcAg and HBsAg which share a similar strategy, i.e. antigen-capture sandwich assay, the amount of core fusion proteins needed to obtain a positive signal was much higher in the HBsAg assay than in the HBcAg assay.

Reversely, if HBcAg fusion proteins were trapped on the solid phase coated with rabbit anti-HBs IgG and then tested for HBc antigenicity on the fusion proteins by using mouse anti-HBc serum and ^{125}I-labelled sheep anti-mouse immunoglobulin as the probe, non-specific binding of the HBcAg fusion proteins to the solid phase allowed anti-HBc serum to bind to the anti-HBc reactive epitope within the HBcAg fusion protein, which resulted in high background of the assay. As a result, the difference in binding between HBcS and HBcAg or $HBcPreS_2$ (as the negative control) was not high enough to differentiate the positive and negative reactions (result not shown).

To investigate whether the specificity of anti-HBs antibody and HBsAg interaction could be inhibited by HBcAg fusion proteins carrying $HBs_{(111-156)}$, an antigen-inhibition RIA was performed in which polystyrene beads coated with HBsAg were incubated at 37°C for 1 hr with rabbit polyclonal anti-HBs antiserum that had been preincubated with HBsAg or HBcAg fusion proteins as the competitors at 37°C for 3 hr, followed by incubation with ^{125}I-labelled second antibody at 37°C for another hour. As shown in Figure 4.4, anti-HBs antibody was inhibited in a concentration-dependent manner from binding to coated HBsAg by the presence of HBsAg made in yeast. Approximately 60% inhibition of binding was achieved with 500 ng of HBsAg as the competitor. On the other hand, only about 20% inhibition was observed with 500 μg of HBcS or $HBcPreS_1PreS_2S$. The inhibition of binding increased slightly when 1 mg of $HBcPreS_1PreS_2S$ was used (data not shown). No inhibition was found with $HBcPreS_1PreS_2$ which served as the negative control of the assay. The results

Figure 4.3 HBs antigenicity of HBcAg fusion proteins. The HBs antigenicity was measured by the sandwich RIA in which HBcAg fusion proteins were trapped with human anti-HBc IgG and the surface exposure of the $HBs_{(111-156)}$ on the particles was measured using rabbit anti-HBs serum and ^{125}I-labelled donkey anti-rabbit immunoglobulin. All samples were assayed concomitantly.


3000
2500
2000
1500
1000
500
0
cpm
HBcS
HBcS145R
HBcS145E
HBcS145K
HBcPreS1 (1-36)
HBcPreS1PreS2S
HBcPreS1PreS2S145R
HBcPreS1PreS2S145E
HBcPreS2
HBcSPreS1PreS2S
HBcSPreS1PreS2S145R
HBcSPreS1PreS2S145E
HBcS145RPreS1PreS2S
HBcS145EPreS1PreS2S
0.5
1
5
10
50
100
500
Concentration (μg)

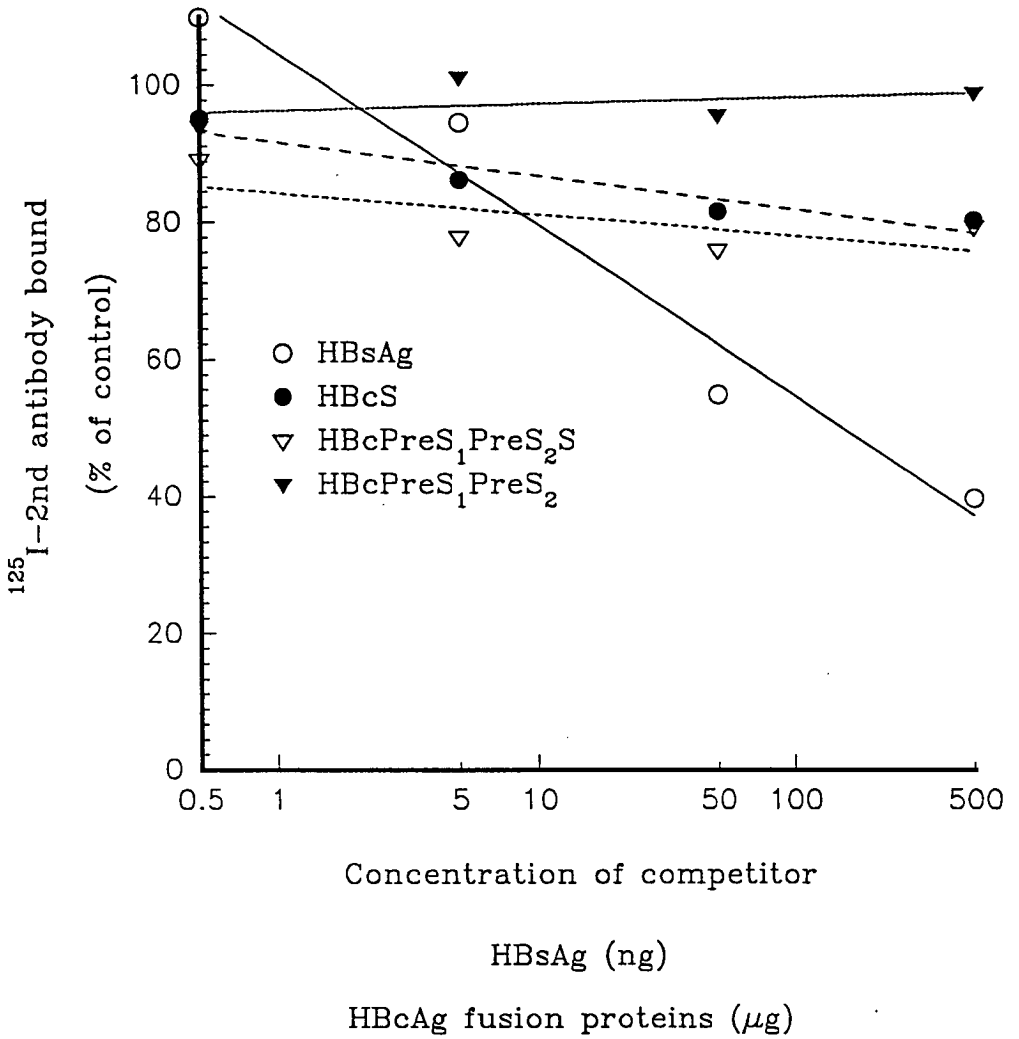


Figure 4.4 The inhibition of anti-HBs antibody binding to HBsAg by HBcAg fusion proteins. The inhibition of the binding of anti-HBs antibody to the solid phase HBsAg in the presence of the competitor was determined using ^{125}I-labelled anti-species immunoglobulin.

obtained from the immunoblot analysis, the antigen-capture sandwich assays and the antigen-inhibition RIA indicate that HBcAg fusion proteins carrying $HBs_{(111-156)}$ display some cross-reactivity with polyclonal anti-HBs antiserum. Thus they appear to contain one or more antigenic determinants related to HBsAg. However, they do not represent all of the epitopes expressed by HBsAg.

4.4 Monoclonal antibody binding studies

Three murine MAbs, RFHBs-1, RFHBS-2 and RFHBS-7, against HBsAg used in these studies were kindly supplied by J.A. Waters and H.C. Thomas of the St Mary's Hospital Medical School, London. RFHBs-1 and RFHBS-2 bind to a cyclic peptide made from amino acids 124-137, and RFHBs-7 to a cyclic peptide from amino acids 139-147 of HBsAg (Carman *et al.*, 1990). Thus the epitopes recognised by these MAbs are conformational, rather than linear peptide sequences of the antigen. In addition, RFHBs-1 has been shown to be capable of neutralising both *ad* and *ay* subtypes of HBV in chimpanzee experiments (Iwarson *et al.*, 1985a) and the antibody that recognises RFHBs-1 epitope is present in the sera of patients who have recovered from acute HBV infection (Waters *et al.*, 1986a).

The antibodies that recognise RFHBs-1 and RFHBs-7 epitopes are also present in the sera of vaccinees who had received the plasma-derived or recombinant HBV vaccines (Waters *et al.*, 1987). Since both sequences, amino acids 124-137 and 139-147 of HBsAg, appear to form part of the *a* determinants of HBsAg, it is reasonable to use these MAbs to examine whether HBcAg fusion proteins carrying $HBs_{(111-156)}$ contain an *a* antigenic determinant, and whether an amino acid substitution of arg, glu or lys for gly at amino acid 145 of HBsAg in HBcAg fusion proteins alters the *a* determinant antigenicity. An antigen-capture sandwich assay was performed in which the test antigen, HBsAg or HBcAg fusion proteins, was captured on RFHBs-1-, RFHBs-2-, or RFHBS-7-coated beads followed by addition of rabbit polyclonal anti-HBs antiserum and ^{125}I-labelled donkey anti-rabbit immunoglobulin which subsequently binds to the captured antigen. As shown in Table 4.3, HBsAg binds to all three MAbs in a

Table 4.3 Binding of HBcAg fusion proteins to HBsAg-specific monoclonal antibodies

Antigen[a]	Concentration (μg)	Binding to MAb (P/N ratio)[b]		
		RFHBs-1	RFHBs-2	RFHBs-7
HBsAg	1	19.5	22.4	15.2
	0.1	6.5	12.7	12.7
	0.01	1.8	2.3	2.7
	0.001	1.2	1.2	1.0
HBcS	1000	3.3	2.7	2.2
	100	1.8	1.6	1.4
$HBcS_{145R}$	1000	2.7	2.1	2.0
	100	1.3	1.1	1.1
$HBcS_{145E}$	1000	1.8	1.6	1.4
	100	1.4	1.1	1.1
$HBcS_{145K}$	1000	2.0	1.4	1.2
	100	1.0	1.0	1.0
$HBcS_{145E}PreS_1PreS_2S$	1000	2.8	2.0	2.0
	100	1.6	1.3	1.1
$HBcPreS_{1(1-36)}$	1000	1.3	1.3	1.2
	100	1.3	1.1	1.0
Buffer[c]	-	1.0	1.0	1.0

[a] The tested antigens were added to polystyrene beads coated with MAb RFHBs-1, RFHBs-2 or RFHBs-7 and detected by rabbit anti-HBs serum and ^{125}I-labelled donkey anti-rabbit immunoglobulin.

[b] Binding results are expressed as cpm ratio of samples measured (P) and the negative control containing the reaction buffer (N).

[c] The binding (cpm) of the reaction buffer (10% donkey serum in PBS) to RFHBs-1, RFHBs-2 and RFHBs-7 is 246, 252 and 380, respectively.

concentration-dependent manner, indicating that the epitopes that the three MAbs recognise are indeed presented by HBsAg. No detectable binding was observed with $HBcPreS_{1(1-36)}$ which bears no HBs sequence and served as a negative control for the assay. All the HBcAg fusion proteins reacted more weakly, if at all, with the three MAbs, compared to HBsAg. HBcS, $HBcS_{145R}$ and $HBcS_{145E}PreS_1PreS_2S$ showed similar binding to RFHBs-1, RFHBs-2 and RFHBs-7, among which, HBcS showed a slight increase in binding to the MAbs when its P/N ratio was compared to a negative control. Both $HBcS_{145E}$ and $HBcS_{145K}$ showed similar binding to RFHBs-1, but to a lesser extent, as compared with HBcS, $HBcS_{145R}$ and $HBcS_{145E}PreS_1PreS_2S$, whereas they reacted poorly or negligibly with RFHBs-2 and RFHBs-7. The observation that HBcAg fusion proteins, either wild-type or mutant proteins, reacted poorly with MAbs specific to the *a* determinant of HBsAg, presumably due to poor surface accessibility, was in agreement with the results of poor reactivity of HBcAg fusion proteins with polyclonal anti-HBs antibody either in solid phase (AUSRIA test) or in liquid phase (DARIP inhibition test). These results indicate the difficulty in comparing the change of the *a* determinant of HBsAg in HBcAg fusion proteins carrying gly_{145} mutation.

4.5 Discussion

The plasmids expressing fusion proteins were based on ptacHpaII (Stahl and Murray, 1989), which was constructed from elements of the plasmid pR1-11 (Stahl *et al.*, 1982). The plasmid pR1-11 encodes a fusion protein containing the first 8 amino acids of β-galactosidase followed to full-length HBcAg with the first 2 amino acids deleted, while plasmid ptacHpaII encodes a similar fusion protein with truncated $HBcAg_{(3-144)}$. All HBcAg fusion proteins carrying insertions at the C-terminus display similar HBc antigenicity as full-length HBcAg (Figures 3.12 and 4.1). Those carrying both N- and C-terminal insertions also show HBc antigenicity when the unpurified samples (ammonium sulphate fractions) were examined by immunoblotting. These results are in accordance with those reported previously (Clarke *et al.*, 1987; Stahl and Murray, 1989; Schodel *et al.*, 1992). Therefore, fusion of foreign sequences to the N-, the truncated C- or both termini of HBcAg preserves the HBc antigenicity. However,

insertion of a foreign sequence between HBcAg residues 75 and 83 has been demonstrated to abrogate recognition of HBcAg by five out of six anti-HBc MAbs and diminish recognition by human polyclonal anti-HBc (Schodel *et al.*, 1992), indicating the importance of this region in B-cell recognition of HBcAg, which is in agreement with previous data from mapping of the immunodominant B-cell epitope (Salfeld *et al.*, 1989). Nevertheless, the inserted epitope between HBcAg residues 75 and 83 was antigenic and could elicit high anti-peptide antibody response in mice. In order to take advantage of HBcAg-specific T-cell and anti-HBc responses that might be induced by HBcAg fusion proteins as suggested from the murine results reported by Milich and colleagues, the integrity of the HBc antigenicity might be of importance.

Predictably, the HBcAg fusion proteins would also display HBe antigenicity. The HBe antigenicity of the HBcAg fusion proteins was not determined in this study. $HBcPreS_{1(1\text{-}20)}$ and truncated HBcAg have been shown to display similar HBeAg reactivity, whereas full-length HBcAg showed very low reactivity (Stahl and Murray, 1989).

Although the sequence of HBeAg is contained within that of HBcAg, the two antigens exhibit fundamentally different biophysical and antigenic properties. Distinct epitopes located exclusively on HBcAg or HBeAg have been detected with polyclonal and monoclonal antibodies (Ferns and Tedder, 1986; Waters *et al.*, 1986b; Colucci *et al.*, 1988; Salfeld *et al.*, 1989; Sallberg *et al.*, 1991). There is a discrepancy regarding the nature of the HBcAg determinant. Earlier studies have indicated that denaturation of core particles destroyed HBcAg specificity with a consequent release of HBeAg, suggesting that HBcAg determinants are sensitive to structural changes (MacKay *et al.*, 1981a; Ohari *et al.*, 1984). However, one report argued against a strict conformation-dependence of HBcAg. An immunodominant HBcAg determinant has been mapped to a linear peptide encompassing amino acids 107-118 (Colucci *et al.*, 1988). Nevertheless, Salfeld *et al.* (1989) reported that HBcAg exposes only a single determinant, which induces numerous anti-HBc antibodies, most of which recognise conformational epitopes, while HBeAg possesses 2 determinants, one linear (HBe1)

and the other conformational (HBe2). Furthermore, the HBcAg epitopes recognised by human anti-HBc antisera are predominantly conformationl. The HBcAg and HBe1 determinants were both mapped to an overlapping hydrophilic sequence around amino acid 80. HBe2 was mapped to a location in the vicinity of amino acid 138, but about 90% of the sequence, between amino acids 10 and 140, was required for its full antigenicity. It has been recently demonstrated that the cysteine residue, which is located within the ten remaining amino acids of the pre-core fragment attached to the HBeAg after cleavage of the leader sequence, determines the quaternary structure and the antigenicity of HBe protein. If this cysteine is lacking, the HBe protein is expressed as a disulphide-linked homodimer showing both HBe and HBc antigenicity (Wasenauer *et al.*, 1992).

The HBcAg fusion proteins containing $HBs_{(111\text{-}156)}$, either wild-type or gly_{145} mutant sequence, display little HBs antigenicity in the DARIP inhibition assay (Table 4.1) or the AUSRIA II RIA (Table 4.2). However, immunoblot analyses of HBcS and the three mutant counterparts confirmed that the $HBs_{(111\text{-}156)}$ was presented on the hybrid HBcAg particles when anti-native HBs or anti-SDS-denatured HBs polyclonal antibodies were used as the probes (Figure 4.2).

In immunoblotting, the HBcAg fusion proteins were presented on a solid support which allowed antibody to bind in a multivalent, affinity-independent manner. However, the proteins would likely not be native, since they were denatured by treatment with an ionic detergent and boiling under reducing conditions. Although electrophoretic transfer probably removes most of the detergent and the reducing agent, the strong non-covalent forces which result in the binding of the polypeptide to the nitrocellulose membrane probably prevent the polypeptide from complete renaturation. Therefore, immunoblotting is likely to favour linear determinants. Since the probes used in the immunoblotting were polyclonal antibodies raised from rabbits immunised with native or denatured HBsAg, it was not surprising that not only the wild-type HBcS but also the three gly_{145} mutants reacted with both polyclonal antibodies containing a number of antibodies against different B-cell epitopes in the

$HBs_{(111-156)}$ sequence.

In the AUSRIA test, the HBsAg is sandwich-captured by two anti-HBs antibodies, one immobilised on the solid phase, the other labelled with ^{125}I as the probe. At high concentrations (100 μg and 500 μg) the HBcAg fusion proteins carrying the $HBs_{(111-156)}$ sequence reacted poorly with anti-HBs antibody with P/N ratio ranging between 2.5 to 3.6.

There are two possibilities for the poor HBs antigenicity displayed in the HBcAg fusion proteins carrying the $HBs_{(111-156)}$ sequence. First, the inserted $HBs_{(111-156)}$ sequence may exhibit conformational changes that alter the accessibility of HBs epitopes on the fusion proteins and hinder them from reacting to anti-HBs antibody. Secondly, the binding of HBs epitopes in HBcAg fusion proteins to the solid phase anti-HBs antibody might result in steric hindrance that prevents the HBs epitopes from reacting efficiently with ^{125}I-labelled anti-HBs antibody. Therefore, if human anti-HBc antibody, instead of anti-HBs antibody, was used as the solid phase antibody and rabbit anti-HBs antibody followed by ^{125}I-labelled donkey anti-rabbit immunoglobulin used as the probe in the antigen-capture sandwich RIA, the possible steric hindrance could be overcome. Surprisingly and importantly, HBs antigenicity was displayed in the HBcAg fusion particles and was demonstrated in a concentration-dependent manner. This assay thus allows the detection of the HBc and HBs antigenicities within the same molecule. Furthermore, only the fusion proteins displaying both HBc and HBs antigenicities could be detected by this assay. However, microgram levels of HBcAg fusion proteins were required in the assay which were 1000-fold higher than those used in a similar assay for HBcAg reactivity. On a weight basis, HBcAg fusion proteins contained approximately three times as much of the HBc sequence as the HBs sequence. Thus assuming the two assays for HBs and HBc reactivities had a similar sensitivity, the HBs antigenicity appeared to be more than 300 fold less reactive than HBc antigenicity in the HBcAg fusion proteins. The poor HBs reactivity in the AUSRIA test appeared to result from steric hindrance. Nevertheless, conformational change might still occur and contribute to the lower HBs antigenicity.

The immunodominant determinants on HBsAg are conformation-dependent, no doubt because of extensive inter- and intra-molecular disulphide bonds. This is particularly true of the *a* determinant of HBsAg. The *a* epitope contained within amino acid residues 139-147 and 122-137 of the S protein appeared to be conformational since cyclic forms of both the corresponding synthetic peptides were recognised with higher affinity than the linear analogues (Dreesman *et al.*, 1982; Brown *et al.*, 1984). In addition, some MAbs against HBsAg bound to cyclic peptides 124-137 and 139-147 of the S protein, but not to the linear peptides (Carman *et al.*, 1990).

Several reports showed that peptides derived from the *a* determinant of HBsAg displayed weaker, if any, HBs antigenicity than native, intact HBsAg. Nevertheless, they elicited anti-HBs antibody, although at a low level as compared with that elicited by native HBsAg, when conjugated to carrier proteins. These results suggest that the peptides constituted do not closely mimic the tertiary structure of the native epitope in HBsAg. The peptide P135-155 of HBsAg showed no reactivity with anti-native HBs (Neurath *et al.*, 1982). The capacity of P138-149 to bind to anti-HBs antibody was also limited (Prince *et al.*, 1982). The peptide P139-147 and cyclic peptide P122-137 have been shown to inhibit the binding of anti-HBs antibody to native HBsAg. However, on a weight basis, a 10^6-fold and $3x10^3$-fold more P138-147 and cyclic P122-137 were required to achieve an equivalent degree of inhibition in comparison with native HBsAg, respectively (Bhatnagar *et al.*, 1982; Ionescu-Matiu *et al.*, 1983). A synthetic peptide, OS[124-147], corresponding to residues 124 to 147 of the S protein self-assembled into a heterogeneous mixture of multiple forms via the disulphide bonds formed by five possible cysteine sulphydryl groups within the peptide and these oligomeric peptides reconstructed a conformational, group-specific antigenic determinant of HBsAg (Manivel *et al.*, 1992b). A polypeptide (MEP-1) comprising multiple epitopes from $preS_{1(1-36)}$, $preS_{2(1-26)}$, $HBs_{(19-28)}$, and $HBs_{(124-147)}$ had been made by Kumar *et al.* (1992). In immunoblot analysis, MEP-1 reacted with anti-$preS_1$ and anti-$preS_2$ peptide antibodies, but did not react or reacted very poorly with anti-$HBs_{(124-147)}$ and anti-HBs antisera. However, when MEP-1 was coated on the solid phase and detected by indirect ELISA, the S-specific epitopes became detectable and

rearrangement of disulphide linkages by thiocyanate treatment seemed to increase their antigenicity.

In the work described here, the HBs antigenicity of the HBcAg fusion proteins is also weak as compared with the native HBsAg. The method employed for the assay of the reactivity of the protein with anti-HBs antibody may influence the result. The HBs antigenicity in the HBcAg fusion protein carrying either wild-type or mutant $HBs_{(111-156)}$ sequences was detectable in the antigen-capture sandwich RIA, using two antibodies with different specificity, anti-HBc and anti-HBs, to detect the HBc and HBs antigenicities simultaneously, whereas little HBs antigenicity was detected in the equivalent assay using anti-HBs antibody for the solid phase and the probe in both sides of the sandwich to capture HBsAg. A 20% inhibition of binding of anti-native HBs antibody to HBsAg was observed after addition of 500 μg of HBcS or $HBcPreS_1PreS_2S$, while 60% inhibition of binding was achieved after addition of 500 ng of HBsAg. These results are in agreement with those described previously, indicating that mimicking of conformational determinants of HBsAg may be more sophisticated, either using free peptide or presentation on a particulate structure, such as HBcAg. Disulphide cyclisation has been shown to increase the antigenicity of synthetic peptides by locking their secondary structures (Dreesman *et al.*, 1982; Brown *et al.*, 1984). It was suggested that a peptide containing a conformational determinant may be locked into the immunodominant regions of heterologous particulate antigens.

Thus, the antigen-capture sandwich RIA and the immunogenicity studies (see Chapter 5) confirm the surface accessibility of the inserted $HBs_{(111-156)}$ epitope. The weak reactivity of HBcAg fusion proteins with anti-HBs antibody might be due to different presentation of the $HBs_{(111-156)}$ segment on the fusion particles from that on the native HBsAg. Nevertheless, the HBcAg fusion proteins carrying the $HBs_{(111-156)}$ fragment were able to induce antibodies cross-reactive with native HBsAg (see Chapter 5).

Monoclonal antibodies, which by definition are directed against a single antigenic determinant, can provide reagents to detect the change of epitope to which they bind.

In the present study, since some HBcAg fusion proteins carry gly_{145} mutants of the $HBs_{(111-156)}$ sequence and the mutation is within the *a* determinant of HBsAg, it is reasonable to use anti-*a* MAbs, eg. RFHBs-1 and RFHBS-2 that bind to a cyclic peptide made from amino acids 124-137, and RFHBs-7 to a cyclic peptide from amino acids 139-147 of HBsAg, to examine the reactivity of these HBcAg fusion proteins. All the HBcAg fusion proteins reacted poorly with these MAbs, compared to HBsAg. The inserted $HBs_{(111-156)}$ sequence was presented in a different conformation which the MAbs against the *a* determinant could not recognise efficiently. These results are in accordance with those of poor reactivity of HBcAg fusion proteins with polyclonal anti-HBs antibody.

Monoclonal antibody binding studies in patients carrying the gly_{145} mutant showed that the HBsAg in the serum at 5 years of age, when anti-HBs induced by HBV vaccine was no longer present, bound significantly less to RFHBs-1, RFHBs-2, and RFHBs-7 MAbs than to the polyclonal anti-HBs (AUSRIA test) (Carman *et al.*, 1990). The reduction in binding of antibodies to the *a* determinant suggests that the mutation has resulted in a conformational change affecting more that one epitope in the *a* determinant. Since arg is much larger residue than gly and is charged, the hydrophilicity of this region is increased, and the HBs specificity may change. It has been shown that cys_{147} and pro_{142} are necessary for the display of full antigenicity (Ashton-Rickardt and Murray, 1989a). It is possible that the substitution of arg for gly may affect the presentation of the amino acids in the neighbourhood that contribute partly to the display of full antigenicity. It has also been demonstrated that accessibility of lys_{141} side chain is important for the presentation of the *a* determinant (Manivel *et al.*, 1992a). It is, therefore, possible that introduction of an amino acid with a bulky and positively charged side chain in the neighbourhood of lys_{141} may result in steric and electrostatic repulsion between the two side chains. Consequently, the lys_{141} side chain may remain buried or at least less accessible in the folded protein, leading to a reduction, or even loss of the *a* determinant. The buried side chains of the amino acids within the antigenic sites may also influence antigenicity as demonstrated in myohemerythrin protein (Alexander *et al.*, 1992).

Recently, Waters *et al.* (1992) demonstrated that the recombinant HBsAg with arginine at amino acid 145 of HBsAg reduced the antigenicity of the HBsAg in a manner similar to that seen in the child infected with $gly_{145} \rightarrow arg$ variant HBV. The anti-HBs found in the pooled sera of convalescent patients or vaccinees which bound to the wild-type HBsAg did not bind to the variant antigen. In addition, the binding of MAbs, RFHBs-1, RFHBs-2 or RFHBs-7, to the variant antigen was also abolished. RFHBs-2 bound to the variant antigen, but at a 10-fold higher concentration as compared to the wild-type, while RFHBs-1 and RFHBs-7 did not at all.

It is generally believed that the minimal requirement of antigenic sites in a native protein is the accessibility of the segment on the protein surface in order to be detected by antibodies binding the protein. Although accessibility is necessary, it is not known whether accessibility of a segment on a native protein surface is sufficient for antibody binding. Segmental mobility or local flexibility of the polypeptide backbone in portions of protein molecules may contribute to the antigenicity of these sites (Getzoff *et al.*, 1987; Geysen *et al.*, 1987). The mobility of a segment in a protein is the degree of atomic motion within the protein structure. Mobility data are only available from x-ray crystallographic or nuclear magnetic resonance studies and had been obtained only for segmental antigenic sites. A clear correlation between the mobility of a segment in a native protein and the ability of antibodies against short peptides corresponding to that segment to bind to the native protein has been demonstrated (Niman *et al.*, 1983). Reciprocally, antibodies (anti-native protein) to more mobile regions of native proteins are more likely to cross-react with short peptides than are antibodies to less mobile regions (Moudallal *et al.*, 1985). It is noteworthy that in these two approaches mobility is important in determining cross-reactivity of either anti-peptide antibodies to native proteins or anti-native protein antibodies to peptides, rather than in determining the immunogenic sites on native proteins.

It has been shown that epitopes fused to the C-terminus of the truncated HBcAg at amino acid residue 144 or at an internal site between amino acid residues 144 and 145

in full-length HBcAg were detected by antigen-capture sandwich ELISA or immunogold electron microscopy (Borisova *et al.*, 1989), thus suggesting external positioning. In contrast, a hybrid HBcAg particle carrying the epitope from simian immunodeficiency virus fused to the C-terminus of the full-length HBcAg failed to induce detectable anti-peptide antibody in guinea pigs (Yon *et al.*, 1992). Because the inserted epitope could not be detected by antigen-capture sandwich ELISA, it was suggested that the arginine-rich C-terminus of HBcAg prevented the foreign peptide from being presented on the surface of the hybrid HBcAg particle. Another explanation might be that the peptide was presented in a different conformation which the MAbs against the peptide could not recognise.

A variety of viral epitopes, eg. FMDV (Clarke *et al.*, 1987), poliovirus, rhinovirus (Clarke *et al.*, 1990) and simian immunodeficiency virus (Yon *et al.*, 1992) have been fused to the N-terminal region of HBcAg through part of the pre-core sequence and the hybrid HBcAg particles proved to be immunogenic in terms of the production of anti-peptide antibody.

In the studies described here, the antigenicity of the inserted $preS_1$ and $preS_2$ fragments in HBcAg fusion proteins carrying both sequences was not examined due to lack of the suitable antibodies. In immunoblotting experiments, $HBcPreS_{1(1-36)}$ (Stahl and Murray, 1989) reacted with anti-$preS_1$ MAb MA18/7 (Heermann *et al.*, 1984), while $HBcPreS_{1(1-20)}$ (Stahl and Murray, 1989) and $HBcPreS_1PreS_2$, containing $preS_{1(1-20)}$ and $preS_{2(1-26)}$ fragments, did not react (results not shown). These results are in agreement with the epitope mapping of MA18/7 which is against a linear epitope encompassing the $preS_{1(27-36)}$ sequence (Dienes *et al.*, 1990; Coursaget *et al.*, 1991). Since the HBcAg fusion proteins constructed in this work contain the $preS_{1(1-20)}$ sequence, MA18/7 could not be used for detection of the $preS_1$ epitope presented on the hybrid HBcAg particle. The anti-$preS_2$ MAb Q19/10 (Heermann *et al.*, 1984) available in this laboratory is against the glycosylated $preS_2$ region. Thus it also was not suitable for the detection of the $preS_2$ epitope derived from *E. coli.* Rabbits and mice immunised with these hybrid particles elicited anti-$preS_{1(1-23)}$ and anti-$preS_{2(1-26)}$

antibodies (see Chapter 5), suggesting that both $preS_{1(1-20)}$ and $preS_{2(1-26)}$ fragments were presented on the hybrid HBcAg particles.

The insertion site in the carrier molecules may influence surface exposure, and hence the immunogenicity of the inserted epitopes. In the HBsAg fusion systems, Delpeyroux and colleagues (1990) have employed two sites, between amino acid residues 50-51 and 113-114, within hydrophilic domains of HBsAg encompassing residues 45 through 72 and 110 through 145, respectively, to insert poliovirus epitopes. The PV-1 fragment, a poliovirus neutralisation epitope, was exposed at the surface of the hybrid HBsAg particles when inserted at position 113 of HBsAg particles, as demonstrated both by reactivity with the MAbs against PV-1 fragment and by trypsin cleavage experiments. In contrast, when inserted at position 50 of HBsAg, the inserted epitope was only poorly accessible. The more intriguing result was that a cooperative effect was observed when the fragment was inserted at both sites of the same HBsAg molecule. The PV-1 antigenicity of the hybrid HBsAg particle carrying the double insertions seemed higher than the expected sum from two hybrid particles carrying a single insertion at the different sites. These results suggested that structural modifications might occur in the carrier part of the hybrid molecule which improved the accessibility and/or conformation of one or both PV-1 fragments.

The periplasmic maltose-binding protein (MalE) from *E. coli* has also been exploited to express foreign peptides (Martineau *et al.*, 1992). It appeared that the antigenic properties of an inserted $preS_{2(13-26)}$ peptide and the neutralising epitope of poliovirus type 1 were different at the two insertion sites within the MalE proteins. Apart from the position of the inserted fragment on the carrier molecules, the intrinsic structural features of the inserted sequences may also influence the antigenicity of the inserted fragment within the hybrid molecules (Delpeyroux *et al.*, 1990; Martineau *et al.*, 1992). Thus, structural constraints might limit the presentation of some antigenic sequences.

FMDV was one of the earliest viruses in which a synthetic peptide approach was attempted for vaccine development. An antigenic fragment of the FMDV (amino acids 141 to 160 of protein VP1) was found to be antigenic as a synthetic peptide and as part of hybrid HBcAg particles (Clarke *et al.*, 1987). Analysis of the three-dimensional structure of the virus showed this $VP1_{(141-160)}$ fragment to be a disordered, flexible, prominent loop (Acharya *et al.*, 1989). These characteristics might allow it to retain antigenicity when expressed in various systems.

In conclusion, the HBcAg fusion proteins carrying the sequences from the $preS_1$, $preS_2$ and S regions at the N- and C-termini of the truncated HBcAg all retained HBc antigenicity. In addition, the HBs antigenicity was detectable in those carrying $HBs_{(111-156)}$ sequence, albeit at a lesser extent. The antigenic determinants within the $preS_1$ and $preS_2$ regions, which do not contain any cysteine residue, are conformation-independent and anti-$preS_1$ and anti-$preS_2$ antibodies were induced in the experimental animals (see Chapter 5), suggesting $preS_1$ and $preS_2$ segments were surface accessible in the HBcAg fusion proteins, and thus the $preS_1$ and $preS_2$ antigenicities were displayed in the hybrid particles. The presentation of conformational epitopes, such as the *a* determinant of HBsAg, to mimic the native epitope on the HBcAg particle appears to be more difficult than presentation of linear epitopes, such as $preS_1$ and $preS_2$ epitopes. However, the immunogenicity studies, discussed in Chapter 5, show that the HBcAg fusion proteins were immunogenic in terms of production of anti-HBs, anti-$preS_1$, and anti-$preS_2$ antibodies.

CHAPTER 5: IMMUNOLOGICAL RESPONSES TO HBcAg FUSION PROTEINS

5.1 Introduction

Two arms of immune response, cellular immune response and humoral immune response, were investigated in rabbits and mice after inoculation with the HBcAg fusion proteins. T-cell proliferative responses to the components of the HBcAg fusion proteins were monitored. Immunogenicity was determined in terms of the production of anti-HBc, anti-HBs and anti-peptide antibodies, which include anti-$preS_{1(1-23)}$, anti-$preS_{2(1-26)}$ and anti-$HBs_{(111-156)}$, in the immunised animals.

5.2 Immune responses of rabbits after inoculation with the HBcAg fusion proteins

Outbred Dutch rabbits were immunised intramuscularly with 0.2, 0.5, or 1 mg of HBcAg fusion proteins in CFA and boosted at least 2 times with the same or smaller doses in IFA at three-week intervals for the first boost and then 28 or 59 days later for the second. Some rabbits were further boosted at various time intervals. Blood samples were collected from each rabbit before and one to two weeks after each injection. Table 5.1 summarises the immunisation regimens for the rabbits.

5.2.1 Cellular immune responses to the HBcAg fusion proteins in rabbits

To determine whether T-cell responses to the components of the HBcAg fusion proteins could be detected in immunised rabbits, lymphocyte-proliferative responses were measured by using PBLs isolated from the rabbit before and after immunisation or boost at the various time intervals. The PBLs were cultured at a cell concentration of 2×10^5 cells/well (for R675, R676, R677, R678, R684 and R685) or 5×10^5 cells/well (for the remaining rabbits) in 96-well microtitre plates in the presence of various antigens for 5 days and their proliferation was measured by $[^3H]$-thymidine incorporation.

Table 5.1 Immunisation regimens of rabbits [Numbers designate days of treatment and those in parentheses designate dose (mg)]

Rabbit	Immunogen	Immunisation	1st boost	2nd boost	3rd boost	4th boost	5th boost	6th boost
R675	HBcS	0 (0.2)	21 (0.2)	80 (0.2)	108 (0.2)	287 (0.2)	315 (0.2)	421 (0.4)
R676	$HBcS_{145R}$	0 (0.2)	21 (0.2)	80 (0.2)	(108 (0.2)	287 (0.2)	315 (0.2)	421 (0.4)
R677	$HBcS_{145E}$	0 (0.2)	21 (0.2)	80 (0.2)	108 (0.2)	287 (0.2)	315 (0.2)	
R678	$HBcS_{145K}$	0 (0.2)	21 (0.2)	80 (0.2)	108 (0.2)	287 (0.2)	421 (0.4)	
R684	HBcS	0 (1)	21 (1)	49 (1)	208 (0.2)	236 (0.2)		
R685	$HBcS_{145R}$	0 (1)	21 (1)	49 (1)	208 (0.2)	236 (0.2)		
R690	$HBcPreS_1PreS_2S$	0 (1)	21 (1)	49 (0.2)	88 (0.2)	137 (0.2)	194 (0.2)	
R697	$HBcPreS_1PreS_2S$	0 (0.5)	21 (0.5)	Sacrificed on day 48 due to long, growing teeth				

Continued ...

Table 5.1 (continued)

Rabbit	Immunogen	Immunisation	1st boost	2nd boost	3rd boost	4th boost	5th boost	6th boost
R691	$HBcPreS_1PreS_2S_{145R}$	0 (1)	21 (1)	Died on day 32				
R692	$HBcPreS_1PreS_2S_{145R}$	0 (0.5)	21 (0.5)	49 (0.2)	98 (0.2)	155 (0.2)		
R699	$HBcPreS_1PreS_2S_{145E}$	0 (0.5)	21 (0.5)	49 (0.2)				
R700	$HBcPreS_1PreS_2S_{145E}$	0 (0.5)	21 (0.5)	49 (0.2)				
R698	$HBcSPreS_1PreS_2S_{145R}$	0 (0.5)	21 (0.5)	49 (0.2)	106 (0.2)			
R703	$HBcSPreS_1PreS_2S_{145R}$	0 (0.5)	22 (0.5)	50 (0.2)				
R702	$HBcSPreS_1PreS_2S_{145E}$	0 (0.5)	22 (0.5)	50 (0.2)				
R701	denatured HBsAg	0 (0.01)	22 (0.01)	50 (0.01)	78 (0.004)			

Representative dose-response curves obtained 13 days after immunisation (day 13) and 13 days after the second boost (day 93) with 0.2 mg $HBcS_{145E}$ in R677 are shown in Figure 5.1. The PBLs from day 13 incorporated 17.4 times and 6.7 times more [^{3}H]-thymidine in response to stimulation with an optimal concentration of $HBcS_{145E}$ (16 μg/ml) and HBcAg (16 μg/ml), respectively, than did non-stimulated PBLs (Figure 5.1a). However, the PBLs did not respond to stimulation with HBsAg at any concentration. In response to $HBcS_{145E}$ (64 μg/ml) and HBcAg (4 μg/ml), the PBLs obtained on day 93 incorporated 101 times and 26 times more [^{3}H]-thymidine than did non-stimulated PBL (Figure 5.1b). Moreover, the PBLs also proliferated in response to stimulation with HBsAg, albeit to a much lesser extent. At an optimal concentration of HBsAg (16 μg/ml), they incorporated 18 times more [^{3}H]-thymidine than did nonstimulated PBLs. No *in vitro* stimulation of the PBLs from R677 was observed with HBcAg fusion protein, HBcAg or HBsAg before immunisation (Figure 5.2c).

The proliferation of PBLs in response to the antigens was monitored in each rabbit. Figure 5.2 summarises the kinetics of the lymphocyte proliferation in response to the optimal doses of the antigens in R675, R676, R677, R678, R684 and R685 immunised and boosted with either HBcS or its gly_{145} mutants. The magnitude of the lymphocyte proliferation in response to HBcAg fusion protein, HBcAg and HBsAg varied among different animals. The lymphocyte-proliferative response to HBcAg fusion protein and HBcAg appeared 7 days after immunisation and maintained but fluctuated during the experiments. In most rabbits, the proliferative responses to HBcAg fusion protein and HBcAg could be greatly augmented after the boosts in those rabbits with declined responses. A weak and delayed specific proliferative response to HBsAg was observed in some rabbits only after the boost. Comparison of proliferative response to the antigens showed that R684 and R685 elicited higher T-cell response than R675, R676, R677 and R678. However, it should be noted that the HBcAg fusion protein used for immunisation and boost of the former were four times the amount compared with that used for the latter. The data from days 49 and 61 of R684 show enormous increases of S.I. in response to HBcAg fusion protein, HBcAg or HBsAg. However,

Figure 5.1 Lymphocyte-proliferative responses in R677 immunised with $HBcS_{145E}$ on (a) day 13 and (b) on day 93, i.e. 13 days after the second boost. The PBLs were cultured with HBcAg, HBsAg or $HBcS_{145E}$ at different concentrations for 5 days. Sixteen hours before harvest, the cultures were pulsed with [^{3}H]-thymidine and the [^{3}H]-thymidine incorporated into the stimulated cells was determined by liquid scintillation counting (mean ± S.D.).

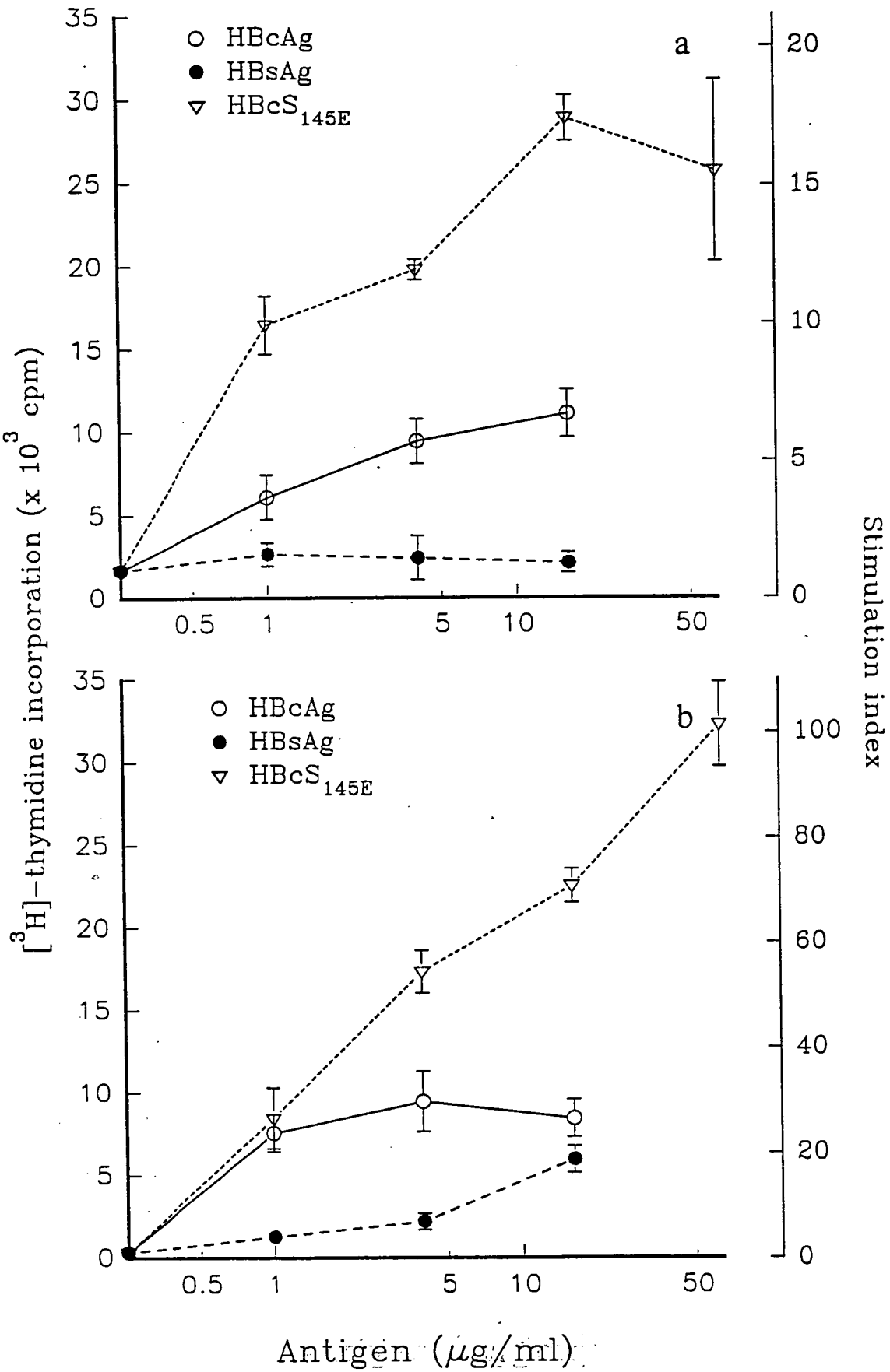
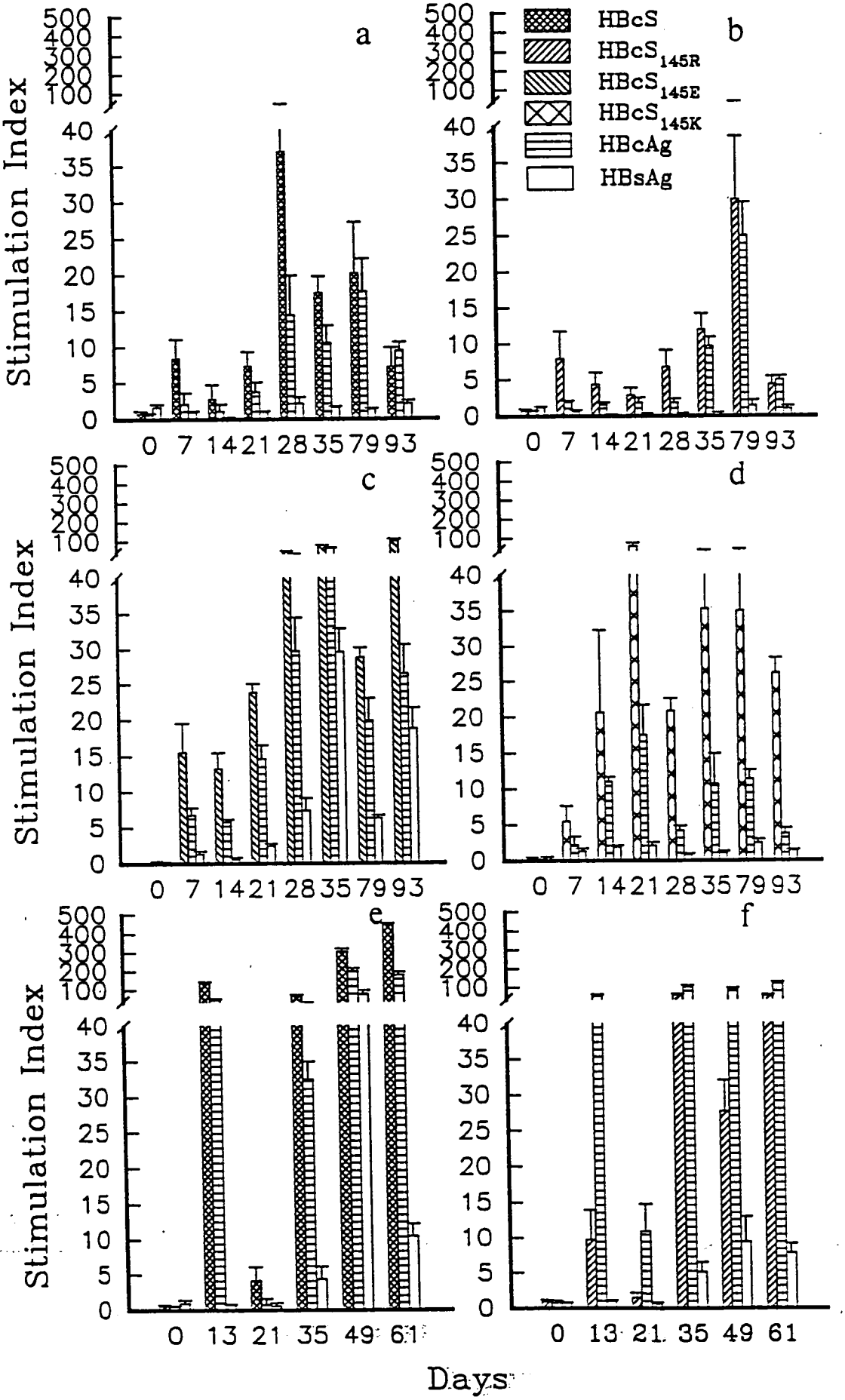

a
HBcAg
HBsAg
HBcS145E
b
HBcAg
HBsAg
HBcS145E
[3H]-thymidine incorporation (x 10^3 cpm)
Stimulation index
Antigen (μg/ml)
0
5
10
15
20
25
30
35
0.5
1
5
10
50
40
60
80
100

Figure 5.2 Kinetics of lymphocyte-proliferative responses in 6 rabbits immunised with HBcS, $HBcS_{145R}$, $HBcS_{145E}$ or $HBcS_{145K}$. (a) R675, (b) 676, (c) R677, and (d) R678 were injected with HBcS, $HBcS_{145R}$, $HBcS_{145E}$, and $HBcS_{145K}$, respectively, on days 0, 21 and 81 etc. (e) R684, and (f) R685 were injected with HBcS and $HBcS_{145R}$, respectively, on days 0, 21 and 49 etc. The PBLs at the indicated days were cultured with the corresponding HBcAg fusion protein (64 μg/ml), HBcAg (16 μg/ml), or HBsAg (16 μg/ml) for 5 days. [^{3}H]-thymidine incorporation in response to these antigens was monitored as described. The stimulation index was calculated by dividing the cpm of [^{3}H]-thymidine incorporated into stimulated cells by the cpm incorporated into nonstimulated cells (mean ± S.D.).

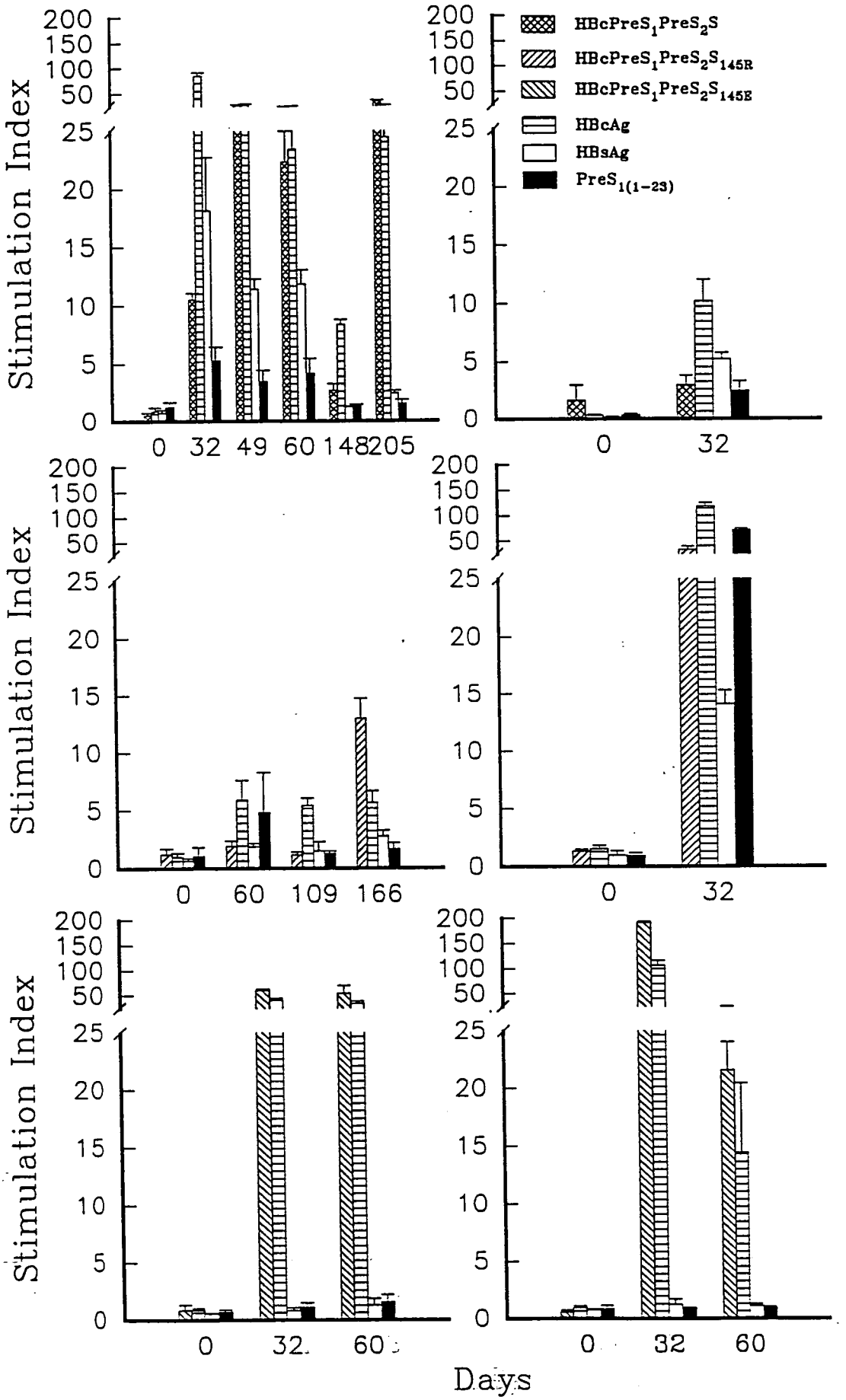

HBcS
HBcS145R
HBcS145E
HBcS145K
HBcAg
HBsAg
a
b
c
d
e
f
Stimulation Index
Stimulation Index
Stimulation Index
0 7 14 21 28 35 79 93
0 7 14 21 28 35 79 93
0 7 14 21 28 35 79 93
0 7 14 21 28 35 79 93
0 13 21 35 49 61
0 13 21 35 49 61
Days

the PBLs from R684 also responded to poly (Na glutamate-alanine-tyrosine), an unrelated polypeptide used to monitor non-specific T-cell activation, with S.I. of 15.9 and 47.2 on days 49 and 61, respectively. Thus, R684 may have had non-specific T-cell activation on these two occasions.

In the following experiments, rabbits were immunised and boosted with the HBcAg fusion proteins containing $preS_1$ and $preS_2$ epitopes as well as either a monomer or a dimer of the $HBs_{(111-156)}$ fragment. The PBLs isolated from the animals were cultured at a cell concentration of 5×10^5 cells/well in the presence of various antigens. The optimal concentrations of HBcAg fusion protein (64 μg/ml) and HBcAg (16 μg/ml) produced only a small increase in proliferation, or even lower proliferation, presumably due to over-proliferation of the cells resulting in nutrient deprivation and thus leading to cell death. Thus, in the following experiments, the maximal concentrations of the antigens used *in vitro* were 16 μg/ml of HBcAg fusion protein and 4 μg/ml of HBcAg. The results in Figure 5.3 expressed as S.I. show the kinetics of lymphocyte proliferation in response to stimulation with HBcAg fusion protein, HBcAg, HBsAg and $preS_{1(1-23)}$ peptide in the rabbits immunised and subsequently boosted with $HBcPreS_1PreS_2S$, either wild-type or gly_{145} mutants. The maximal proliferative responses of the PBLs from R690 to HBcAg, HBsAg and $preS_{1(1-23)}$ peptide occurred after the first boost, except to HBcAg fusion protein in which the highest response developed at a later stage. The proliferative response could be elicited following several boosts and maintained for longer than 6 months (Figure 5.3a).

No *in vitro* stimulation of the PBLs from these rabbits was observed with the antigens before immunisation. After immunisation, the response varied considerably from rabbit to rabbit. There were significant increases in lymphocyte proliferation in response to HBcAg and HBcAg fusion protein in all rabbits except R692 in which a weak proliferation was found on days 60 and 109, which corresponded to 11 days after the second and third boosts, respectively. In R692, IFA, rather than CFA, was used as the adjuvant for immunisation by mistake, which might contribute to the weak

Figure 5.3 Kinetics of lymphocyte-proliferative responses in 6 rabbits immunised with HBcPreS$_1$PreS$_2$S, HBcPreS$_1$PreS$_2$S$_{145R}$ or HBcPreS$_1$PreS$_2$S$_{145E}$. (a) R690 was injected with HBcPreS$_1$PreS$_2$S on days 0, 21, 49, 88, 137 and 194. (b) R697 was injected with HBcPreS$_1$PreS$_2$S on days 0 and 21. (c) R692 was injected with HBcPreS$_1$PreS$_2$S$_{145R}$ on days 0, 21, 49, 98 and 155. (d) R691 was injected with HBcPreS$_1$PreS$_2$S$_{145R}$ on days 0 and 21. (e) R699 and (f) R700 were injected with HBcPreS$_1$PreS$_2$S$_{145E}$ on days 0, 21 and 49. The PBLs at the indicated days were cultured with the corresponding HBcAg fusion protein (16 μg/ml), HBcAg (4 μg/ml), HBsAg (16 μg/ml) or preS$_{1(1-23)}$ synthetic peptide (100 μg/ml) for 5 days. [^{3}H]-thymidine incorporation in response to these antigens was monitored as described. The stimulation index was calculated by dividing the cpm of [^{3}H]-thymidine incorporated into stimulated cells by the cpm incorporated into nonstimulated cells (mean ± S.D.).

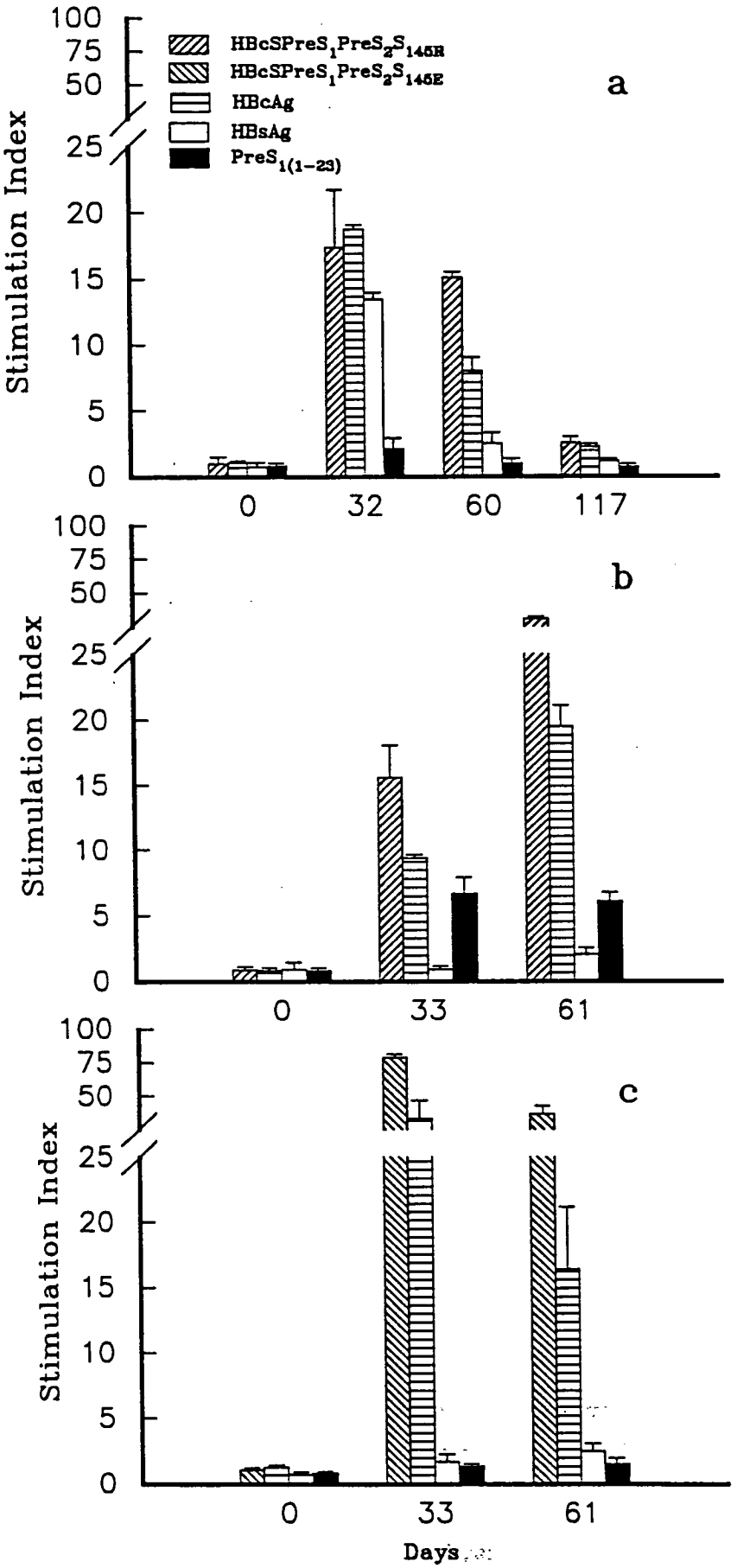

Stimulation Index
200
150
100
50
25
20
15
10
5
0
0 32 49 60 148 205
0 32
0 60 109 166
0 32
0 32 60
0 32 60
Days
HBcPreS1PreS2S
HBcPreS1PreS2S145R
HBcPreS1PreS2S145E
HBcAg
HBsAg
PreS1(1-23)

lymphocyte proliferation (Figure 5.3c). Some rabbits responded to HBsAg or $preS_{1(1-23)}$ peptide, among which R691 showed the highest response (Figure 5.3d). Due to the unavailability of $preS_{2(1-26)}$ peptide until the later stage of the experiments, only a limited number of PBL samples were tested in response to $preS_{2(1-26)}$ peptide and no proliferative response was found (data not shown).

The results of lymphocyte proliferation in response to various antigens in the rabbits immunised with HBcAg fusion protein carrying a dimer of $HBs_{(111-156)}$ sequence, $HBcSPreS_1PreS_2S_{145R}$ in R698 and R703 or $HBcSPreS_1PreS_2S_{145E}$ in R702, are given in Figure 5.4. Similar to the results in Figure 5.3, the PBLs of the three rabbits proliferated in response to stimulation with HBcAg or HBcAg fusion protein. In comparison with the response to HBsAg, the PBLs from R698 showed the highest response (Figure 5.4a) and those from R703 and R702 showed only marginal responses (Figures 5.4b and 5.4c). In regard to $preS_{1(1-23)}$ and $preS_{2(1-26)}$ peptides, only the PBLs from R703 responded to $preS_{1(1-23)}$ peptide (Figure 5.4b), but no response was found to $preS_{2(1-26)}$ peptide (data not shown).

In the rabbit experiments, R701 was exclusively immunised and boosted with 10 μg of SDS-denatured HBsAg, rather than HBcAg fusion protein, for the preparation of anti-denatured HBs antibody. The PBLs isolated 11 days after the first and second boosts proliferated in response to stimulation with HBsAg at concentrations of 4 μg/ml (S.I. = 5.0 and 6.0, respectively) and 16 μg/ml (S.I. = 5.6 and 8.2, respectively) (Figure 5.5). The HBsAg-specific T-cell response in R701 was lower than HBcAg-specific T-cell response in those immunised with the HBcAg fusion proteins. Similarly, the HBsAg-specific T-cell response was also consistently lower than the HBcAg-specific response in the rabbits immunised with HBcAg fusion proteins (Figures 5.2 to 5.4). Cumulatively, the results are in agreement with previous data indicating that HBcAg was more immunogenic than HBsAg in terms of T-cell activation in mice (Milich *et al.*, 1987b).

Figure 5.4 Kinetics of lymphocyte-proliferative responses in 3 rabbits immunised with HBcSPreS$_1$PreS$_2$S$_{145R}$ or HBcSPreS$_1$PreS$_2$S$_{145E}$. (a) R698 was injected with HBcSPreS$_1$PreS$_2$S$_{145R}$ on days 0, 21, 49 and 106. (b) R703 was injected with HBcSPreS$_1$PreS$_2$S$_{145R}$ on days 0, 22 and 50. (c) R702 was injected with HBcSPreS$_1$PreS$_2$S$_{145E}$ on days 0, 22 and 50. The PBLs at the indicated days were cultured with the corresponding HBcAg fusion protein (16 μg/ml), HBcAg (4 μg/ml), HBsAg (16 μg/ml) or preS$_{1(1-23)}$ synthetic peptide (100 μg/ml) for 5 days. [^{3}H]-thymidine incorporation in response to these antigens was monitored as described. The stimulation index was calculated by dividing the cpm of [^{3}H]-thymidine incorporated into stimulated cells by the cpm incorporated into nonstimulated cells (mean ± S.D.).


HBcSPreS1PreS2S145R
HBcSPreS1PreS2S145E
HBcAg
HBsAg
PreS1(1-23)
a
b
c
Stimulation Index
100
75
50
25
20
15
10
5
0
0
32
60
117
33
61
Days

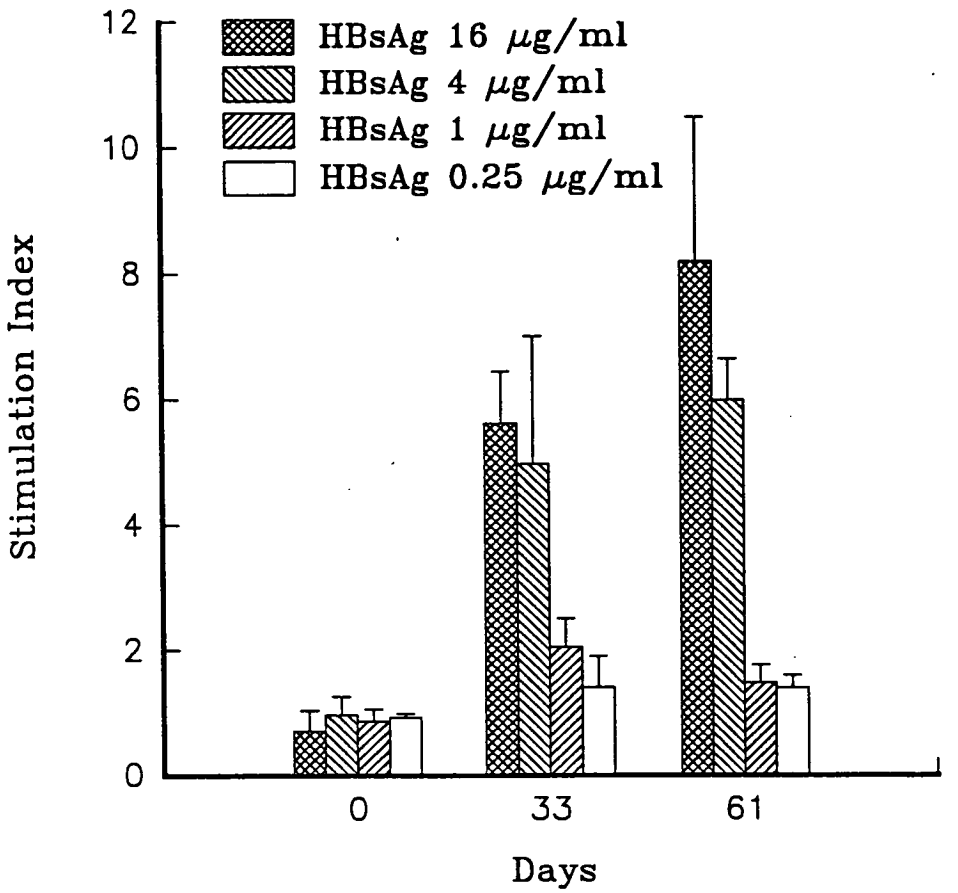


Figure 5.5 Kinetics of lymphocyte-proliferative responses in R701 injected with SDS-denatured HBsAg on days 0, 22 and 50. The PBLs at the indicated days were cultured with various concentrations of native HBsAg for 5 days. [^{3}H]-thymidine incorporation in response to the antigen was monitored as described. The stimulation index was calculated by dividing the cpm of [^{3}H]-thymidine incorporated into stimulated cells by the cpm incorporated into nonstimulated cells (mean ± S.D.).

In summary, the results of the lymphocyte proliferation measurements indicate that immunisation of rabbits with the HBcAg fusion proteins carrying the $HBs_{(111-156)}$ fragment with or without $preS_1$ and $preS_2$ fragments elicited high T-cell proliferative responses to HBcAg and HBcAg fusion proteins in all rabbits, while a moderate to low response to HBsAg was observed in most rabbits, with variable responses to $preS_{1(1-23)}$ (5 out of 9 rabbits with S.I. between 2.4 and 71.7), and no response to the $preS_{2(1-26)}$ peptide. However, there was considerable variation in the T-cell proliferative responses among the rabbits. Comparison of R684 and R685 (Figures 5.2e and 5.2f) with R675, R676, R677 and R678 (Figures 5.2a to 5.2d), in which the former two rabbits received 4 times more antigen than the latter, indicates that the doses of immunisation and boost might influence the T-cell proliferative responses to HBcAg, HBcAg fusion protein and HBsAg, as higher responses were found in R684 and R685. Moreover, comparison of R690 and R691 (Figures 5.3a and 5.3d) with R684 and R685 (Figures 5.2e and 5.2f), in which the former received $HBcPreS_1PreS_2S$ and the latter received HBcS, but with the same dose, suggests the inclusion of $preS_1$ and $preS_2$ regions in the HBcAg fusion protein might enhance the HBsAg-specific T-cell response. Since lymphocyte-proliferative responses to HBsAg and $preS_{1(1-23)}$ in the rabbits immunised with $HBcSPreS_1PreS_2S_{145R}$ or $HBcSPreS_1PreS_2S_{145E}$ varied (Figure 5.4), it was not possible to determine the influence of the number of the $HBs_{(111-156)}$ fragments, ie. a monomer or a dimer, on the HBsAg- or $preS_1$-specific T-cell response.

5.2.2 Humoral immune responses to the HBcAg fusion proteins in rabbits

Blood samples were taken from immunised rabbits at regular intervals, 7 to 14 days after immunisation and each boost, and sera were assessed for the presence of anti-HBc, anti-HBs and anti-peptide antibodies. Tables 5.2 to 5.16 summarise the humoral immune responses of the rabbits to the HBcAg fusion proteins and Table 5.17 shows anti-HBs response in R701 which was immunised with SDS- and DTT-denatured HBsAg.

Table 5.2 Antibody responses of R675 to HBcS

Treatment[a]	Days postimmunisation	Anti-HBc titre[b]	Anti-HBs		
			DARIP (%)[c]		AUSAB[d] (RIA unit)
			adw	*ayw*	
Immunisation	0	0	5.7	27.6	92
	14	20	8.0	ND[e]	54
1st boost on day 21	35	1280	41.8	ND	92
2nd boost on day 79	93	5120	57.8	ND	92
3rd boost on day 108	120	5120	57.9	46.1	92
4th boost on day 287	297	2560	ND	42.9	36
5th boost	315	2560	ND	36.8	ND
	322	5120	ND	48.1	ND
	329	5120	ND	50.4	54
6th boost on day 421	435	2560	ND	7.5	0

a Rabbit 675 was immunised with 0.2 mg of HBcS and boosted 6 times on the indicated days with the same dose of antigen except for the 6th boost in which 0.4 mg was given.
b Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.
c Anti-HBs was determined by the DARIP test using either *adw* or *ayw* subtype of ^{125}I-labelled HBsAg and the titres are expressed as % of ^{125}I-HBsAg co-precipitated with anti-HBs and anti-rabbit IgG.
d Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.
e Not determined.

Table 5.3 Antibody responses of R676 to $HBcS_{145R}$

Treatment[a]	Days postimmunisation	Anti-HBc titre[b]	Anti-HBs		
			DARIP (%)[c]		AUSAB[d] (RIA unit)
			adw	*ayw*	
Immunisation	0	0	7.5	9.8	8
	14	160	8.4	ND[e]	16
1st boost on day 21	35	5120	29.7	ND	54
2nd boost on day 79	93	20480	38.2	ND	16
3rd boost on day 108	120	20480	35.6	33.1	8
4th boost on day 287	297	10240	ND	43.9	36
5th boost	315	10240	ND	34.9	ND
	322	10240	ND	37.1	ND
	329	10240	ND	39.8	16
6th boost on day 421	435	10240	ND	47.5	54

a Rabbit 676 was immunised with 0.2 mg of $HBcS_{145R}$ and boosted 6 times on the indicated days with the same dose of antigen except for the 6th boost in which 0.4 mg was given.
b Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.
c Anti-HBs was determined by the DARIP test using either *adw* or *ayw* subtype of ^{125}I-labelled HBsAg and the titres are expressed as % of ^{125}I-HBsAg co-precipitated with anti-HBs and anti-rabbit IgG.
d Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.
e Not determined.

Table 5.4 Antibody responses of R677 to $HBcS_{145E}$

Treatment[a]	Days postimmunisation	Anti-HBc titre[b]	Anti-HBs		
			DARIP (%)[c]		AUSAB[d] (RIA unit)
			adw	*ayw*	
Immunisation	0	0	5.3	10.8	8
	14	160	4.7	ND[e]	8
1st boost on day 21	35	2560	7.4	ND	8
2nd boost on day 79	93	20480	13.9	ND	16
3rd boost on day 108	120	20480	14.0	24.3	36
4th boost on day 287	297	5120	ND	16.3	54
5th boost	315	10240	ND	9.7	ND
	322	5120	ND	17.9	ND
	329	10240	ND	30.0	72

[a] Rabbit 677 was immunised with 0.2 mg of $HBcS_{145E}$ and boosted 5 times on the indicated days with the same dose of antigen.
[b] Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.
[c] Anti-HBs was determined by the DARIP test using either *adw* or *ayw* subtype of ^{125}I-labelled HBsAg and the titres are expressed as % of ^{125}I-HBsAg co-precipitated with anti-HBs and anti-rabbit IgG.
[d] Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.
[e] Not determined.

Table 5.5 Antibody responses of R678 to $HBcS_{145K}$

Treatment[a]	Days postimmunisation	Anti-HBc titre[b]	Anti-HBs		
			DARIP (%)[c]		AUSAB[d] (RIA unit)
			adw	*ayw*	
Immunisation	0	0	3.9	9.3	0
	14	40	4.4	ND[e]	ND
1st boost on day 21	35	640	36	ND	ND
2nd boost on day 79	93	5120	6.8	ND	ND
3rd boost on day 108	120	10240	6.3	12.3	0
4th boost on day 287	297	2560	ND	13.3	ND
5th boost on day 421	435	5120	ND	58.3	54

[a] Rabbit 678 was immunised with 0.2 mg of $HBcS_{145K}$ and boosted 5 times on the indicated days with the same dose of antigen except for the 5th boost in which 0.4 mg was given.

[b] Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.

[c] Anti-HBs was determined by the DARIP test using either *adw* or *ayw* subtype of ^{125}I-labelled HBsAg and the titres are expressed as % of ^{125}I-HBsAg co-precipitated with anti-HBs and anti-rabbit IgG.

[d] Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.

[e] Not determined.

Table 5.6 Antibody responses of R684 to HBcS

Treatment[a]	Days postimmunisation	Anti-HBc titre[b]	Anti-HBs		
			DARIP (%)[c]		AUSAB[d] (RIA unit)
			adw	*ayw*	
Immunisation	0	0	4.2	8.7	0
	13	320	4.0	ND[e]	0
1st boost	21	1280	6.2	ND	ND
	35	2560	48.6	ND	92
2nd boost	49	10240	ND	ND	ND
	61	40960	ND	42.4	54
3rd boost on day 208	218	20480	ND	59.0	72
4th boost	236	20480	ND	52.8	16
	243	20480	ND	48.9	8
	250	20480	ND	51.9	8

[a] Rabbit 684 was immunised with 1 mg of HBcS and boosted 4 times on the indicated days with 1 mg of antigen in the first two boosts and 0.2 mg in the rest.
[b] Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.
[c] Anti-HBs was determined by the DARIP test using either *adw* or *ayw* subtype of ^{125}I-labelled HBsAg and the titres are expressed as % of ^{125}I-HBsAg co-precipitated with anti-HBs and anti-rabbit IgG.
[d] Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.
[e] Not determined.

Table 5.7 Antibody responses of R685 to $HBcS_{145R}$

Treatment[a]	Days postimmunisation	Anti-HBc titre[b]	Anti-HBs		
			DARIP (%)[c]		AUSAB[d] (RIA unit)
			adw	*ayw*	
Immunisation	0	0	4.6	10.5	0
	13	320	6.4	ND[e]	0
1st boost	21	1280	14.2	ND	ND
	35	5120	63.0	ND	36
2nd boost	49	20480	ND	ND	ND
	61	40960	ND	63.8	36
3rd boost on day 208	218	10240	ND	68.7	72
4th boost	236	10240	ND	64.4	36
	243	10240	ND	74.7	54
	250	10240	ND	80.9	72

[a] Rabbit 685 was immunised with 1 mg of $HBcS_{145R}$ and boosted 4 times on the indicated days with 1 mg of antigen in the first two boosts and 0.2 mg in the rest.
[b] Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.
[c] Anti-HBs was determined by the DARIP test using either *adw* or *ayw* subtype of ^{125}I-labelled HBsAg and the titres are expressed as % of ^{125}I-HBsAg co-precipitated with anti-HBs and anti-rabbit IgG.
[d] Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.
[e] Not determined.

Table 5.8 Antibody responses of R690 to $HBcPreS_1PreS_2S$

Treatment[a]	Days postimmunisation	Anti-HBc titre[b]	Anti-HBs		Anti-peptide (P/N ratio)[e]		
			DARIP[c] (%)	AUSAB[d] (RIA unit)	$HBs_{(111-156)}$	$PreS_{1(1-23)}$	$PreS_{2(1-26)}$
Immunisation	0	0	8.6	0	0.7	0.4	0.4
1st boost	21	1280	23.6	262	ND[f]	4.2	1.0
	32	2560	75.1	352	12.8	10.8	7.6
2nd boost	49	10240	80.8	ND	ND	12.5	14.7
	60	10240	81.6	292	ND	12.4	13.4
3rd boost	88	20480	60.6	ND	ND	12.5	10.5
	99	20480	52.8	112	ND	12.3	8.8
4th boost on day 137	149	10240	73.2	112	ND	10.2	9.0
5th boost on day 194	205	5120	86.5	72	13.8	6.6	10.9

[a] Rabbit 690 was immunised with 1 mg of $HBcPreS_1PreS_2S$ and boosted 5 times on the indicated days with 1 mg of antigen in the first boost and 0.2 mg in the rest.

[b] Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.

[c] Anti-HBs was determined by the DARIP test using *ayw* subtype of ^{125}I-labelled HBsAg and the titres are expressed as % of ^{125}I-HBsAg co-precipitated with anti-HBs and anti-rabbit IgG.

[d] Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.

[e] Anti-peptide antibodies were determined by the indirect antibody RIA in which the indicated synthetic peptides were used as the solid-phase ligand and the titres are expressed as P/N ratio which represents cpm ratio of samples measured (P) and negative control (N).

[f] Not determined.

Table 5.9 Antibody responses of R697 to $HBcPreS_1PreS_2S$

Treatment[a]	Days postimmunisation	Anti-HBc titre[b]	Anti-HBs		Anti-peptide (P/N ratio)[e]		
			DARIP[c] (%)	AUSAB[d] (RIA unit)	$HBs_{(111-156)}$	$PreS_{1(1-23)}$	$PreS_{2(1-26)}$
Immunisation	0	0	12.0	54	1.0	0.9	1.9
1st boost	21	1280	15.8	72	ND[f]	3.3	2.2
	32	2560	51.7	262	ND	9.5	7.1
	48	2560	68.1	ND	6.8	3.7	12.3

[a] Rabbit 697 was immunised and boosted once with 0.5 mg of $HBcPreS_1PreS_2S$. The rabbit was sacrificed on day 48 due to long, growing teeth.

[b] Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.

[c] Anti-HBs was determined by the DARIP test using *ayw* subtype of ^{125}I-labelled HBsAg and the titres are expressed as % of ^{125}I-HBsAg co-precipitated with anti-HBs and anti-rabbit IgG.

[d] Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.

[e] Anti-peptide antibodies were determined by the indirect antibody RIA in which the indicated synthetic peptides were used as the solid-phase ligand and the titres are expressed as P/N ratio which represents cpm ratio of samples measured (P) and negative control (N).

[f] Not determined.

Table 5.10 Antibody responses of R691 to $HBcPreS_1PreS_2S_{145R}$

Treatment[a]	Days postimmunisation	Anti-HBc titre[b]	Anti-HBs		Anti-peptide (P/N ratio)[e]		
			DARIP[c] (%)	AUSAB[d] (RIA unit)	$HBs_{(111-156)}$	$PreS_{1(1-23)}$	$PreS_{2(1-26)}$
Immunisation	0	0	14.2	0	0.8	0.7	1.0
1st boost	21	1280	49.3	16	ND[f]	2.2	15.9
	32	2560	80.6	36	10.5	3.0	11.5

a Rabbit 691 was immunised and boosted once with 1 mg of $HBcPreS_1PreS_2S_{145R}$. The rabbit died on day 32.

b Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.

c Anti-HBs was determined by the DARIP test using *ayw* subtype of ^{125}I-HBsAg and the titres are expressed as % of ^{125}I-HBsAg co-precipitated with anti-HBs and anti-rabbit IgG.

d Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.

e Anti-peptide antibodies were determined by the indirect antibody RIA in which the indicated synthetic peptides were used as the solid-phase ligand and the titres are expressed as P/N ratio which represents cpm ratio of samples measured (P) and negative control (N).

f Not determined.

Table 5.11 Antibody responses of R692 to $HBcPreS_1PreS_2S_{145R}$

Treatment[a]	Days postimmunisation	Anti-HBc titre[b]	Anti-HBs		Anti-peptide (P/N ratio)[e]		
			DARIP[c] (%)	AUSAB[d] (RIA unit)	$HBs_{(111-156)}$	$PreS_{1(1-23)}$	$PreS_{2(1-26)}$
Immunisation	0	0	10.4	8	5.5	0.6	0.7
1st boost	21	640	ND	54	ND[f]	0.8	1.2
	32	5120	73.3	54	ND	3.4	7.8
2nd boost	49	20480	73.1	ND	ND	1.4	1.8
	60	40960	74.4	72	ND	1.3	2.1
3rd boost on day 98	109	5120	74.9	36	ND	1.1	1.1
4th boost on day 155	166	10240	40.4	36	7.0	1.6	1.0

a Rabbit 692 was immunised with 0.5 mg of $HBcPreS_1PreS_2S_{145R}$ and boosted 4 times on the indicated days with 0.2 mg of antigen except for the 1st boost in which 0.5 mg was given.

b Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.

c Anti-HBs was determined by the DARIP test using *ayw* subtype of ^{125}I-HBsAg and the titres are expressed as % of ^{125}I-HBsAg co-precipitated with anti-HBs and anti-rabbit IgG.

d Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.

e Anti-peptide antibodies were determined by the indirect antibody RIA in which the indicated synthetic peptides were used as the solid-phase ligand and the titres are expressed as P/N ratio which represents cpm ratio of samples measured (P) and negative control (N).

f Not determined.

Table 5.12 Antibody responses of R699 to $HBcPreS_1PreS_2S_{145E}$

Treatment[a]	Days postimmunisation	Anti-HBc titre[b]	Anti-HBs		Anti-peptide (P/N ratio)[e]		
			DARIP[c] (%)	AUSAB[d] (RIA unit)	$HBs_{(111-156)}$	$PreS_{1(1-23)}$	$PreS_{2(1-26)}$
Immunisation	0	0	7.4	0	0.9	0.6	0.7
1st boost	21	1280	63.8	36	ND[f]	2.1	1.2
	32	1280	67.8	16	ND	7.4	1.4
2nd boost	49	1280	ND	ND	ND	10.6	2.5
	60	2560	62.3	16	10.7	14.1	2.4

[a] Rabbit 699 was immunised with 0.5 mg of $HBcPreS_1PreS_2S_{145E}$ and boosted twice on the indicated days with 0.5 mg and 0.2 mg of antigen, respectively.

[b] Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.

[c] Anti-HBs was determined by the DARIP test using *ayw* subtype of ^{125}I-labelled HBsAg and the titres are expressed as % of ^{125}I-HBsAg co-precipitated with anti-HBs and anti-rabbit IgG.

[d] Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.

[e] Anti-peptide antibodies were determined by the indirect antibody RIA in which the indicated synthetic peptides were used as the solid-phase ligand and the titres are expressed as P/N ratio which represents cpm ratio of samples measured (P) and negative control (N).

[f] Not determined.

Table 5.13 Antibody responses of R700 to $HBcPreS_1PreS_2S_{145E}$

Treatment[a]	Days postimmunisation	Anti-HBc titre[b]	Anti-HBs		Anti-peptide (P/N ratio)[e]		
			DARIP[c] (%)	AUSAB[d] (RIA unit)	$HBs_{(111-156)}$	$PreS_{1(1-23)}$	$PreS_{2(1-26)}$
Immunisation	0	0	11.9	8	2.7	0.7	1.0
1st boost	21	1280	47.3	36	ND[f]	1.1	1.2
	32	2560	68.2	36	ND	3.7	2.5
2nd boost	49	2560	ND	ND	ND	6.4	7.4
	60	2560	67.8	72	9.1	7.4	13.1

a Rabbit 700 was immunised with 0.5 mg of $HBcPreS_1PreS_2S_{145E}$ and boosted twice on the indicated days with 0.5 mg and 0.2 mg of antigen, respectively.

b Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.

c Anti-HBs was determined by the DARIP test using *ayw* subtype of ^{125}I-labelled HBsAg and the titres are expressed as % of ^{125}I-HBsAg co-precipitated with anti-HBs and anti-rabbit IgG.

d Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.

e Anti-peptide antibodies were determined by the indirect antibody RIA in which the indicated synthetic peptides were used as the solid-phase ligand and the titres are expressed as P/N ratio which represents cpm ratio of samples measured (P) and negative control (N).

f Not determined.

Table 5.14 Antibody responses of R698 to HBcSPreS$_1$PreS$_2$S$_{145R}$

Treatment[a]	Days postimmunisation	Anti-HBc titre[b]	Anti-HBs		Anti-peptide (P/N ratio)[e]		
			DARIP[c] (%)	AUSAB[d] (RIA unit)	$HBs_{(111-156)}$	$PreS_{1(1-23)}$	$PreS_{2(1-26)}$
Immunisation	0	0	13.9	8	1.0	1.8	1.7
1st boost	21	160	7.8	54	ND[f]	0.5	0.8
	32	2560	15.8	54	ND	1.8	3.5
2nd boost	49	10240	35.4	ND	ND	11.3	18.3
	60	10240	36.6	8	ND	4.0	6.2
3rd boost on day 106	117	10240	40.4	16	1.0	0.9	1.0

[a] Rabbit 698 was immunised with 0.5 mg of HBcSPreS$_1$PreS$_2$S$_{145R}$ and boosted 3 times on the indicated days with 0.5 mg of antigen in the first boost and 0.2 mg in the rest.

[b] Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.

[c] Anti-HBs was determined by the DARIP test using *ayw* subtype of ^{125}I-labelled HBsAg and the titres are expressed as % of ^{125}I-HBsAg co-precipitated with anti-HBs and anti-rabbit IgG.

[d] Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.

[e] Anti-peptide antibodies were determined by the indirect antibody RIA in which the indicated synthetic peptides were used as the solid-phase ligand and the titres are expressed as P/N ratio which represents cpm ratio of samples measured (P) and negative control (N).

[f] Not determined.

Table 5.15 Antibody responses of R703 to HBcSPreS$_1$PreS$_2$S$_{145R}$

Treatment[a]	Days postimmunisation	Anti-HBc titre[b]	Anti-HBs		Anti-peptide (P/N ratio)[e]		
			DARIP[c] (%)	AUSAB[d] (RIA unit)	HBs$_{(111-156)}$	PreS$_{1(1-23)}$	PreS$_{2(1-26)}$
Immunisation	0	0	6.2	16	0.5	0.5	0.6
1st boost	22	320	8.0	8	ND[f]	0.5	0.6
	33	2560	12.6	8	ND	0.8	1.1
2nd boost	50	10240	27.6	16	ND	0.6	0.8
	61	10240	27.9	183	1.9	1.0	0.8

[a] Rabbit 703 was immunised with 0.5 mg of HBcSPreS$_1$PreS$_2$S$_{145R}$ and boosted twice on the indicated days with 0.5 mg and 0.2 mg of antigen, respectively.

[b] Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.

[c] Anti-HBs was determined by the DARIP test using *ayw* subtype of ^{125}I-labelled HBsAg and the titres are expressed as % of ^{125}I-HBsAg co-precipitated with anti-HBs and anti-rabbit IgG.

[d] Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.

[e] Anti-peptide antibodies were determined by the indirect antibody RIA in which the indicated synthetic peptides were used as the solid-phase ligand and the titres are expressed as P/N ratio which represents cpm ratio of samples measured (P) and negative control (N).

[f] Not determined.

Table 5.16 Antibody responses of R702 to HBcSPreS$_1$PreS$_2$S$_{145E}$

Treatment[a]	Days postimmunisation	Anti-HBc titre[b]	Anti-HBs		Anti-peptide (P/N ratio)[e]		
			DARIP[c] (%)	AUSAB[d] (RIA unit)	HBs$_{(111-156)}$	PreS$_{1(1-23)}$	PreS$_{2(1-26)}$
Immunisation	0	0	7.4	0	0.7	1.0	1.2
1st boost	22	320	6.6	8	ND[f]	0.4	0.5
	33	2560	19.8	16	ND	1.1	1.5
2nd boost	50	ND	53.6	16	ND	1.0	1.1
	61	10240	52.8	16	11.2	1.1	1.2

[a] Rabbit 702 was immunised with 0.5 mg of HBcSPreS$_1$PreS$_2$S$_{145E}$ and boosted twice on the indicated days with 0.5 mg and 0.2 mg of antigen, respectively.

[b] Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.

[c] Anti-HBs was determined by the DARIP test using *ayw* subtype of ^{125}I-labelled HBsAg and the titres are expressed as % of ^{125}I-HBsAg co-precipitated with anti-HBs and anti-rabbit IgG.

[d] Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.

[e] Anti-peptide antibodies were determined by the indirect antibody RIA in which the indicated synthetic peptides were used as the solid-phase ligand and the titres are expressed as P/N ratio which represents cpm ratio of samples measured (P) and negative control (N).

[f] Not determined.

Table 5.17 Antibody responses of R701 to denatured HBsAg

Treatment[a]	Days postimmunisation	Anti-HBs		Anti-HBs$_{(111-156)}$ peptide[d] (P/N ratio)
		DARIP[c] (%)	AUSAB[d] (RIA unit)	
Immunisation	0	5.4	0	0.5
1st boost	22	9.2	54	ND[e]
	33	60.1	92	14.0
2nd boost	50	55.6	54	ND
	61	55.6	16	ND
3rd boost on day 78	92	50.6	ND	13.0
	100	49.8	ND	16.3

[a] Rabbit 701 was immunised with 10 μg of SDS-denatured HBsAg and boosted 3 times on the indicated days with the same dose of antigen except for the last boost in which 4 μg was given.

[b] Anti-HBs was determined by the DARIP test using *ayw* subtype of ^{125}I-labelled HBsAg and the titres are expressed as % of ^{125}I-HBsAg co-precipitated with anti-HBs and anti-rabbit IgG.

[c] Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.

[d] Anti-HBs$_{(111-156)}$ peptide antibody was determined by the indirect antibody RIA in which HBs$_{(111-156)}$ synthetic peptide was used as the solid-phase ligand and the titres are expressed as P/N ratio which represents cpm ratio of samples measured (P) and negative control (N).

[e] Not determined.

5.2.2.1 Anti-HBc response

Serum samples were serially diluted two-fold and assayed for anti-HBc antibody by the competitive RIA. Figure 5.6 shows the kinetics of the production of anti-HBc antibody in R675, R676, R677 and R678 after immunisation and subsequent boost with HBcS carrying wild-type or gly_{145} mutant in $HBs_{(111-156)}$. The titres reached maximal levels 12 to 22 days after the second boost, except in R678 which peaked 12 days after the third boost, after which the levels of anti-HBc antibody persisted or declined to half or quarter of the maximal levels finally, although the animals were further boosted a few times. The levels of anti-HBc antibody in all rabbits remained in high titres at final bleeding on days 435, 329 or 250 (Tables 5.2 to 5.7). The production of anti-HBc antibody in rabbits immunised with the HBcAg fusion proteins carrying $HBs_{(111-156)}$ as well as $preS_1$ and $preS_2$ fragments is shown in Tables 5.8 to 5.16. The kinetics of the anti-HBc production in these rabbits was similar to that shown in Figure 5.6. The titres continued to rise to a plateau level after the first or second boost, which persisted but did not appear to rise further by subsequent boosts in most rabbits.

In conclusion, these results demonstrated that a high anti-HBc response could be elicited by the HBcAg fusion proteins, which was very similar to that elicited by HBcAg, and the anti-HBc levels maintained for a long time, up to 435 days postimmunisation.

5.2.2.2 Anti-HBs antibody response

Anti-HBs antibody in the sera from rabbits immunised with the HBcAg fusion proteins were analysed by both DARIP and the AUSAB RIA tests. In the DARIP assay, 10-fold dilution of tested sera were incubated with ^{125}I-labelled HBs (*adw* or *ayw* subtype) followed by addition of donkey anti-rabbit IgG to precipitate any ^{125}I-labelled antigen-antibody complex. It should be noted that the HBV DNA used in the original cloning experiments (Burrell *et al.*, 1979; Pasek *et al.*, 1979), which led to the construction

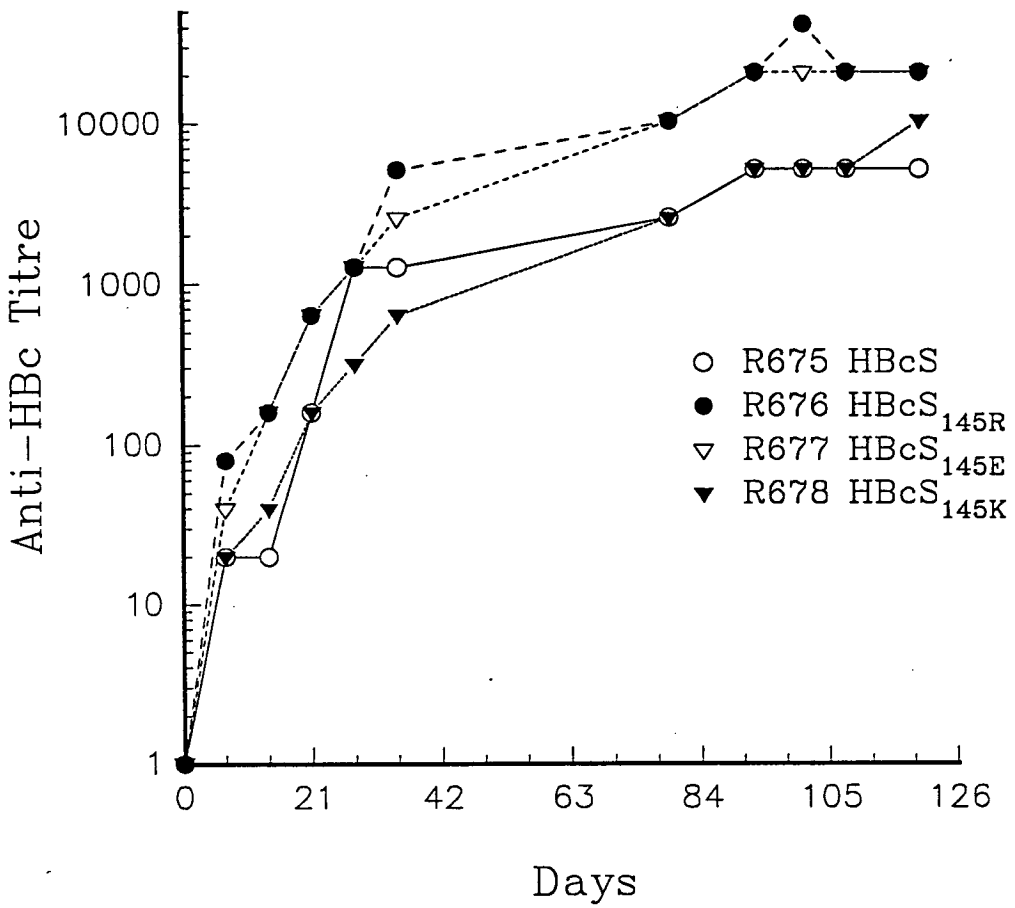


Figure 5.6 Anti-HBc titres in 4 rabbits immunised with HBcS or its gly_{145} mutant proteins. R675, R676, R677 and R678 were injected with HBcS, $HBcS_{145R}$, $HBcS_{145E}$ and $HBcS_{145K}$, respectively, on days 0, 21, 80 and 108 etc. Serum samples obtained at indicated days were serially diluted and tested for anti-HBc antibody by the competitive RIA. Titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled anti-HBc binding to the solid phase HBcAg.

of pHinG2 expressing HBsAg in yeast, exhibited the complex serotype *adyw*. However, in a DARIP competition assay, the yeast-derived HBsAg showed a specificity for monospecific anti-*y* serum, but not for monospecific anti-*d* serum (Ashton-Rickardt and Murray, 1989b). Although the HBsAg coding region in the HBcAg fusion constructs was derived from the same source as in pHinG2, anti-HBs antibody raised against the HBcAg fusion proteins could react equally with both *adw* and *ayw* subtypes of ^{125}I-HBsAg in the DARIP assay, suggesting that these antibodies were primarily directed toward the *a* group-specific determinant on HBsAg. The results in Figure 5.7 were obtained by using ^{125}I-HBsAg (*adw*) in the DARIP assay. In R675 immunised with HBcS, a low level of anti-HBs antibody was produced 21 days after immunisation. After the first and second boosts, the titre continued to rise, reaching peak activity within 14 days after the second boost (day 93), after which the titre did not rise further. In R676 immunised with $HBcS_{145R}$, no anti-HBs antibody was detectable before day 21. After boosting, the response was similar to that observed in R675, although to a lesser extent. In R677 immunised with $HBcS_{145E}$, anti-HBs antibody was not detectable until 14 days after the second boost (day 93), after which the titre remained unchanged even when further boosted. In R678 immunised with $HBcS_{145K}$, no anti-HBs antibody was detectable in all samples, although three boosts were given. In R675, R676 and R677 samples bled after day 120, the anti-HBs antibody titres maintained or declined slightly, but could be boosted to the same levels as the day 120 samples, except that no anti-HBs antibody was found in the R675 day 435 samples (Tables 5.2 to 5.4). In R678, the anti-HBs antibody was still not detectable after the fourth boost (day 287); however, on day 435, 14 days after the fifth boost, antibody was detected with a titre comparable to that in the R675 day 329 sample (Table 5.5). The anti-HBs antibody response in R684 and R685 immunised with HBcS and $HBcS_{145R}$, respectively, at a higher dose, are shown in Tables 5.6 and 5.7. R685 elicited a higher anti-HBs response than R684. However, the responses of the anti-HBs production were similar to those observed in R675 and R676.

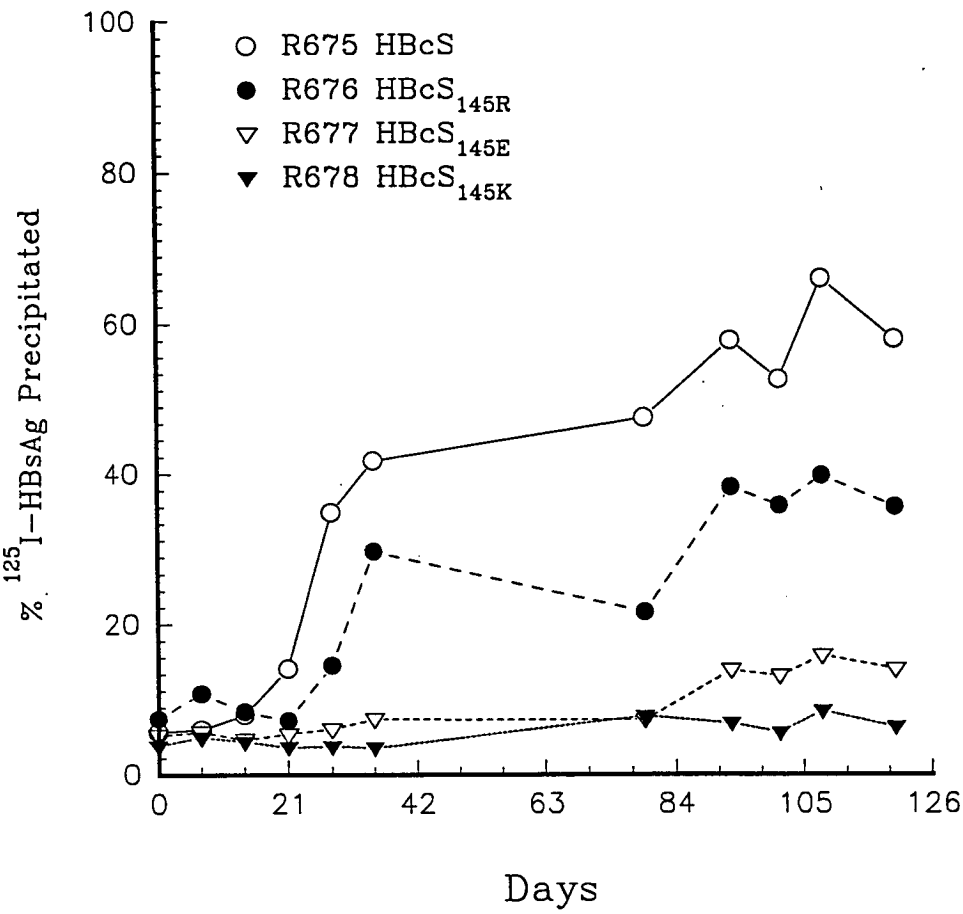
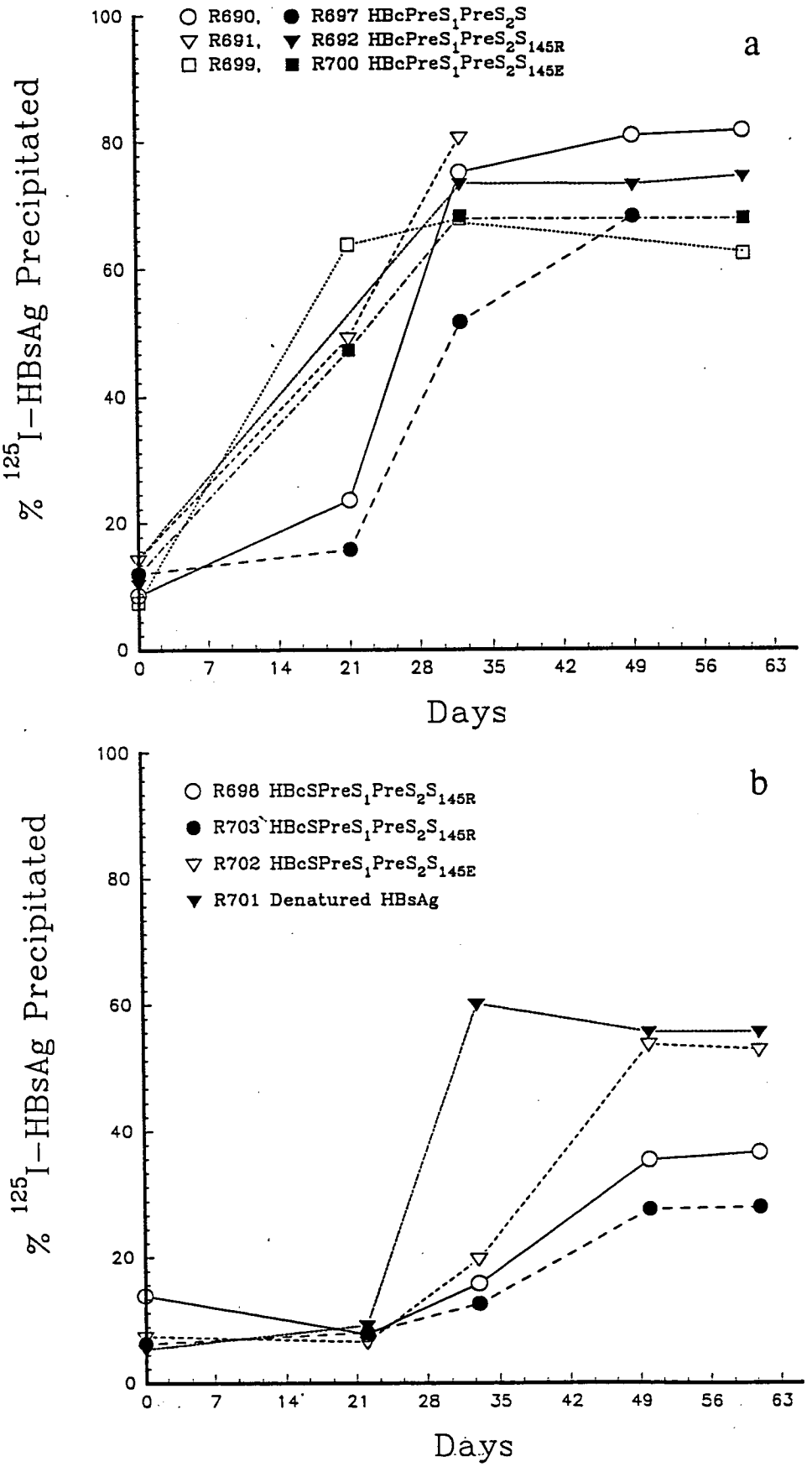


Figure 5.7 Anti-HBs production in 4 rabbits immunised with HBcS or its gly_{145} mutant proteins. R675, R676, R677 and R678 were injected with HBcS, $HBcS_{145R}$, $HBcS_{145E}$ and $HBcS_{145K}$, respectively, on days 0, 21, 81 and 108 etc. Serum samples obtained at indicated days were tested for anti-HBs antibody by the DARIP assay using ^{125}I-labelled HBsAg (*adw*) as the reagent. The amounts of anti-HBs antibody present in the sera were expressed as a percentage of ^{125}I-bound in the precipitate compared to total ^{125}I added in the reaction.

The anti-HBs responses in rabbits immunised with $HBcPreS_1PreS_2S$ or $HBcSPreS_1PreS_2S$ carrying wild-type or a gly_{145} mutant in $HBs_{(111-156)}$ are shown in Figure 5.8. $HBcPreS_1PreS_2S$ carrying either wild-type or gly_{145} mutants provoked a more rapid and greater anti-HBs antibody response than HBcS or its mutant counterparts in rabbits (Figure 5.7). The anti-HBs titres in these rabbits (R690, R691, R692, R697, R699 and R700) began to rise following immunisation, and reached a plateau after the first boost, following which the titres persisted (Figure 5.8a). The responses and the titres of anti-HBs production in these rabbits were similar to those observed in R701 which had been immunised with denatured HBsAg (Figure 5.8b). In R698, R702 and R703, immunised with $HBcSPreS_1PreS_2S_{145R}$ or $HBcSPreS_1PreS_2S_{145E}$, the induction of anti-HBs antibody was slower and to a lower level than in those immunised with $HBcPreS_1PreS_2S$, carrying wild-type or gly_{145} mutants in $HBs_{(111-156)}$ (Figure 5.8b). Anti-HBs antibody could be detected with a moderate titre only after the first boost and was maintained at the same level after the second boost.

The anti-HBs titres were also determined by the AUSAB RIA in some sera from rabbits immunised with the HBcAg fusion proteins . The serum samples were placed in contact with the plastic beads that had been coated with both *ad* and *ay* subtypes of HBsAg, and during incubation, antibody, if present, was fixed to the solid phase and was detected following the addition of ^{125}I-labelled HBsAg. RIA units were estimated according to the semiquantitative method recommended by the manufacturer. The specificity in the AUSAB test was in agreement with that obtained from the DARIP assay. Sera negative for anti-HBs antibody by the DARIP assay were also negative in the AUSAB test. Nevertheless, the degree of reactivity between these two tests varied (Tables 5.2 to 5.17). Comparion of the RIA units obtained from the AUSAB test shows that R690 and R697, which were immunised with $HBcPreS_1PreS_2S$, elicited the highest anti-HBs response with 352 and 262 units/0.2 ml serum on day 32 (Tables 5.8 and 5.9). All preimmunisation sera were negative for anti-HBs antibody, except R675 and R697 (Tables 5.2 and 5.9) which reacted strongly in the AUSAB test. These false positive results were presumably due to non-

Figure 5.8 Anti-HBs production in 10 rabbits immunised with (a) $HBcPreS_1PreS_2S$ or (b) $HBcSPreS_1PreS_2S$, carrying wild-type or a gly_{145} mutant in $HBs_{(111\text{-}156)}$, or denatured HBsAg on days 0, 21 (22) or/and 49 (50) *etc.* R697 and R691 died on days 48 and day 32, respectively. Serum samples obtained at indicated days were tested for anti-HBs antibody by the DARIP assay using ^{125}I-labelled HBsAg (*ayw*) as the reagent. The amounts of anti-HBs antibody present in the sera were expressed as a percentage of ^{125}I-bound in the precipitate compared to total ^{125}I added in the reaction.


a
R690, R697 HBcPreS1PreS2S
R691, R692 HBcPreS1PreS2S145R
R699, R700 HBcPreS1PreS2S145E
% 125I-HBsAg Precipitated
Days
b
R698 HBcSPreS1PreS2S145R
R703 HBcSPreS1PreS2S145R
R702 HBcSPreS1PreS2S145E
R701 Denatured HBsAg
% 125I-HBsAg Precipitated
Days

specificity of cross-reacting substances or components in the rabbit sera. However, in the DARIP tests, the preimmunisation sera from R675 and R697 were both anti-HBs negative when ^{125}I-HBsAg of *adw* or *ayw* was used as the test reagent.

Collectively these data indicate that HBcAg fusion proteins carrying $HBs_{(111-156)}$ fragments, either wild-type or gly_{145} mutants in $HBs_{(111-156)}$, whether including $preS_{1(1-20)}$ and $preS_{2(1-26)}$ fragments or not, elicited antibodies that could cross-react with native HBsAg. $HBcS_{145E}$ and especially, $HBcS_{145K}$ appeared to induce less antibodies recognised the native HBsAg. However, there was animal to animal variation in producing anti-HBs antibody, which could be expected from outbred animals. It should be noted here that R701, which was immunised with HBsAg that had been reduced and denatured with DTT, SDS and boiling, also elicited antibody cross-reactive with native HBsAg (Table 5.8b), the levels of which were comparable to those induced from the HBcAg fusion proteins.

5.2.2.3 Anti-peptide antibodies

The sera from rabbits immunised with the HBcAg fusion proteins were assayed for reactivity against the corresponding peptide by the indirect antibody RIA. These assays were carried out using synthetic $preS_{1(1-23)}$, $preS_{2(1-26)}$ or $HBs_{(111-156)}$ peptide bound directly to the solid phase, and ^{125}I-labelled second antibody to probe anti-peptide antibody bound to peptide-coated beads. Data are expressed as P/N ratios which represent the cpm ratio of samples measured (P) and negative control (N) at the same dilution (10^{-2} or 10^{-3}). All rabbits immunised with $HBcPreS_1PreS_2S$, either wild-type or gly_{145} mutants in $HBs_{(111-156)}$, elicited anti-$preS_1$ and anti-$preS_2$ antibodies (Tables 5.8 to 5.13). Surprisingly, $HBcSPreS_1PreS_2S$ carrying a dimer of $HBs_{(111-156)}$ fragment induced anti-$preS_1$ and anti-$preS_2$ antibodies in only one (R698) out of three rabbits (Tables 5.14 to 5.16). The highest levels of the anti-$preS_1$ and anti-$preS_2$ responses were obtained 11 to 28 days after the first boost and persisted for 205 days, but either did not appear to alter significantly after further boosts (R690), or decreased with time (R692 and R698). Although the same concentration of $preS_{1(1-23)}$ and $preS_{2(1-}$

$_{26)}$ peptides was used for preparing the peptide-coated beads, the binding efficiency of the two peptides to the solid phase might be different. It was not possible, therefore, to compare the titres between anti-$preS_1$ and anti-$preS_2$ antibodies in the rabbit sera. Moreover, the heterogeneity of the response did not allow meaningful comparison of the $preS_1$- and $preS_2$-specific immunogenicity between $HBcPreS_1PreS_2S$ and $HBcSPreS_1PreS_2S$ in rabbits. Anti-$HBs_{(111-156)}$ antibody was also examined by the indirect antibody RIA in some rabbit sera (Tables 5.8 to 5.17). The levels of anti-$HBs_{(111-156)}$ antibody parallelled those of anti-HBs antibody obtained by the DARIP assay.

5.3 Immune responses of mice after inoculation with HBcAg fusion proteins

In Section 5.2, the immunogenicity studies of the HBcAg fusion proteins were carried out in outbred rabbits. High-titred, anti-HBc antibodies were produced in all rabbits immunised with HBcAg fusion proteins. However, the immune responses to $preS_1$, $preS_2$ and HBs peptides were variable. These results from rabbits were in agreement with the observations in humans. Regardless of clinical disease, high-titred, anti-HBc antibodies are produced by virtually 100% of HBV-infected individuals. In contrast, the response to HBsAg varies, which may account for the spectrum of clinical manifestations associated with HBV infection. Although the variation of the genetic make-up in the outbred rabbits reflected the outbred human population, the use of inbred strains of mice to compare the immunogenicity of the HBcAg fusion proteins may provide more information about the immunological features of these HBcAg fusion proteins carrying different epitopes.

5.3.1 Immunogenicity of HBcAg fusion proteins in terms of antibody responses

To evaluate and compare the immunogenicities of HBcAg fusion proteins carrying $HBs_{(111-156)}$ with or without $preS_{1(1-20)}$ and $preS_{2(1-26)}$ fragments in inbred strains of mice, groups of 5 BALB/c mice were immunised intraperitoneally (I.P.) with equal doses (20 μg) of 6 different HBcAg fusion proteins and subsequently boosted with 20 μg,

50 μg and 50 μg of the same immunogen on days 28, 57 and 104, respectively. Mice were bled and sera from each group were pooled at 2 or 3 weeks after each injection. As shown in Table 5.18, anti-HBc antibody was detected in all groups of mice after the first boost, which was similar to the results obtained from rabbit experiments. As expected, there was no significant difference in their abilities to elicit anti-HBc antibody in the mice immunised with HBcS, its three gly_{145} mutants, $HBcPre_1PreS_2S$ or $HBcSPreS_1PreS_2S$. In contrast, these HBcAg fusion proteins induced negligible or low-titred anti-HBs antibody, except $HBcPreS_1PreS_2S$ elicited 82 RIA units (per 0.2 ml serum) of anti-HBs antibody on day 118 following three boosts. The anti-HBs response was delayed since little anti-HBs antibody was detected until 118 days after immunisation followed by the three boosts. In rabbits, however, anti-HBs antibody was found earlier in some rabbits after immunisation and before boostings (day 21). In addition, low levels of anti-$preS_1$ and anti-$preS_2$ antibodies (P/N ratio $\geq$2.1) were detected on days 71 and 118 in mice immunised with $HBcPreS_1PreS_2S$ and only anti-$preS_2$, but not anti-$preS_1$, antibody detected on 118 days in those immunised with $HBcSPreS_1PreS_2S$; no anti-$preS_1$ and anti-$preS_2$ antibodies were induced in the earlier samples (Table 5.19). Although the dose of injection relative to the body weight for the mice was higher than that for the rabbit, the anti-HBs, anti-$preS_1$ and anti-$preS_2$ antibody responses induced by the HBcAg fusion proteins were lower in mice than in rabbits, whereas high-titred anti-HBc antibody was induced in both species. The discrepancy in the former may be due to the differences in the immune responses among species or in the injection routes.

Higher doses of the HBcAg fusion proteins were used in the following experiment in which 40 μg of immunogens were inoculated into groups of 5 BALB/c mice for immunisation and two boosts on days 28 and 64. As shown in Table 5.20, high-titred anti-HBc antibody was again produced in the mice immunised with HBcAg or the HBcAg fusion proteins. Mice immunised with $HBcS_{145R}PreS_1PreS_2S$ or $HBcS_{145E}PreS_1PreS_2S$ also produced 72 and 26 RIA units (per 0.2 ml serum) of anti-HBs antibodies, respectively. Despite the fact that the HBcAg fusion proteins induced only low anti-HBs response in BALB/c mice, the antibody response to native HBsAg

Table 5.18 Anti-HBc and anti-HBs antibody responses of BALB/c mice to the HBcAg fusion proteins

Immunogen[a]	Day 13		Day 22		Day 42		Day 71		Day 118	
	Anti-HBc[b]	Anti-HBs[c]	Anti-HBc	Anti-HBs	Anti-HBc	Anti-HBs	Anti-HBc	Anti-HBs	Anti-HBc	Anti-HBs
HBcS	80	ND[d]	640	16	2560	8	2560	<8	2560	8
$HBcS_{145R}$	160	ND	160	<8	1280	8	5120	<8	5120	8
$HBcS_{145E}$	40	ND	320	<8	2560	8	5120	12	10240	26
$HBcS_{145K}$	80	ND	320	0	5120	<8	10240	<8	5120	<8
$HBcPreS_1PreS_2S$	40	ND	320	<8	1280	16	2560	<8	5120	82
$HBcSPreS_1PreS_2S$	40	ND	320	0	5120	16	5120	8	5120	<8

[a] Groups of 5 BALB/c mice were immunised with 20 μg of the indicated immunogens and boosted thrice with 20 μg, 50 μg, and 50 μg of the same antigens on days 28, 57 and 107, respectively.

[b] Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.

[c] Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.

[d] Not determined.

Table 5.19 Anti-$preS_{1(1-23)}$ and anti-$preS_{2(1-26)}$ antibody responses of BALB/c mice to various immunogens

Immunogen	Dose (μg)	Day 22 or 20		Day 42		Day 71, 77 or 79		Day 118	
		Anti-$preS_1$[c]	Anti-$preS_2$[c]	Anti-$preS_1$	Anti-$preS_2$	Anti-$preS_1$	Anti-$preS_2$	Anti-$preS_1$	Anti-$preS_2$
$HBcPreS_{1(1-36)}$[a]	40	0.8	0.7	3.4	0.8	1.5	1.6	ND	ND
$HBcPreS_2$[a]	40	0.8	1.4	0.8	2.9	1.0	5.4	ND	ND
$HBcPreS_1PreS_2$[a]	40	1.0	1.2	4.9	2.3	5.6	2.3	ND	ND
$HBcS_{145R}PreS_1PreS_2S$[a]	40	1.0	1.2	0.8	0.9	3.2	2.5	ND	ND
$HBcS_{145E}PreS_1PreS_2S$[a]	40	1.0	1.3	1.4	1.1	1.7	1.1	ND	ND
$PreS_{1(1-23)}$ peptide[a]	100	0.7	0.8	0.7	0.8	0.9	1.1	ND	ND
$PreS_{2(1-26)}$ peptide[a]	100	0.8	1.5	0.7	2.0	0.9	1.4	ND	ND
$HBcPreS_1PreS_2S$[b]	20	1.1	1.1	0.9	1.2	2.3	2.2	3.4	3.7
$HBcSPreS_1PreS_2S$[b]	20	0.7	0.9	0.9	1.1	1.1	1.2	1.3	2.8

[a] Groups of 5 BALB/c mice were immunised with the indicated immunogens and doses and boosted twice on days 28 and 62 (or 64) with the same amount of antigens. Mice were bled on days 20 (or 22), 42 and 77 (or 79).

[b] Groups of 5 BALB/c mice were immunised with the indicated immunogens and doses and boosted thrice with 20 μg, 30 μg and 50 μg of the same antigens on days 28, 57 and 107, respectively. Mice were bled on days 13, 22, 42, 71 and 118.

[c] Anti-peptide antibody was determined by the indirect antibody RIA in which $preS_{1(1-23)}$ or $preS_{2(1-26)}$ synthetic peptide was used as the solid-phase ligand and the titres are expressed as P/N ratio, which represents cpm of samples measured (P) and negative control (N).

Table 5.20 Anti-HBc and anti-HBs antibody responses of BALB/c mice to various immunogens

Immunogen[a]	Dose (μg)	Day 22 or 20		Day 42		Day 79 or 77	
		Anti-HBc[b]	Anti-HBs[c]	Anti-HBc	Anti-HBs	Anti-HBc	Anti-HBs
HBcAg	4	80		2560		5120	
HBsAg	2	0	144	0	>2048	0	>10240
$HBcPreS_{1(1-36)}$	40	20		640		640	
$HBcPreS_2$	40	80		1280		5120	
$HBcPreS_1PreS_2$	40	320		1280		2560	
$HBcS_{145R}PreS_1PreS_2S$	40	160	16	2560	8	2560	72
$HBcS_{145E}PreS_1PreS_2S$	40	320	8	2560	8	5120	26
$PreS_{1(1-23)}$ peptide	100	0		0		0	
$PreS_{2(1-26)}$ peptide	100	0		0		0	

[a] Groups of 5 BALB/c mice were immunised with the indicated immunogens and doses and boosted twice on days 28 and 64 (or 62) with the same amount of antigens except HBcAg and HBsAg, in which only 1 μg and 0.5 μg were used for the second boost, respectively.

[b] Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.

[c] Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.

was extremely high in the mice. Two μg of HBsAg induced more than 10240 RIA units of anti-HBs antibody. Anti-$preS_1$ or/and anti-$preS_2$ antibodies were detected in mice injected with $HBcPreS_{1(1\text{-}36)}$, $HBcPreS_2$, $HBcPreS_1PreS_2$, $HBcS_{145R}PreS_1PreS_2S$, $HBcPreS_1PreS_2S$, or $HBcSPreS_1PreS_2S$ on days 42, 71 (or 79), and 118 (Table 5.19). The HBcAg fusion proteins carrying the $preS_1$ or/and $preS_2$ fragments only appear to elicit higher anti-$preS_1$ or/and $preS_2$ antibodies than those carrying $preS_1$ and $preS_2$ as well as a monomer or a dimer of $HBs_{(111\text{-}156)}$ fragments. The $HBs_{(111\text{-}156)}$ fragment might affect the surface accessibility of the $preS_1$ and $preS_2$ epitopes in the hybrid particles. When mice were immunised with 100 μg of $preS_{1(1\text{-}23)}$ or $preS_{2(1\text{-}26)}$ peptide in the absence of carrier proteins, no anti-peptide antibodies were detected in any of samples (Table 5.19). The enhanced immunogenicity of peptide epitopes presented on the HBcAg particles is in accordance with the previous studies of Clarke *et al.* (1989).

In further mouse experiments, $HBcPreS_1PreS_2S_{145R}$ or $HBcSPreS_1PreS_2S_{145R}$ was inoculated at a range of concentrations from 10 μg to 500 μg into groups of BALB/c mice, which were subsequently boosted with the same dose on day 28. As shown in Table 5.21, the levels of anti-HBc antibody increased enormously 14 days after the first boost (day 42). Similar levels of anti-HBs antibody were found in the mice as those elicited by some rabbits (R691, R692, R698, R699, R700, R702 and R703) immunised with similar HBcAg fusion proteins. However, regarding the dose of immunisation relative to the body weight and the kinetics of the response, the anti-HBs response in BALB/c mice appeared to be reduced and delayed as compared to that in rabbits, and no dose-response correlation was observed in either set of experiments which made it difficult to compare the immunogenicity of these two HBcAg fusion proteins. Furthermore, low levels of anti-$preS_1$ or/and anti-$preS_2$ antibodies were detectable in some samples on days 42 and 92 (data not shown).

As illustrated in Table 5.22, when a wide range of concentrations of HBcS (from 0.005 μg to 250 μg) were inoculated into groups of BALB/c mice, which were subsequently boosted with the same dose on days 28 and 56, the levels of anti-HBc

Table 5.21 Anti-HBc and anti-HBs antibody responses of BALB/c mice to $HBcPreS_1PreS_2S_{145R}$ and $HBcSPreS_1PreS_2S_{145R}$

Immunogen[a]	Dose (μg)	Day 24		Day 42		Day 92	
		Anti-HBc[b]	Anti-HBs[c]	Anti-HBc	Anti-HBs	Anti-HBc	Anti-HBs
$HBcPreS_1PreS_2S_{145R}$	10	40	<8	1280	8	ND[d]	16
	50	80	12	2560	36	ND	54
	250	320	16	5120	36	ND	92
	500	320	<8	5120	16	ND	54
$HBcSPreS_1PreS_2S_{145R}$	10	20	<8	640	12	ND	16
	50	160	12	10240	12	ND	54
	250	160	<8	10240	<8	ND	<8
	500	160	8	10240	26	ND	72

[a] Groups of 4 BALB/c mice were immunised with the indicated immunogens and doses and boosted on day 28 with the same amount of antigens.

[b] Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.

[c] Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.

[d] Not determined.

Table 5.22 Anti-HBc and anti-HBs antibody responses of BALB/c mice to HBcS

Immunogen[a]	Dose (μg)	Day 21		Day 42		Day 70	
		Anti-HBc[b]	Anti-HBs[c]	Anti-HBc	Anti-HBs	Anti-HBc	Anti-HBs
HBcS	0.005	<5	<8	<5	8	<5	12
	0.05	<5	16	<5	12	<5	8
	0.4	<5	36	40	36	20	54
	2	40	<8	1280	12	640	12
	10	160	16	2560	12	5120	12
	50	320	36	5120	26	10240	16
	250	640	<8	10240	<8	10240	<8

[a] Groups of 5 BALB/c mice were immunised with the indicated doses and boosted twice on days 28 and 56 with the same amount of antigens. Mice were bled on days 21, 42 and 70.

[b] Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.

[c] Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.

antibody elicited by the animals could be correlated with the amount of injected antigen. HBcS at a 0.05 μg dose or less did not elicit the production of anti-HBc antibody, and 0.4 μg induced only marginal response, while 10 μg of the antigen elicited high anti-HBc antibody on days 42 and 70. Further 2-fold and 4-fold increases in anti-HBc titre were observed when 5-fold and 25-fold concentrations of the antigen were inoculated, respectively. However, the levels of anti-HBs response were still similar to those found in the previous experiments (Table 5.21). The highest anti-HBs response (54 RIA units) was found in mice inoculated with 0.4 μg of HBcS. Higher doses of HBcS did not stimulate higher anti-HBs response. There was little direct correlation between the anti-HBs response and the immunisation dose.

In conclusion, HBcAg fusion proteins induced anti-HBc antibody in mice as high as in rabbits, and the induction was regulated in a dose-dependent manner. There was no significant difference in inducing anti-HBc antibody among different HBcAg fusion proteins. In terms of the production of anti-HBs antibody, the response appeared to be higher in mice immunised with HBcPreS_1PreS_2S than in those immunised with HBcS or HBcSPreS_1PreS_2S (Table 5.18). These results were consistent with the results obtained from the rabbits. Nevertheless, the anti-HBs response induced by HBcAg fusion proteins in mice was lower and later than in rabbits. It is noteworthy that the anti-HBs response induced by HBcAg fusion proteins, in either mice or rabbits, was much lower than that induced by native HBsAg.

5.3.2 Immunogenicity of HBcAg fusion proteins in B10.S mice which are genetically non-responsive to HBsAg

The humoral immune response to HBsAg varies considerably from patient to patient during infection and after vaccination. In contrast, high titres of anti-HBc are produced by all HBV-infected patients. The non-responsiveness to HBsAg in man results in the chronic carrier state which is an important reservoir for persistence and transmission of HBV. It has been documented that the murine humoral immune response to HBsAg is regulated by H-2-linked immune response (Ir) genes (Milich and

Chisari, 1982). The availability of HBsAg non-responder strains, e.g. H-2^s and H-2^f strains, has provided a model to study the methods of circumventing HBsAg non-responsiveness. The H-2-restricted humoral immune response to HBsAg in mice has been demonstrated to be antigen dose-dependent (Milich and Chisari, 1982). Non-responsive SJL (H-2^s) mice immunised with 40 μg, a 10-fold higher dose than that for the responder strain, did produce a primary anti-HBs response, although the magnitude was reduced as compared to that of high-responder strains received 4 μg of the antigen. Moreover, the immunisation route also influenced the responsiveness to HBsAg. Immunisation of SJL mice with HBsAg through the hind footpads, instead of I.P. injection, also resulted in a primary anti-HBs response, in contrast to I.P. immunisation, which did not. However, the magnitude and kinetics of the humoral responses were markedly reduced and delayed.

To investigate the feasibility of using HBcAg fusion proteins carrying the $HBs_{(111\text{-}156)}$ fragment to provide HBc-specific T-helper cell activity for anti-HBs antibody production in mice with HBsAg non-responder haplotypes, hence circumventing the non-responsiveness to HBsAg, three groups of five B10.S (H-2^s) mice were immunised with a mixture of HBcAg and HBsAg, or with HBcAg fusion proteins (HBcS or $HBcS_{145R}$) alone. Two other groups were immunised with HBcAg or HBsAg alone to serve as control groups. All groups of mice received a further 3 boosts on days 28, 58 and 100. They were bled 10 and 24 days after immunisation as well as 14 or 15 days after boostings. As shown in Table 5.23, except for the HBsAg-immunised mice, all groups produced anti-HBc antibody as early as 10 days after immunisation and the titres continued to rise, peaked on day 43, then maintained the same level or declined to a half or a quarter of the peak level, although two further boosts were given. The anti-HBc response among these groups did not differ significantly. With respect to the anti-HBs response, B10.S mice immunised with 4 μg of HBsAg produced no anti-HBs until 14 days after the second boost (day 72). Surprisingly, those immunised with a mixture of HBcAg and HBsAg produced anti-HBs antibody as early as 10 days; the titre peaked on day 24 and could not be augmented by the boostings. In contrast, no anti-HBs response was detected in B10.S mice immunised with HBcS or

Table 5.23 Anti-HBc and anti-HBs antibody responses of B10.S mice to various immunogens

Immunogen[a] (μg)	Day 10		Day 24		Day 43		Day 72		Day 114	
	Anti-HBc[b]	Anti-HBs[c]	Anti-HBc	Anti-HBs	Anti-HBc	Anti-HBs	Anti-HBc	Anti-HBs	Anti-HBc	Anti-HBs
HBcAg (16)	320	ND[d]	640	ND	5120	0	2560	0	1280	0
HBsAg (4)	<2	0	<2	0	<2	0	<2	288	<2	1614
HBcAg (16) + HBsAg (4)	320	59	1280	1468	5120	558	5120	638	2560	302
HBcS (20)	160	ND	320	ND	2560	0	2560	0	1280	0
$HBcS_{145R}$(20)	640	ND	640	ND	2560	0	2560	0	2560	0

[a] Groups of 5 B10.S mice were immunised with the indicated doses of immunogens and boosted thrice on days 28, 58 and 100.

[b] Anti-HBc was determined by the competitive RIA and the titres are expressed as the reciprocal of the serum dilution that gave 50% inhibition of ^{125}I-labelled antibody binding.

[c] Anti-HBs was determined by the AUSAB RIA and the titres are expressed as RIA units per 0.2 ml of serum.

[d] Not determined.

$HBcS_{145R}$ throughout the observation period. The lack of an anti-HBs response after primary immunisation of 4 μg of HBsAg in B10.S mice is consistent with the results previously described by Milich and Chisari (1982). However, repeated boostings of HBsAg could circumvent the non-responsiveness, since 288 and 1614 RIA units (per 0.2 ml serum) of anti-HBs antibody were detected on days 72 and 114, respectively, which corresponded to 14 days after the second and third boosts. It should be noted that the anti-HBs responses in B10.S mice were consistently inferior to those in BALB/c mice. The results from B10.S mice immunised with a mixture of HBcAg and HBsAg are of interest and imply that HBcAg-specific T cells can function to help anti-HBs production even when HBcAg and HBsAg were not present within the same molecule or particle. The results appear to contradict the previous results published by Milich *et al.* (1987c), who demonstrated that B10.S mice primed with HBcAg or $HBc_{(120-140)}$ peptide and subsequently challenged with a suboptimal dose of virions (HBV) 3 weeks post-priming, produced anti-HBs antibody, whereas HBcAg-primed mice challenged with a mixture of HBcAg and HBsAg did not. Their results indicate that HBcAg-primed T-cells can help anti-HBs antibody production, and the T-helper cell activity required that HBcAg and HBsAg be present within the same particle (virion), since the mixture was ineffective. The discrepancy between the two experiments could result from the different immunisation regimen used. In the work described here, instead of priming with HBcAg (4 μg), HBcAg (16 μg) and HBsAg (4 μg) were concomitantly administered to mice for the immunisation and the first boost, and 1/4 and 1/8 of the original doses were used for the second and the third boosts, respectively. The mechanism underlying circumvention of non-responsiveness to HBsAg is unclear, however, it seems that HBcAg-activated T-cells non-specifically stimulated HBsAg-specific B-cells to produce anti-HBs, which differed from intermolecular-intrastructural T-helper cell-B-cell interactions (Milich *et al.*, 1987c). However, two groups of B10.S mice immunised with 20 μg of HBcS or $HBcS_{145R}$ produced no anti-HBs antibody, which was different from that obtained from BALB/c mice (Table 5.18). The results indicate that the immunisation of HBcS or $HBcS_{145R}$ did not circumvent the non-responsiveness to HBsAg through HBcAg-specific Th cell function.

When BALB/c or B10.S mice were primed with HBcAg (4 μg) and challenged 3 weeks later (day 21) with a lower dose (2 μg) of HBcPreS$_1$PreS$_2$S, a considerable level of anti-HBc antibody was elicited on days 28 and 35, whereas little or no anti-HBs antibody was detected (data not shown). The lymphocyte-proliferative response by the splenocytes was also assessed on day 35 after sacrifice of the mice. Low levels of lymphocyte proliferation to HBcAg and the HBc fusion protein were detected in all mice injected with HBcAg (priming) alone, HBcPreS$_1$PreS$_2$S (challenge) alone, or HBcAg (priming) followed by HBcPreS$_1$PreS$_2$S (challenge), while no lymphocyte-proliferative response to HBsAg, preS$_{1(1\text{-}23)}$ or preS$_{2(1\text{-}26)}$ peptide was observed (data not shown). The failure to elicit anti-HBs antibody in either the BALB/c or B10.S mice primed with HBcAg and challenged later with HBcPreS$_1$PreS$_2$S might be due to low HBcAg-specific T-cell response resulting in no functional help for anti-HBs production, or due to no HBs$_{(111\text{-}156)}$-specific B cell response, as anti-HBs antibody response to HBcAg fusion proteins carrying the HBs$_{(111\text{-}156)}$ fragment is low in BALB/c mice as shown in this work. Furthermore, the dose for challenge may be quite critical for such experiments, and this has not been fully explored in this study.

5.4 HBcAg fusion proteins carrying HBs$_{(111\text{-}156)}$ fragment elicit antibodies cross-reactive with native HBsAg

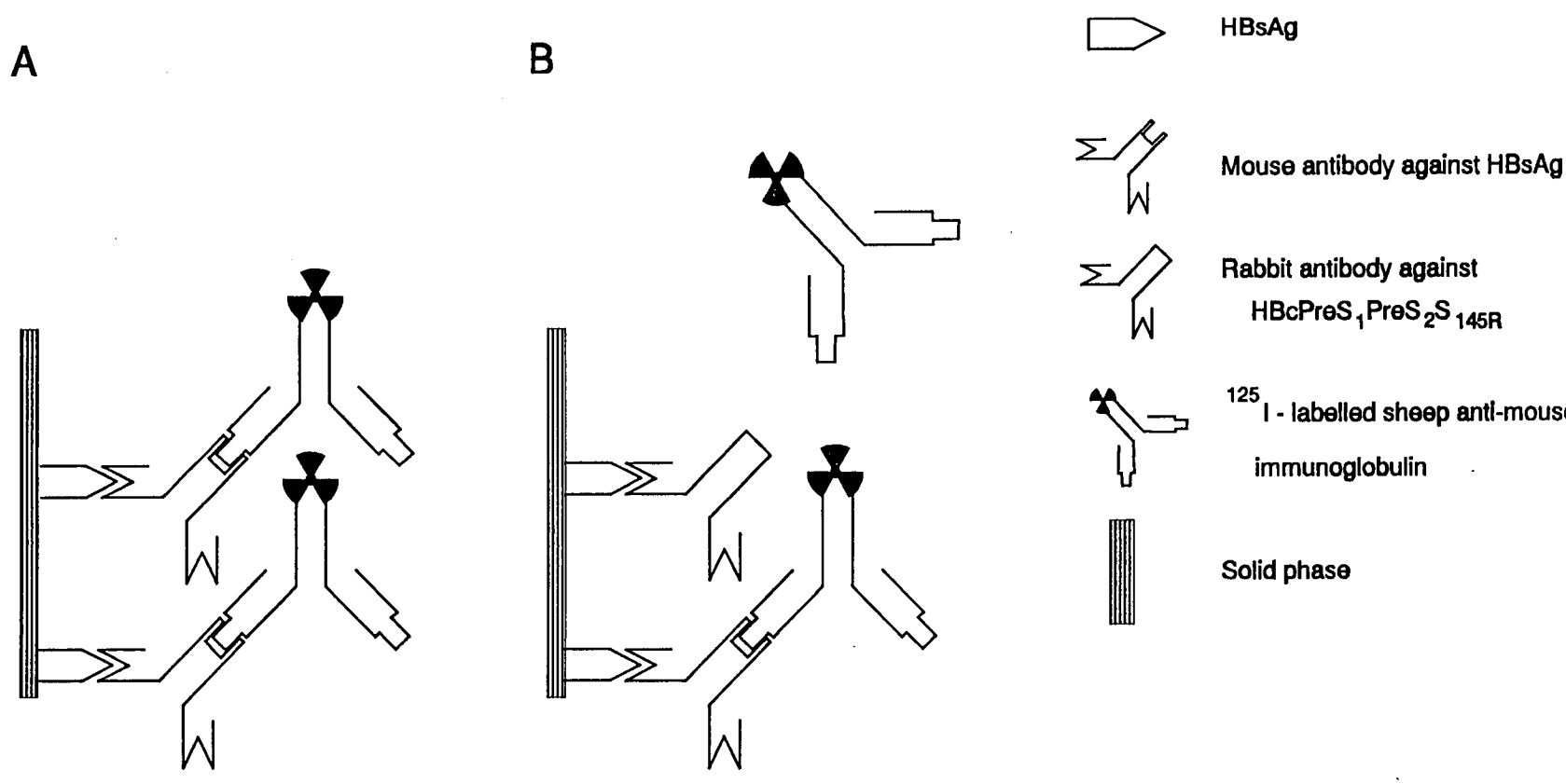
The results obtained from Sections 5.2.2 and 5.3.1 show that rabbits and BALB/c mice immunised with HBcAg fusion proteins carrying the HBs$_{(111\text{-}156)}$ fragment elicited antibodies which could react with native HBsAg, albeit at lower levels than those immunised with intact, native HBsAg, as measured by the AUSAB test or the DARIP assay (Figures 5.7 and 5.8, Tables 5.18, 5.20, 5.21 and 5.22). On the other hand, anti-HBs antiserum from rabbits or mice raised against native HBsAg reacted with HBs$_{(111\text{-}156)}$ peptide in the solid-phase indirect antibody RIA. To further compare the anti-HBs and anti-HBcAg fusion protein sera, a competition test was set up in which mouse polyclonal antibody against native HBsAg, with a certain dilution to saturate HBsAg in the solid phase, was incubated with HBsAg-coated beads along with various dilutions of rabbit polyclonal antibody against the HBcAg fusion protein as the

competitor and was probed with ^{125}I-labelled sheep anti-mouse immunoglobulin (Figure 5.9). Figure 5.10 shows that binding of anti-HBs antiserum to the solid phase HBsAg was inhibited in a concentration-dependent manner by increasing concentrations of antiserum to HBcAg fusion protein. A 62.8% inhibition of binding was observed after addition of undiluted antiserum raised against HBcPreS$_1$PreS$_2$S$_{145R}$ from R692 (day 92), whereas preimmunised serum from the same rabbit failed to inhibit the binding. On the other hand, binding of mouse polyclonal anti-HBs antiserum to the solid phase HBs$_{(111-156)}$ peptide was also inhibited by rabbit polyclonal antiserum against HBcPreS$_1$PreS$_2$S$_{145R}$ by 36.3% (Figure 5.11). In addition, antisera raised against HBcS, HBcS$_{145R}$, HBcS$_{145E}$ and HBcS$_{145K}$ all inhibited the binding of anti-native HBsAg antiserum to HBsAg to at least some extent in similar assays (data not shown). Therefore, the results indicate that polyclonal antibody against HBcAg fusion proteins carrying either wild-type or gly$_{145}$ mutant HBs$_{(111-156)}$ fragments and polyclonal antibody against native, intact HBsAg recognise either a common or overlapping determinant on HBs$_{(111-156)}$ sequence.

5.5 Discussion

In the work described in this chapter, the immunogenicities of the HBcAg fusion proteins were determined in terms of T-cell response and antibody production. The experiments demonstrate that immunisation of rabbits with hybrid HBcAg carrying the S or/and preS$_1$ and preS$_2$ fragments resulted in the generation of HBcAg-specific and envelope protein-specific antibodies and that the PBLs from such rabbits proliferated after *in vitro* stimulation with HBcAg, HBcAg fusion protein, HBsAg or preS$_{1(1-23)}$ peptide. The T-cell responses to HBcAg or HBcAg fusion protein were much higher than those to HBsAg or preS$_{1(1-23)}$. Moreover, all immunised rabbits responded highly to HBcAg and HBcAg fusion protein, while most rabbits responded moderately to weakly to HBsAg. Responses to the preS$_{1(1-23)}$ peptide was variable and no response was observed to the preS$_{2(1-26)}$ peptide. In regard to the humoral response, rabbits immunised with HBcAg fusion proteins (wild-type or gly$_{145}$ mutants) with or without preS$_1$ and preS$_2$ fragments, elicited high-titred anti-HBc antibody. They also elicited

Figure 5.9 The diagram illustrating the competitive RIA. (A) Mouse anti-HBs antiserum binds to the solid phase HBsAg following the binding of ^{125}I-labelled sheep anti-mouse immunoglobulin. (B) Inhibition of mouse anti-HBs antiserum bound to the solid phase HBsAg in the presence of the competitor, rabbit antiserum against $HBcPreS_1PrS_2S_{145R}$, as detected by ^{125}I-labelled sheep anti-mouse immunoglobulin.


A
B
HBsAg
Mouse antibody against HBsAg
Rabbit antibody against
HBcPreS1PreS2S145R
125I - labelled sheep anti-mouse
immunoglobulin
Solid phase

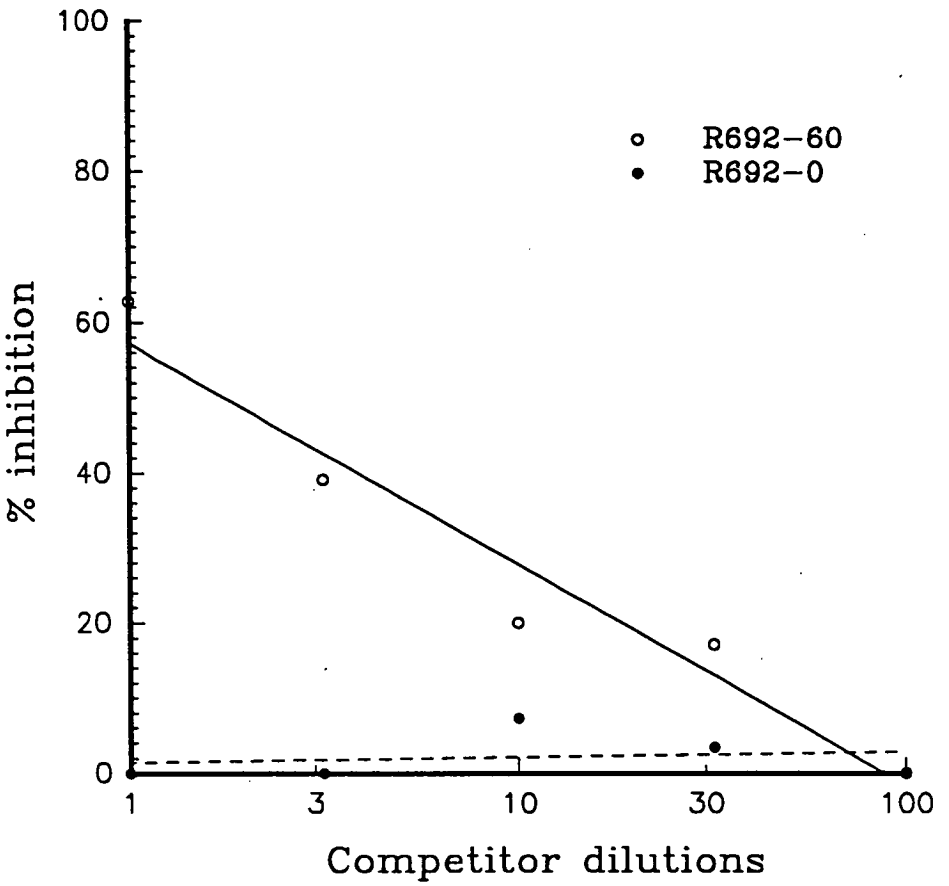


Figure 5.10 Competition between anti-HBcPreS$_1$PreS$_2$S$_{145R}$ and anti-HBs antibodies for the HBs$_{(111\text{-}156)}$-related epitope on HBsAg. Polystyrene beads coated with HBsAg were incubated with 100 μl of $^1/_{12.5}$ dilution of mouse anti-HBs serum along with 100 μl of the indicated dilution of R692-60 (11 days after the second boost with HBcPreS$_1$PreS$_2$S$_{145R}$) or R692-0 (preimmunised) serum as the competitor. The binding of mouse anti-HBs serum to HBsAg was monitored by addition of ^{125}I-labelled sheep anti-mouse immunoglobulin.

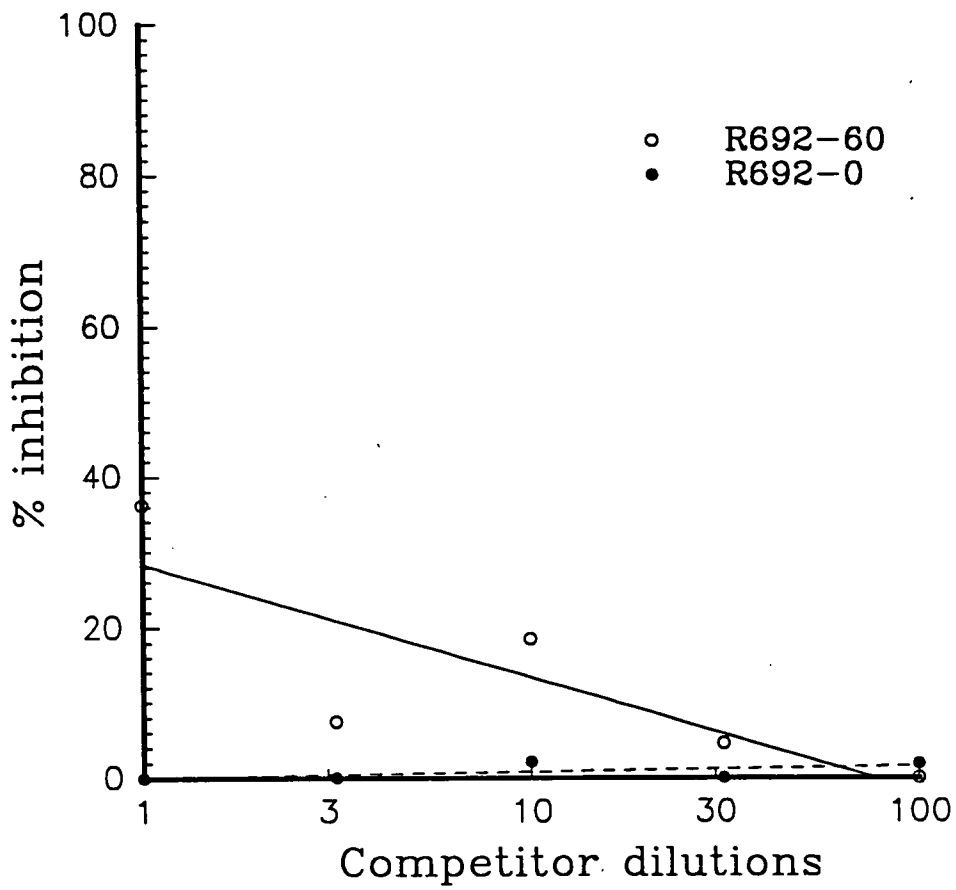


Figure 5.11 Competition between anti-HBcPreS$_1$PreS$_2$S$_{145R}$ and anti-HBs antibodies for the HBs$_{(111\text{-}156)}$ peptide epitope. Polystyrene beads coated with HBs$_{(111\text{-}156)}$ peptide were incubated with 100 μl of $^1/_{12.5}$ dilution of mouse anti-HBs serum along with 100 μl of the indicated dilution of R692-60 (11 days after the second boost with HBcPreS$_1$PreS$_2$S$_{145R}$) or R692-0 (preimmunised) serum as the competitor. The binding of mouse anti-HBs serum to the HBs$_{(111\text{-}156)}$ was monitored by addition of ^{125}I-labelled sheep anti-mouse immunoglobulin.

antibodies cross-reactive with native HBsAg at a lesser extent. Anti-$preS_{1(1-23)}$ and anti-$preS_{2(1-26)}$ antibodies could also be detected in those antibody. They also elicited antibodies cross-reactive with native HBsAg at a lesser extent. Anti-$preS_{1(1-23)}$ and anti-$preS_{2(1-26)}$ antibodies could also be detected in those immunised with HBcAg fusion proteins carrying $preS_1$ and $preS_2$ fragments.

5.5.1 T-cell epitopes within the HBcAg fusion protein

In mice, several synthetic peptides derived from the HBcAg sequence induced proliferation of HBcAg-primed T lymphocytes from several congenic strains, but HBcAg was more efficient in eliciting proliferation of HBcAg-specific T lymphocytes than were the synthetic peptides (Milich *et al.*, 1987b). Reciprocally, Immunisation of mice with the synthetic peptides elicited T-cells that recognised native HBcAg. However, the efficiency of the recognition varied from peptide to peptide and within responding strains, some peptide-primed T cells responded preferentially to the peptide. T-cell epitopes are localised between amino acid residues 85 and 140, depending on the H-2 haplotype of mouse strain. For example, $H\text{-}2^s$ and $H\text{-}2^b$ strains recognised amino acids 120-140, $H\text{-}2^f$ and $H\text{-}2^q$ strains recognised amino acids 100-120, and $H\text{-}2^d$ strain recognised 85-100 predominantly. In humans, the dominant T-cell epitopes of HBcAg were identified within amino acids 111-130 and 51-70 from patients with chronic HBV infection (Daniels *et al.*, 1992). Significantly more patients with CAH than with CPH or normal controls showed T-cell proliferative response to amino acids 111-130. In contrast, three out of six patients with CPH and none of those with CAH responded to amino acids 51-70.

The results obtained in the studies described here show that in rabbits the hybrid HBcAg particle consisting of amino acids 3-144 of HBcAg as well as the epitopes from HBV envelope proteins was an efficient immunogen in terms of T-cell activation as measured by the *in vitro* lymphocyte-proliferative response to HBcAg and HBcAg fusion protein. Since the HBsAg-specific T-cell response was much weaker and less consistent than HBcAg-specific T-cell response, the high response to the HBcAg

fusion protein was most likely attributable to the HBcAg sequence within the hybrid particle. It has been shown that HBcAg was approximately 100-fold more efficient than HBsAg in eliciting T-cell response in mice (Milich *et al.*, 1987b). In high HBcAg-responsive strains of mice, HBcAg-primed T-cells isolated from the popliteal lymph nodes responded to HBcAg *in vitro* at a concentration as low as 0.03 ng/ml, which was equivalent to 0.0014 nM. In the work described here, rabbits immunised with the HBcAg fusion proteins responded *in vitro* to HBcAg at a concentration only as low as 0.0625 μg/ml, which corresponded to 2.98 nM. Although the T-cell response to HBcAg seemed to be less efficient in rabbits than in mice, the different T-cell response might be due to the differences in species, the dose for immunisation, the injection route, or the source of T cells for the *in vitro* assay. It is also noteworthy that HBcAg-specific T-cell response was much higher than HBsAg- or $preS_1$-specific T-cell response in rabbits immunised with the HBcAg fusion proteins, which is consistent with the previous results from mice (Milich *et al.*, 1987b) and humans (Ferrari *et al.*, 1990).

Ferrari *et al.* (1990) found that patients with acute hepatitis B showed high levels of proliferative response to HBcAg and HBeAg, while in patients with chronic HBV infection the levels of T-cell response to HBcAg and to HBeAg were lower than those observed during acute infection. However, T-cell responses to the S protein, $preS_1$ and $preS_2$ peptides were only rarely and transiently present, and then only at low levels, during acute and chronic infections. Furthermore, that the time of appearance of a detectable T cell response to HBcAg is correlated with the time of clearance of HBsAg from the serum suggests that the amplification of the anti-HBs B cell response might occur via a direct cell-cell interaction of HBcAg-specific T cells with HBsAg-specific B cells through the "intermolecular-intrastructural" mechanism described by Milich *et al.* (1987c). In addition to this mechanism, HBsAg-specific T and B cell responses might be augmented by the release of antigen-nonspecific lymphokines by activated HBcAg-specific T cells. This mechanism was also observed in B10.S mice, a HBsAg non-responder strain, immunised with HBcAg and HBsAg simultaneously in the work herein (Table 5.23). In mouse experiments, splenocytes from BALB/c or

B10.S mice primed with HBcAg followed by challenge with HBcPreS_1PreS_2S elicited weak lymphocyte-proliferative responses to HBcAg and the HBcAg fusion protein, as did the mice injected with HBcAg or HBcPreS_1PreS_2S only (data not shown). Nevertheless, no lymphocyte-proliferative response to HBsAg, preS_1 or preS_2 peptide was observed in any group of mice examined (data not shown).

Unlike the conformational B-cell epitopes, T-cell epitopes on the major protein are sequential. Reduced, alkylated, formic acid-denatured HBsAg as well as denatured major protein (P25) isolated from HBsAg and tryptic fragments of P25 were all recognised by HBsAg-specific T cells, when used for immunisation, for they elicited an HBsAg-specific T-cell response (Milich *et al.*, 1985c). In attempts to identify the sequences recognised by HBsAg-primed T cells from congenic strains of mice, several synthetic peptide analogues were screened for their ability to induce proliferation of these cells. The active peptides were distinct for different congenic strains of mice. The peptides encompassing amino acids 38-52, 95-109, 110-137 and 140-154 of the major protein were found to elicit T cell proliferation (Milich *et al.*, 1985c). However, these peptides were much less efficient in eliciting T-cell proliferation than were equimolar quantities of HBsAg. The same authors also reported that peptides S(110-137) and S(38-52) stimulated T cells isolated from some HBV vaccinees.

In humans, Celis *et al.* (1988) used $CD4^+$ cell lines isolated from HBV vaccinees to localise the dominant T cell epitope near the N-terminus of the major protein. A single peptide S(4-33) contained a dominant T cell epitope and was highly efficient in eliciting T cell proliferation to the same extent as equimolar concentrations of formic acid-denatured HBsAg. The shortest peptide carrying a dominant epitope recognised by both helper and cytotoxic T cells was S(19-28) having the sequence with 9 amino acids common to all HBV subtypes. Recently, Greenstein *et al.* (1992) established 54 human T cell lines from individuals immunised with recombinant HBsAg vaccines. It was found that the peptide S(19-33) elicited significant proliferation in T cell lines derived from 50 donors. This universal T-cell epitope, which was recognised in donors of many different HLA-DR and HLA-DQ haplotypes,

was employed to construct a chimaeric peptide containing S(19-33) and the third V region loop structure (V3 loop) of HIV-1 envelope gp120. The BALB/c mice immunised with this chimaeric peptide were found to elicit potent S(19-33)-specific T-cell responses and anti-V3 loop antibodies that bound to the native gp120 (Greenstein *et al.*, 1992). In the work described here, ten out of thirteen rabbits immunised with HBcAg fusion proteins carrying the $HBs_{(111-156)}$ fragment responded to native HBsAg (S.I.≥2.1) *in vitro* in some stage of the experiments. However, the HBsAg-specific T-cell responses were variable among rabbits. That the $HBs_{(111-156)}$-primed rabbits elicited an HBsAg-specific T-cell response suggests that the T-cell epitope for rabbits, whether dominant or not, existed within $HBs_{(111-156)}$ sequence. Reciprocally, whether immunisation with native HBsAg in rabbits could elicit T cells that would recognise $HBs_{(111-156)}$ peptide remains unknown. The result from R701 also shows that denatured HBsAg-primed T-cells could recognise native HBsAg *in vitro*.

Several synthetic peptides [$preS_{1(1-21)}$, $preS_{1(12-32)}$, $preS_{1(32-53)}$, $preS_{1(53-74)}$ and $preS_{1(94-117)}$] from the $preS_1$ region of the *adw*$_2$ subtype, which contains 11 more amino acids at the N-terminus compared with the *adyw* subtype used in the studies described here, elicited the proliferation of T cells from mice primed with HBsAg containing the $preS_1$ sequence (Milich *et al.*, 1986a). The highest proliferation was elicited by peptide $preS_{1(12-32)}$. In reciprocal experiments, T cells from mice primed with $preS_{1(12-32)}$ or $preS_{1(94-117)}$ were induced to proliferate by both the homologous peptide and HBsAg containing the $preS_1$ sequence (Milich *et al.*, 1987a). The smallest peptide that elicited an efficient, $preS_1$-specific, T-cell proliferative response *in vitro* was $preS_{1(12-21)}$. In human studies, 7 out of 11 individuals immunised with plasma-derived HBsAg vaccines containing low amounts of $preS_1$ and $preS_2$ antigens showed detectable levels of T-cell response to $preS_1$ (Ferrari *et al.*, 1992). Two peptides spanning residues 21-48 of the $preS_1$ region (21-30 and 29-48) were stimulatory for 4 and 3 distinct subjects, respectively. Two further T-cell recognition sites within $preS_1$ for humans were defined by peptides 81-95 and 94-108. The T-cell epitope defined by $preS_{1(94-108)}$ in humans was also identified in the murine study (Milich *et al.*, 1985c). In the work described here, 5 out of 9 rabbits immunised with HBcAg fusion proteins carrying the

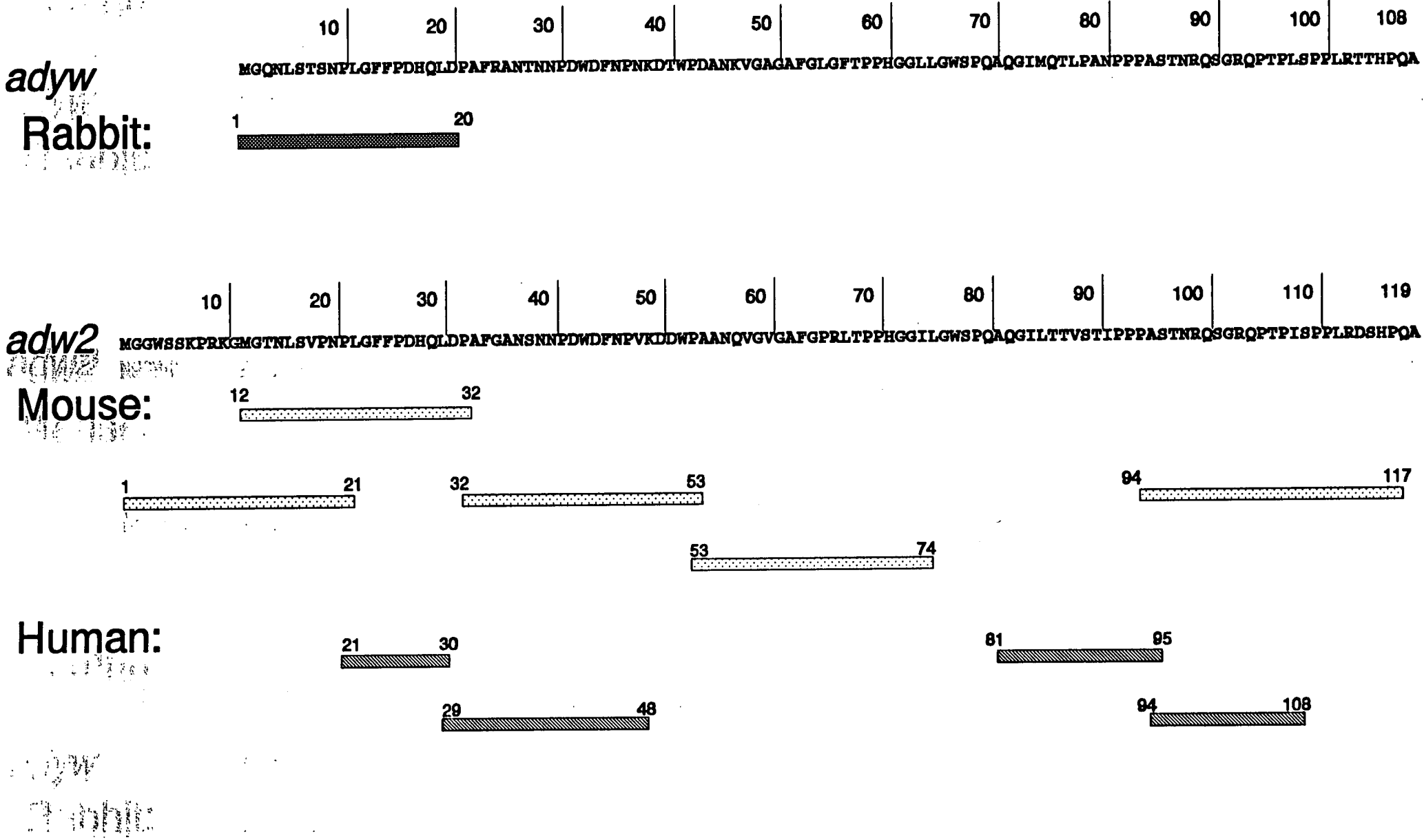
$preS_{1(1-20)}$ fragment, which corresponded to $preS_{1(12-31)}$ of *adw*$_2$ subtype used in the previous reports (Milich *et al.*, 1986a and 1987a; Ferrari *et al.*, 1992), elicited T-cell responses to the $preS_{1(1-23)}$ peptide. These results indicate the existence of the T-cell epitope for rabbits within $preS_{1(1-20)}$, which was identified in the murine system (Milich *et al.*, 1987a). The T-cell epitopes described above are shown in Figure 5.12.

In regard to $preS_2$ region, HBsAg/p33-primed T cells from a number of murine strains were induced to proliferate by peptides from the C-terminal half of the $preS_2$ region encompassing residues 29-55, within which 17 distinct T-cell sites were identified (Milich *et al.*, 1990a). The fine specificity of T-cell recognition of $preS_2$ was dependent on the H-2 haplotype of the responding strain and was subtype specific. Reciprocally, the ability of these synthetic peptides to elicit T-cells cross-reactive with the native HBsAg/p33 was variable and depended on the nature of the immunising peptide. Although $preS_{2(1-26)}$ peptide contains the neutralising B-cell epitope (Neurath *et al.*, 1986) and the T-cell epitope in mice (Milich *et al.*, 1986c), the inability of $preS_{2(1-26)}$-primed T cells to recognise the native $preS_2$ region indicates that T-cell recognition sites on the native $preS_2$ region are not identical to those on the synthetic $preS_{2(1-26)}$ peptide. In the work described here, none of the rabbits immunised with the HBcAg fusion proteins carrying the $preS_{2(1-26)}$ fragment elicited T-cell response to the corresponding peptide *in vitro*, suggesting that the T-cell epitopes recognised by rabbits and by mice may be different or T-cell epitopes on the $preS_{2(1-26)}$ sequence within the HBcAg fusion proteins may not be identical to those on the synthetic $preS_{2(1-26)}$ peptide. The immunodominant T-cell sites within $preS_2$ region for humans remain to be identified.

5.5.2 B-cell epitopes within the HBcAg fusion proteins

All the HBcAg fusion proteins elicited high levels of anti-HBc antibody, indicating the fusion of peptide epitopes at the C-terminus of the truncated HBcAg consisting of residues 3-144 did not change the antigenicity (see Chapter 4) and immunogenicity of HBcAg. Furthermore, the high anti-HBc response was correlated with high

Figure 5.12 T-cell epitopes of the $preS_1$ region. The amino acid sequences of the $preS_1$ region for *adyw* and *adw2* subtypes are shown in the single-letter code. Lines below the sequences denote T-cell epitopes identified in rabbit (this study), mouse (Milich *et al.*, 1985c) and humans (Ferrari *et al.*, 1992).


adyw
10 20 30 40 50 60 70 80 90 100 108
MGQNLSTSNPLGFFPDHQLDPAFRANTNNPDWDFNPNKDTWPDANKVGAGAFGLGFTPPHGGLLGWSPQAQGIMQTLPANPPPASTNRQSGRQPTPLSPPLRTTHPQA
Rabbit:
1 20
adw2
10 20 30 40 50 60 70 80 90 100 110 119
MGGWSSKPRKGMGTNLSVPNPLGFFPDHQLDPAFGANSNNPDWDFNPVKDDWPAANQVGVGAFGPRLTPPHGGILGWSPQAQGILTTVSTIPPPASTNRQSGRQPTPISPPLRDSHPQA
Mouse:
12 32
1 21
32 53
94 117
53 74
Human:
21 30
81 95
29 48
94 108

HBcAg-specific T cell response in rabbits, which is in agreement with previous results in mice (Milich and McLachlan, 1986, Milich *et al.*, 1987b). An immunodominant, linear HBcAg determinant was mapped to amino acids 107-118, as the corresponding peptide reacted with human anti-HBc sera and mouse anti-HBc MAbs. In addition, the peptide elicited antibodies that blocked the binding of polyclonal human anti-HBc to HBcAg (Colucci *et al.*, 1988). A single conformational determinant around amino acid 80 of HBcAg was also identified (see Section 4.5; Salfeld *et al.*, 1989).

Most HBsAg B-cell epitopes, either group or subtype specific, are dependent on the conformation of HBsAg particles, because they are destroyed by reduction and alkylation (Sukeno *et al.*, 1972; Vyas *et al.*, 1972). The major protein is rich in cysteine residues (in residues 48, 65, 69, 76, 90, 107, 121, 124, 137, 138, 139, 147, 149 and 221), some of which are believed to contribute to the conformation of HBsAg particles and also to that of the P25 molecule by making inter- and intra-molecular disulphide bonds. A number of synthetic peptides derived from the major protein have been evaluated for the ability to raise antibodies that could recognise native HBsAg, in attempts to develop synthetic vaccines. Most of the studies with synthetic peptides have focused on the region encompassing residues 110-150 of the major protein as this represents one of the two relatively more hydrophilic domains on the protein. The HBs sequence used for fusion to HBcAg in the work described here also encompasses residues 111-156. The peptide containing residues 138-149 of the major protein, with four cysteine residues replaced by α-aminobutyric acid in order to prevent polymerisation and other side reactions, when conjugated onto human erythrocytes and injected in mice, was found to induce anti-HBs antibody (Prince *et al.*, 1982). Moreover, it was shown that residues 139-147 represented the total or an essential part of the *a* determinant of HBsAg (Bhatnagar *et al.*, 1982). However, the antigenicity and immunogenicity (after coupling to KLH) of the peptide were very low in comparison with native HBsAg. The level of anti-HBs antibody elicited by peptide 135-155 conjugated to a carrier protein was also low as compared with anti-HBs level elicited by native HBsAg (Neurath *et al.*, 1982). In the studies described here, the levels of anti-HBs antibody response elicited by HBcAg fusion proteins carrying the

$HBs_{(111-156)}$ fragment in rabbits and in BALB/c mice were also lower than those elicited by native HBsAg. It proved difficult to mimic these conformational epitopes by linear synthetic peptides. This problem was partially overcome by using cyclic peptides generated by intra-molecular disulphide bond formation between cysteine residues present in the peptides (Dreesman *et al.*, 1982; Brown *et al.*, 1984).

Two cyclic peptides containing disulphide bonds in the region between residues 117-137 and 122-137 were shown to elicit an anti-HBs response in mice after a single injection without linkage to a protein carrier (Dreesman *et al.*, 1982). The cyclic peptide 122-137 was also demonstrated to contain an *a* antigenic determinant that was dependent on conformation and a sequential *y* subtype epitope (Ionescu-Matiu *et al.*, 1983). The *a* epitope contained within residues 139-147 appears to be a conformational one since a cyclic form of the corresponding synthetic peptide was recognised with higher affinity than the linear analogue (Brown *et al.*, 1984). Although cyclic peptides as putative *a* epitope mimetics have been reported, there is evidence to suggest that this may not represent the whole picture. Gerin *et al.* (1983) found that the *a* determinant was composed of at least three nonoverlapping sequences. Recently, Manivel *et al.* (1992a and 1992b) showed that a synthetic peptide containing 124-147 of the major protein spontaneously self-oligomerised to reconstitute three distinct *a* epitopes that included one between residues 124-137, another between residues 139-147, and the third epitope including residues met_{133} and lys_{141}.

Studies in the $preS_1$ region (adw_2 subtype with 119 residues) localised residues 32-53 and 94-117 as antibody binding sites for humans and mice, as well as 1-21 and 12-32 for additional sites for humans, as the corresponding synthetic peptides, when used as solid phase antigens, bound sera from mice immunised with P39/HBsAg or from humans collected during or soon after acute HBV infection (Milich *et al.*, 1986a). Attachment of purified HBsAg particles to cultured hepatoma cells (Neurath *et al.*, 1985) or isolated plasma membranes from fresh human liver (Pontisso *et al.*, 1989a) has been described and attributed to amino acids 21-47 in the $preS_1$ domain of the

large protein (Neurath *et al.*, 1986; Pontisso *et al.*, 1989b). When conjugated to a carrier protein, $preS_{1(21-47)}$ elicited antibodies protective against HBV infection in chimpanzees (Neurath *et al.*, 1989; Thornton *et al.*, 1989).

The unconjugated peptide containing the complete 55 amino acid residues of the $preS_2$ region (*adw* subtype) has been shown to protect 4 out of 4 chimpanzees from HBV infection of either the *ad* or *ay* subtypes (Emini *et al.*, 1989). Residues 1-26 of the $preS_2$ region have been shown to represent a dominant antibody binding site (Neurath *et al.*, 1984; Milich *et al.*, 1985d), and Thornton *et al.* (1987), using $preS_{2(1-26)}$ conjugated to KLH for immunisation, reported that chimpanzees were protected against HBV infection. Itoh *et al.* (1986), using $preS_{2(14-32)}$ also conjugated to KLH, demonstrated protection in two chimpanzees. T-cell studies by Milich *et al.* (1986c) with these two peptides showed that the T-cell epitope resided within residues 1-13 and the B-cell epitope was located in the 14-26 region, indicating that $preS_{2(1-26)}$ contained both T- and B-cell epitopes, whereas $preS_{2(14-32)}$ contained only a B-cell epitope and would require a carrier protein. Indeed, it has been shown that free $preS_{2(1-26)}$ emulsified in oil was protective against HBV infection in 2 chimpanzees (Thornton *et al.*, 1989). Cumulatively, these results indicate that synthetic peptides derived from either the $preS_1$ or the $preS_2$ region can individually elicit protection against HBV infection. The $preS_1$ fragment used in the studies described here encompasses residues 1-20 of an *adyw* subtype with 108 residues, which corresponds to residues 12-31 of an adw_2 subtype. The rabbits and BALB/c mice immunised with HBcAg fusion proteins carrying the $preS_{1(1-20)}$ and $preS_{2(1-26)}$ fragments elicited antibodies against the corresponding peptides.

5.5.3 Enhanced immunogenicity of the HBcAg fusion proteins

HBcAg-specific T cell response may provide functional help for the production of the anti-envelope antibodies. The inclusion of $preS_1$ and $preS_2$ epitopes to the HBcAg fusion proteins appeared to enhance the immunogenicity in terms of anti-HBs production.

5.5.3.1 T-cell help for antibody production

In the studies described here, very efficient T-cell responses specific to HBcAg and HBcAg fusion protein were elicited by the rabbits immunised with the HBcAg fusion proteins. However, only moderate to weak HBsAg- and $preS_1$-specific T cell responses were induced, and no $preS_2$-specific T cell response was observed. Nevertheless, antibodies against $preS_{1(1\text{-}23)}$ and $preS_{2(1\text{-}26)}$ peptides were produced in these rabbits. These results suggest that the source of T-cell help for the anti-$preS_2$ production was attributed to the HBcAg-specific T cells. Furthermore, in R699 and R700, anti-$preS_{1(1\text{-}23)}$ antibody was produced despite the failure to activate $preS_1$-specific T cells; the HBcAg-specific T cells might contribute Th-cell function for anti-$preS_1$ antibody production. Similarly, the results from some rabbits, eg. R675, R676, R700, R792 and R703, also suggest that the HBcAg-specific T cells might provide Th function for the production of anti-HBs antibody. However, in R703, a $preS_1$-specific T cell response was induced, but no anti-$preS_{1(1\text{-}23)}$ antibody was produced. In R702, the absence of $preS_1$-specific T cells correlated with the absence of anti-$preS_1$ antibody. The variable responses were most likely attributed to the different haplotypes among outbred rabbits used in the studies. Recently, it was reported that immunisation of mice with hybrid HBc/preS particles exclusively primed Th cells specific for HBcAg and not for the inserted epitopes, even if the inserted sequence contained a T-cell epitope for that strain of mice (Schodel *et al.*, 1992). This result suggests that the mere inclusion of a T-cell epitope in the inserted sequence does not guarantee that it will be functional in the context of the hybrid particles. One possible explanation is that the T-cell epitope within the hybrid HBcAg particles may be processed differently from that within the native protein. The other explanation is that intramolecular competition between T-cell epitopes might occur (Perkins *et al.*, 1991).

It has been indicated that immunodominance of the T cell response is not simply a function of the primary amino acid sequence, but is a function of the context of the epitope within the protein molecule (Perkins *et al.*, 1991). It has also been suggested that immunogens are probably polar, since the orientation between the T- and B-cell

epitopes in a synthetic immunogen could affect immunogenicity (Golvano *et al*,. 1990; Milich *et al.*, 1990c). Therefore, the orientation between the T- and B-cell epitopes within the HBcAg fusion proteins might result in the difference in immunogenicity. Nevertheless, it appears that the ability of the HBcAg to induce a strong HBcAg-specific Th-cell response in the context of multimeric B-cell epitopes may explain the carrier effects of HBcAg particles.

The T cell-independent nature of HBcAg raises the possibility of immunisation to immunodeficient patients with HBcAg fusion proteins carrying peptide epitope(s). It was demonstrated that anti-HBc and anti-HRV2 (Human rhinovirus type 2) antibodies were elicited in outbred nude mice immunised with HBcAg fusion proteins carrying peptide epitope HRV2, although the immunogenicity was much poorer than that seen in euthymic mice (Francis *et al.*, 1990). HBcAg fusion proteins carrying $preS_{1(12-47)}$ sequence at the N-terminus through a pre-core linker sequence also elicited low levels of anti-HBc and anti-$preS_1$ antibodies in BALB/c (*nu/nu*) athymic mice (Schodel *et al.*, 1992).

5.5.3.2 Inclusion of $preS_1$ and $preS_2$ epitopes to the HBcAg fusion proteins

The results presented in this work indicate that $HBcPreS_1PreS_2S$ was superior to HBcS in terms of the titres and onset of production of anti-HBs antibody, suggesting that the inclusion of $preS_1$ and $preS_2$ epitopes to the HBcAg fusion proteins enhanced the immunogenicity with regard to anti-HBs production. However, HBcAg fusion proteins carrying the $preS_1$ and $preS_2$ as well as a dimer of $HBs_{(111-156)}$ sequences, ie. $HBcSPreS_1PreS_2S$ did not enhance, and even decreased the immunogenicity in eliciting anti-HBs antibody compared with those carrying $preS_1$ and $preS_2$ as well as a single $HBs_{(111-156)}$ sequence, ie. $HBcPreS_1PreS_2S$. The possible explanation for the enhanced immunogenicity of $HBcPreS_1PreS_2S$ might be that the $HBs_{(111-156)}$ fragment in the context of the fusion particle might be more surface accessible than that in HBcS, as $HBcPreS_1PreS_2S$ appeared to be more potent than HBcS in terms of HBs antigenicity, which rendered $HBcPreS_1PreS_2S$ more immunogenic than HBcS in inducing anti-HBs

antibody. The double insertion of the $HBs_{(111-156)}$ sequence in the HBcAg fusion proteins might also result in a different arrangement of the antigenic sites, such as a decrease of the surface exposure, which did not favour the induction of the anti-HBs antibody, since the surface exposure of the antigenic fragment usually correlates well with its capacity to induce antibody against the corresponding antigenic site. With regard to the anti-peptide antibodies against $preS_{1(1-23)}$ and $preS_{2(1-26)}$ induced by the HBcAg fusion proteins containing $preS_1$ and $preS_2$ fragments, only one out of three rabbits immunised with HBcAg fusion proteins carrying a dimer of the $HBs_{(111-156)}$ as well as the $preS_1$ and $preS_2$ fragments produced anti-$preS_1$ and anti-$preS_2$ antibodies, while all rabbits immunised with HBcAg fusion proteins carrying a monomer of the $HBs_{(111-156)}$ as well as the $preS_1$ and $preS_2$ fragments produced anti-peptide antibodies. Again, the $preS_1$ and $preS_2$ sequences in the context of $HBcSPreS_1PreS_2S$ might cause structural constraints that limit surface exposure of these antigenic sites.

It has been reported that proteins containing multiple copies of the peptide encompassing amino acids 137 to 162 of VP1 from FMDV fused to the N-terminus of β-galactosidase were greatly superior to that containing a single copy of the peptide in eliciting anti-peptide and neutralising antibody (Broekhuijsen *et al.*, 1987). The addition of a cysteine residue to the C-terminus of uncoupled synthetic FMDV peptides resulted in the formation of dimers, and also enhanced their immunogenicity (Francis *et al.*, 1987a). Therefore, the enhanced immunogenicity of the peptide fused to HBcAg could also result from the increased epitope density presented on the core particles. However, double copies of the $HBs_{(111-156)}$ sequence within HBcAg fusion proteins did not further improve the immunogenicity of the particles in the work described here.

Clarke *et al.* (1987) compared the immunogenicity of the peptide encompassing amino acids 141-160 of the VP1 protein of FMDV in different presenting systems. On a weight for weight basis, the peptide on HBV core particles was about 30-40 times more immunogenic than a peptide/β-galactosidase fusion protein containing tandem copies of the FMDV epitope, 500 times more immunogenic than free synthetic

peptide, and almost as immunogenic as inactivated FMDV particles. It has also been demonstrated that rhinovirus peptide/HBcAg fusion proteins were 10 times more immunogenic than the peptide coupled to KLH and 100 times more immunogenic than uncoupled peptide with an added Th-cell epitope (Francis *et al.*, 1990). Within the HBcAg particles, the position of the inserted epitope affected the immunogenicity of the hybrid particles. Recently, Schodel *et al.* (1992) showed that insertion of a $preS_1$ epitope between amino acids 75 and 83 of HBcAg, which had previously been suggested to represent a dominant antibody-binding site, resulted in the most efficient anti-$preS_1$ antibody response compared with C- and N-terminal fusions. In addition, the N-terminal fusion through a pre-core linker sequence was more immunogenic than the C-terminal fusion to the truncated HBcAg at amino acid 156. A $preS_1$ epitope fused directly to the N-terminus of the full-length HBcAg was not surface accessible and not immunogenic. It has also been shown that the HBcAg fusion proteins carrying insertions at the N-terminus through a pre-core sequence and internal fusions between amino acid 80 and 81 of HBcAg both induced good anti-peptide antibody responses, whereas those carrying insertions at the C-terminus of the full-length HBcAg did not induce anti-peptide antibody (Yon *et al.*, 1992). Since different epitopes were fused to the HBcAg particles as well as different species and immunisation regimens were used, it is difficult to compare the immunogenicity data presented in this work and those described earlier.

Apart from the use of CFA and IFA as adjuvants, Francis *et al.* (1990) compared the immunogenicity of HBcAg fusion proteins in IFA, aluminum hydroxide, which is acceptable for use as an adjuvant in humans, and in the absence of adjuvant. Although IFA gave the greatest response after primary immunisation, aluminum hydroxide was a suitable adjuvant after single reinoculation. Furthermore, the hybrid HBcAg particles were also immunogenic in the absence of adjuvant although the primary response was about 10-fold lower than the responses to the adjuvanted preparations. Therefore, adjuvantation probably does not cause problems for the practical application of HBcAg fusion protein as vaccines. It has been reported that HBcAg fusion proteins carrying rhinovirus epitopes could elicit antibody responses

against HBcAg and the fused peptide after nasal or oral administration to BALB/c mice (Francis *et al.*, 1990). Schodel *et al.* (1990) also showed that mice orally fed with the recombinant *Salmonella* expressing $HBc_{(111-155)}/preS_{2(14-24)}$ fusion protein developed high titres of serum antibodies against HBcAg and lower titres of antibodies to $preS_2$. These results suggest that it may be possible to use HBcAg as a carrier for the development of synthetic peptide-based oral vaccines.

5.5.4 Antibody responses to fusions carrying wild-type and mutant HBs segments

The HBcAg fusion proteins carrying the $HBs_{(111-156)}$ fragments (derived from *adyw* subtype, but exhibiting *y* subtype), either wild-type or 3 gly_{145} mutants, in a monomer or a dimer, induced anti-peptide response that cross-reacted equally with native HBsAg of both *ayw* and *adw* subtypes, suggesting that the anti-$HBs_{(111-156)}$ peptide antibodies were directed toward a group-specific epitope on HBsAg. Furthermore, rabbit anti-HBs antibody raised against native HBsAg reacted with $HBs_{(111-156)}$ peptide corresponding to the inserted HBs fragment in the hybrid HBcAg particles. The immunological cross-reactivity between antisera raised against HBcAg fusion proteins carrying the $HBs_{(111-156)}$ fragment and against native HBsAg was further assessed by two competition RIAs. First, antiserum raised against HBcAg fusion protein carrying the $HBs_{(111-156)}$ fragment inhibited the binding of anti-HBs antibody to HBsAg. Secondly, antiserum raised against HBcAg fusion protein carrying the $HBs_{(111-156)}$ fragment also inhibited the binding of anti-HBs antibody to $HBs_{(111-156)}$ peptide. Collectively, these results indicate that polyclonal anti-HBs antibody and polyclonal anti-$HBs_{(111-156)}$ antibody raised against HBcAg fusion protein carrying the $HBs_{(111-156)}$ fragment recognise either a common or overlapping determinant on the $HBs_{(111-156)}$ sequence. Since HBV cannot be propagated easily *in vitro* and standard virological techniques of detecting neutralising antibody are not available, chimpanzee-challenge studies are the only way to evaluate whether the anti-peptide antibodies induced by HBcAg fusion proteins containing the $HBs_{(111-156)}$ or/and $preS_{1(1-20)}$ and $preS_{2(1-26)}$ fragments are virus-neutralising.

The emergence of an escape mutant that has undergone glycine to arginine substitution at amino acid 145 in the major protein of the HBsAg, under pressure of an HBV vaccine-induced response, indicates that the current vaccines may induce a host response that is lacking in some component(s) induced during recovery from natural HBV infection. Recently, this escape mutant has been shown to be capable of causing hepatitis in chimpanzees inoculated with serum (10^{-6} dilution) from the infected patient, which contained only the mutant virus detected by PCR (Ogata *et al.*, 1993). Waters *et al.* (1992) demonstrated that immunisation of mice with recombinant HBsAg carrying this mutation, although eliciting a high titres of antibody that recognised the mutant HBsAg, produced 100-fold lower antibodies recognised the wild-type HBsAg. The HBcAg fusion proteins containing gly_{145} mutant sequence of $HBs_{(111\text{-}156)}$, eg. $HBcS_{145R}$ and $HBcPreS_1PreS_2S_{145R}$, might be useful for evaluation of their efficacy to protect against this gly_{145} mutant virus. In addition, an ideal vaccine should induce a broader repertoire of protective antibodies against both wild-type and mutant viruses. One approach to achieve this goal would be combining various epitopes or different sequences of the same epitope, either on the same particle or in a cocktail of particles. In this respect, the HBcAg fusion proteins carrying one wild-type and one mutant sequence of the $HBs_{(111\text{-}156)}$ fragment, eg. $HBcSPreS_1PreS_2S_{145R}$, $HBcSPreS_1PreS_2S_{145E}$, $HBcS_{145R}PreS_1PreS_2S$, and $HBcS_{145E}PreS_1PreS_2S$, could be evaluated for their efficacy to protect chimpanzees against both wild-type and mutant HBV. By cotransformation with different plasmids carrying different fusion genes, it is possible to obtain mixed HBcAg particles presenting both wild-type and mutant fragments fused to different molecules of HBcAg, but assembled into the same particle. With the HBsAg system, it has been demonstrated that mixed particles presenting both authentic HBsAg and HBsAg fusion protein carrying poliovirus epitope elicited much higher antibody responses to HBsAg and poliovirus epitope than those elicited by the hybrid HBsAg particles consisting of the fusion protein only (Delpeyroux *et al.*, 1988). It is tempting to speculate that the enhanced immunogenicity of the mixed particles consisting of both HBsAg and HBsAg/polio fusion protein might be partly attributed to the "intermolecular-intrastructural" helper mechanism provided by the HBsAg-specific T cells.

In an attempt to induce a broadly cross-reactive neutralising antibody, another approach would be changing the antigenic specificity of the neutralising epitope by substitution of amino acid residue(s). However, this approach appears to be quite empirical. Ashton-Rickardt and Murray (1989b) demonstrated that the antigenic subtype of HBsAg altered from y^+d^- to y^+d^+ by combining the substitution of serine$_{113}$ by threonine with the replacement of arginine$_{122}$ by lysine, or of tyrosine$_{134}$ by phenylalanine, or both of these changes in the major protein of HBsAg. Recently, broadly cross-reactive CTLs capable of effector function against a wide range of HIV isolates have been generated by using substituted peptides within the V3 loop of gp160 of HIV-1, which contains an immunodominant determinant for CTLs, as *in vitro* stimulants for the spleen cells from mice immunised with gp160 (Takahashi *et al.*, 1992). The competitive binding studies described in this work show that the antibodies raised against HBcS, HBcS$_{145R}$, HBcS$_{145E}$, HBcS$_{145K}$ or HBcPreS$_1$PreS$_2$S$_{145R}$ recognised a common or overlapping epitope within the HBs$_{(111\text{-}156)}$ sequence as compared with anti-native HBs antibody. It would be of interest to examine the cross-reactivity of the antisera raised against HBcAg fusion proteins carrying gly$_{145}$ mutant sequence of the HBs$_{(111\text{-}156)}$ fragment with the gly$_{145}$→arg escape mutant HBsAg. The HBcAg fusion proteins carrying glu$_{145}$ or lys$_{145}$ mutant HBs$_{(111\text{-}156)}$ sequences, eg. HBcS$_{145E}$, HBcS$_{145K}$ and HBcPreS$_1$PreS$_2$S$_{145E}$ might be exploited in this direction. However, whether these HBcAg fusion proteins can induce a broadly cross-reactive neutralising antibody remains to be answered.

The work described here shows the enhanced immunogenicities of the HBV envelope epitopes fused to the C-terminus of the truncated HBcAg. The highest anti-HBs responses of the HBcAg fusion proteins were seen in rabbits immunised with HBcAg fusion protein carrying the HBs$_{(111\text{-}156)}$ fragment accompanied with preS$_{1(1\text{-}20)}$ and preS$_{2(1\text{-}26)}$ fragments. This HBcAg fusion protein, HBcPreS$_1$PreS$_2$S, consists of HBcAg as well as three different envelope epitopes from preS$_1$, preS$_2$, and S regions, which may induce an immune response that more closely mimics that induced after natural HBV infection. The high HBc-specific T-cell response may also augment the production of the antibodies against the three envelope epitopes. Although there is

no evidence from clinical trials to prove that HBV vaccines containing the S and preS regions are superior to those containing only the S protein, the use of HBcAg as a carrier for HBV envelope epitopes with or without introducing mutant sequences provides a tempting approach for the development of a multivalent vaccine against wild-type HBV and its escape mutants.

CHAPTER 6: CONCLUSION

HBcAg is a potent immunogen in terms of T cell activation and antibody production. Furthermore, HBcAg can induce antibody production via both T-cell dependent and T-cell independent pathways (Millich and McLachlan, 1986). It has been shown that HBcAg contains epitopes that stimulate Th cells in mice and that animals primed with HBcAg or particular peptides within this sequence when challenged with HBV produced antibodies to HBsAg, including the preS epitopes, as well as HBcAg (Milich *et al.*, 1987c). This result may well explain the rapid appearance of high levels of anti-HBs, which is virus neutralising, and elevated levels of anti-HBe in chimpanzees that had been immunised with HBcAg or HBcAg and HBeAg and subsequently challenged with HBV (Murray *et al.*, 1984 and 1987). HBcAg-specific Th cells could induce anti-S antibody through "intermolecular-intrastructural" Th cell-B cell interaction in mice that did not respond to the S protein provided that HBcAg and HBsAg be present within the same particle. Therefore, HBcAg may serve as a particulate, carrier protein for presentation of peptide epitopes.

Several gene fusions were constructed in which coding sequences for epitopes from the envelope proteins of HBV, including the S, $preS_1$ and $preS_2$ regions, were linked to those for the C- or both termini of truncated HBcAg. All plasmids expressing fusion proteins contain the coding sequence for amino acid residues 3-144 of HBcAg fused to C-terminus of the first 8 amino acid residues of β-galactosidase via a tripeptide linker sequence. The HBcAg fusion proteins carrying the epitopes at the C-terminus were expressed efficiently in *E. coli* and the products assembled to form particles morphologically similar to HBcAg itself and were soluble, which allowed easy purification. However, when HBcAg carried both the S epitope at the N-terminus as well as the $preS_1$ and $preS_2$ or/and the S epitopes at the C-terminus, ie., $SHBcPreS_1PreS_2$ or $SHBcPreS_1PreS_2S$, the fusion proteins, although still formed particles, were aggregated which hampered their purification. The largest HBcAg fusion protein containing 318 amino acid residues, in which 165 residues from the sequences of the S, $preS_1$ and $preS_2$ domains were attached at the C-terminus of residue 144 of HBcAg, compared with 183 of the normal HBcAg polypeptide, could still be tolerated to form core-like particles.

The emergence of an escape mutant with an amino acid substitution from glycine to arginine at amino acid residue 145 of HBsAg which cannot be neutralised by vaccine-induced anti-HBs antibody provided the impetus for constructing the fusions with the immunodominant region of the S protein, $HBs_{(111-156)}$, carrying mutations at residue 145 of HBsAg in which glycine was replaced by arginine, glutamic acid or lysine. On the basis of these mutant sequences, fusions were also constructed carrying a dimer of $HBs_{(111-156)}$, one from wild-type and the other from a mutant sequence, accompanied by $preS_1$ and $preS_2$ sequences, with the aim of creating multivalent immunogens based on HBcAg which might induce antibody with a wider specificity to confer immunity against HBV infection, including antigenic variants.

All HBcAg fusion proteins retained HBc antigenicity and immunogenicity in terms of T-cell activation and antibody response. In all cases animals immunised with the fusion proteins mounted strong T-cell-proliferative responses to HBcAg, which may provide Th cells for augmentation of antibody production against the inserted epitopes.

The HBcAg fusion proteins containing $HBs_{(111-156)}$, either wild-type or gly_{145} mutants, displayed HBs antigenicity in immunoblot analysis. The HBs antigenicity could also be detected, although weaker than the native HBsAg, within the HBcAg fusion particles in the antigen-capture RIA in which the particles were first trapped on the solid phase with human anti-HBc antibody and then examined for the presence of anti-HBs reactive epitopes, ie., the HBs antigenicity, on the surface of the particles using rabbit anti-HBs antiserum and radiolabelled second antibody (anti-rabbit immunoglobulin) as the probe. However, they showed little HBsAg antigenicity in a standard solid-phase RIA in which the HBsAg present on the fusion particles is sandwich-captured by two anti-HBs antibodies, one immobilised on the solid phase, the other radiolabelled as the probe.

The weak HBs reactivity of the HBcAg fusion proteins might be due to different presentation of the $HBs_{(111-156)}$ segment on the fusion particles from that on the native HBsAg. The $HBs_{(111-156)}$ segment within the hybrid HBcAg particles, however, could

elicit T-cells capable of recognising native HBsAg, although weaker and less consistent than those specific to HBcAg. Furthermore, the HBcAg fusion proteins carrying $HBs_{(111-156)}$ segment, not only wild-type but also three gly_{145} mutants, induced antibody responses cross-reactive with wild-type HBsAg determined by a standard solid-phase RIA in which anti-HBs present in the immune serum is sandwich-captured by two HBsAg molecules, one immobilised on the solid phase, the other radiolabelled as the probe. The competitive RIA demonstrates that polyclonal antibody against the HBcAg fusion proteins carrying $HBs_{(111-156)}$, either wild-type or gly_{145} mutants, and polyclonal antibody against native, intact HBsAg recognise either a common or overlapping determinant on the $HBs_{(111-156)}$ sequence. It would be of interest, therefore, to examine the cross-reactivity of the antibody raised against HBcAg fusion proteins carrying wild-type or gly_{145} mutant sequence of the $HBs_{(111-156)}$ segment with the naturally occurring gly_{145}→arg mutant HBsAg.

The HBcAg fusion proteins carrying the $preS_1$ and $preS_2$ or/and the S epitopes also elicited antibodies to the corresponding peptides, which might be useful for providing a broader repertoire of protective antibodies. Some immunised animals also elicited T-cell-proliferative responses to the $preS_1$ peptide. The HBcAg fusion proteins carrying the S as well as $preS_1$ and $preS_2$ epitopes, ie., $HBcPreS_1PreS_2S$, were superior to those carrying only the S domain in terms of the quantity and onset of anti-HBs production. Nevertheless, the fusions carrying a dimer of the S domain accompanied by the $preS_1$ and $preS_2$ epitopes, ie., $HBcSPreS_1PreS_2S$, appeared to be relatively poor immunogens in terms of antibody production against the inserted epitopes.

These experiments show that the sequence and position of the epitope fused to HBcAg may influence the physical and chemical properties of the hybrid particles as well as immunogenicity of the inserted epitope. Attempts to induce a broadly cross-reactive neutralising antibody against HBV, the use of HBcAg to present multiple epitopes from the S, $preS_1$ and $preS_2$ regions or to present altered neutralising epitope of the S protein may therefore be a practical approach. The results illustrate the potential value of such fusion proteins as vaccines against wild-type HBV and its variants.

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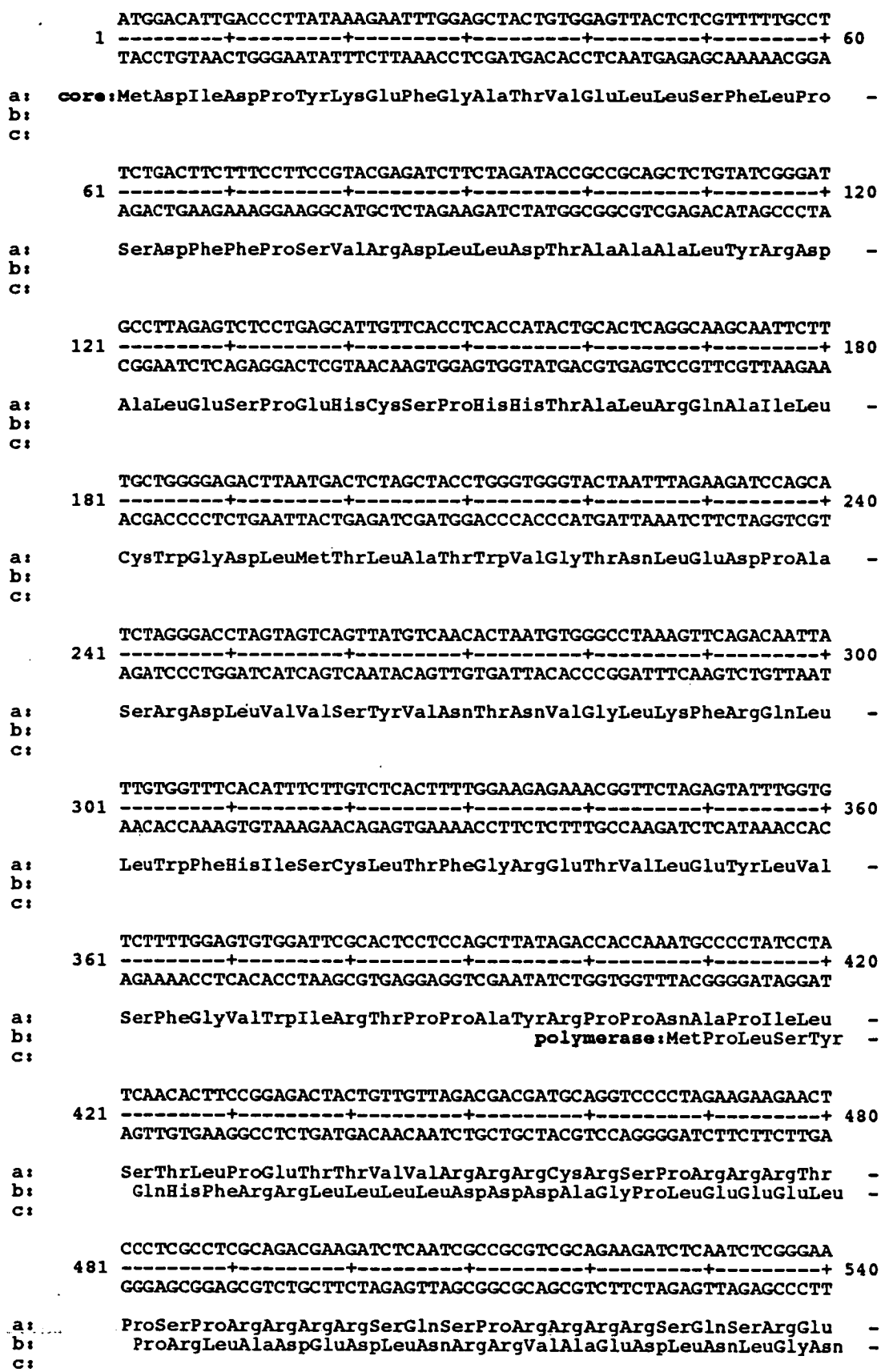
Appendix: Nucleotide sequence of HBV subtype *adyw*

The nucleotide sequence of HBV subtype *adyw* contained in plasmid pHBV130 (Pasek *et al.*, 1979; Gough and Murray, 1982; Pugh *et al.*, 1986) is shown. Both strands of the complete genome (3182 nucleotides) are shown with the sense strand on top. Numbering is by the convention of Pasek *et al.* (1979), which set number 1 at the start of the core ORF. The amino acid sequence of the viral ORFs is shown in their respective translational frames.

ORFs:

Proteins	nucleotide sequences	amino acid sequences
pre-core	3096-3182	frame c
core	1-549	frame a
polymerase	407-2903	frame b
$preS_1$	948-1271	frame c
$preS_2$	1272-1436	frame c
surface	1437-2114	frame c
X	2656-3117	frame c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1
TACCTGTAACTGGGAATATTTCTTAAACCTCGATGACACCTCAATGAGAGCAAAAACGGA
a: core:MetAspIleAspProTyrLysGluPheGlyAlaThrValGluLeuLeuSerPheLeuPro -
b:
c:
TCTGACTTCTTTCCTTCCGTACGAGATCTTCTAGATACCGCCGCAGCTCTGTATCGGGAT
61
AGACTGAAGAAAGGAAGGCATGCTCTAGAAGATCTATGGCGGCGTCGAGACATAGCCCTA
a: SerAspPhePheProSerValArgAspLeuLeuAspThrAlaAlaAlaLeuTyrArgAsp -
b:
c:
GCCTTAGAGTCTCCTGAGCATTGTTCACCTCACCATACTGCACTCAGGCAAGCAATTCTT
121
CGGAATCTCAGAGGACTCGTAACAAGTGGAGTGGTATGACGTGAGTCCGTTCGTTAAGAA
a: AlaLeuGluSerProGluHisCysSerProHisHisThrAlaLeuArgGlnAlaIleLeu -
b:
c:
TGCTGGGGAGACTTAATGACTCTAGCTACCTGGGTGGGTACTAATTTAGAAGATCCAGCA
181
ACGACCCCTCTGAATTACTGAGATCGATGGACCCACCCATGATTAAATCTTCTAGGTCGT
a: CysTrpGlyAspLeuMetThrLeuAlaThrTrpValGlyThrAsnLeuGluAspProAla -
b:
c:
TCTAGGGACCTAGTAGTCAGTTATGTCAACACTAATGTGGGCCTAAAGTTCAGACAATTA
241
AGATCCCTGGATCATCAGTCAATACAGTTGTGATTACACCCGGATTTCAAGTCTGTTAAT
a: SerArgAspLeuValValSerTyrValAsnThrAsnValGlyLeuLysPheArgGlnLeu -
b:
c:
TTGTGGTTTCACATTTCTTGTCTCACTTTTGGAAGAGAAACGGTTCTAGAGTATTTGGTG
301
AACACCAAAGTGTAAAGAACAGAGTGAAAACCTTCTCTTTGCCAAGATCTCATAAACCAC
a: LeuTrpPheHisIleSerCysLeuThrPheGlyArgGluThrValLeuGluTyrLeuVal -
b:
c:
TCTTTTGGAGTGTGGATTCGCACTCCTCCAGCTTATAGACCACCAAATGCCCCTATCCTA
361
AGAAAACCTCACACCTAAGCGTGAGGAGGTCGAATATCTGGTGGTTTACGGGGATAGGAT
a: SerPheGlyValTrpIleArgThrProProAlaTyrArgProProAsnAlaProIleLeu -
b: polymerase:MetProLeuSerTyr -
c:
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421
AGTTGTGAAGGCCTCTGATGACAACAATCTGCTGCTACGTCCAGGGGATCTTCTTCTTGA
a: SerThrLeuProGluThrThrValValArgArgArgCysArgSerProArgArgArgThr -
b: GlnHisPheArgArgLeuLeuLeuLeuAspAspAspAlaGlyProLeuGluGluGluLeu -
c:
CCCTCGCCTCGCAGACGAAGATCTCAATCGCCGCGTCGCAGAAGATCTCAATCTCGGGAA
481
GGGAGCGGAGCGTCTGCTTCTAGAGTTAGCGGCGCAGCGTCTTCTAGAGTTAGAGCCCTT
a: ProSerProArgArgArgArgSerGlnSerProArgArgArgArgSerGlnSerArgGlu -
b: ProArgLeuAlaAspGluAspLeuAsnArgArgValAlaGluAspLeuAsnLeuGlyAsn -
c:

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      541 ---------+---------+---------+---------+---------+---------+ 600
          AGAGTTACAATCATAGGGAACCTGAGTATTCCACCCTTTAAAATGACCCGAAATAAGAAG

a:        SerGlnCysEndTyrProLeuAspSerEnd(core end)
b:         LeuAsnValSerIleProTrpThrHisLysValGlyAsnPheThrGlyLeuTyrSerSer  -
c:

          TACTGTACCTGTCTTTAACCCTCATTGGAAAACGCCCTCTTTTCCTAATATACATTTACA
      601 ---------+---------+---------+---------+---------+---------+ 660
          ATGACATGGACAGAAATTGGGAGTAACCTTTTGCGGGAGAAAAGGATTATATGTAAATGT

a:
b:         ThrValProValPheAsnProHisTrpLysThrProSerPheProAsnIleHisLeuHis  -
c:

          CCAAGATATTATCAAAAAATGTGAACAGTTTGTAGGGCCGCTCACAGTCAATGAGAAAAG
      661 ---------+---------+---------+---------+---------+---------+ 720
          GGTTCTATAATAGTTTTTTACACTTGTCAAACATCCCGGCGAGTGTCAGTTACTCTTTTC

a:
b:         GlnAspIleIleLysLysCysGluGlnPheValGlyProLeuThrValAsnGluLysArg  -
c:

          AAGGTTAAAATTGATCATGCCTGCTAGGTTTTATCCTAATTTTACCAAATATTTGCCCTT
      721 ---------+---------+---------+---------+---------+---------+ 780
          TTCCAATTTTAACTAGTACGGACGATCCAAAATAGGATTAAAATGGTTTATAAACGGGAA

a:
b:         ArgLeuLysLeuIleMetProAlaArgPheTyrProAsnPheThrLysTyrLeuProLeu  -
c:

          GGATAAGGGTATTAAACCTTATTATCCAGAACATCTAGTTAATCATTACTTCCAAACTAG
      781 ---------+---------+---------+---------+---------+---------+ 840
          CCTATTCCCATAATTTGGAATAATAGGTCTTGTAGATCAATTAGTAATGAAGGTTTGATC

a:
b:         AspLysGlyIleLysProTyrTyrProGluHisLeuValAsnHisTyrPheGlnThrArg  -
c:

          ACACTATTTACACACTCTATGGAAGGCGGGTGTTTTATATAAGAGAGTATCAACACATAG
      841 ---------+---------+---------+---------+---------+---------+ 900
          TGTGATAAATGTGTGAGATACCTTCCGCCCACAAAATATATTCTCTCATAGTTGTGTATC

a:
b:         HisTyrLeuHisThrLeuTrpLysAlaGlyValLeuTyrLysArgValSerThrHisSer  -
c:

          CGCCTCATTTTGTGGGTCACCATATTCTTGGGAACAAGAGCTACAGCATGGGGCAGAATC
      901 ---------+---------+---------+---------+---------+---------+ 960
          GCGGAGTAAAACACCCAGTGGTATAAGAACCCTTGTTCTCGATGTCGTACCCCGTCTTAG

a:
b:         AlaSerPheCysGlySerProTyrSerTrpGluGlnGluLeuGlnHisGlyAlaGluSer  -
c:                                                  preS1:MetGlyGlnAsnLeu -

          TTTCCACCAGCAATCCTCTGGGATTCTTTCCCGACCACCAGTTGGATCCAGCCTTCAGAG
      961 ---------+---------+---------+---------+---------+---------+ 1020
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a:
b:         PheHisGlnGlnSerSerGlyIleLeuSerArgProProValGlySerSerLeuGlnSer  -
c:          SerThrSerAsnProLeuGlyPhePheProAspHisGlnLeuAspProAlaPheArgAla -

          CAAACACCAACAATCCAGATTGGGACTTCAATCCCAACAAGGACACCTGGCCAGACGCCA
     1021 ---------+---------+---------+---------+---------+---------+ 1080
          GTTTGTGGTTGTTAGGTCTAACCCTGAAGTTAGGGTTGTTCCTGTGGACCGGTCTGCGGT

a:
b:         LysHisGlnGlnSerArgLeuGlyLeuGlnSerGlnGlnGlyHisLeuAlaArgArgGln  -
c:          AsnThrAsnAsnProAspTrpAspPheAsnProAsnLysAspThrTrpProAspAlaAsn -
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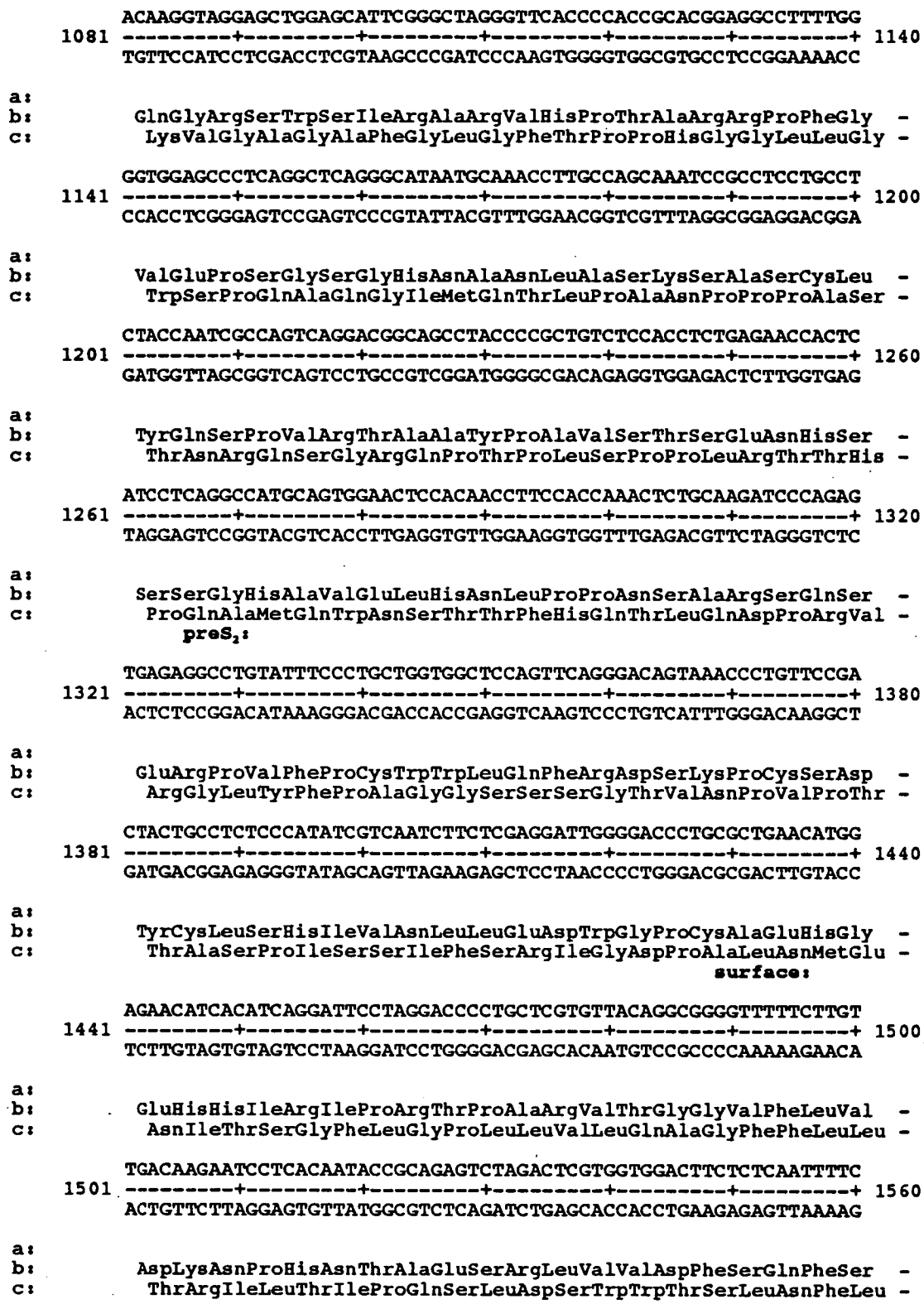
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1200
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1261
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1320
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1321
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1440
a:
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c: ThrAlaSerProIleSerSerIlePheSerArgIleGlyAspProAlaLeuAsnMetGlu -
surface:
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1441
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c:            ThrSerCysProProThrCysProGlyTyrArgTrpMetCysLeuArgArgPheIleIle -

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     1681 ---------+---------+---------+---------+---------+---------+ 1740
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b:          LeuProLeuHisProAlaAlaMetProHisLeuLeuValGlySerSerGlyLeuSerArg  -
c:            PheLeuPheIleLeuLeuLeuCysLeuIlePheLeuLeuValLeuLeuAspTyrGlnGly -

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     1801 ---------+---------+---------+---------+---------+---------+ 1860
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c:            ThrCysThrThrProAlaGlnGlyIleSerMetTyrProSerCysCysCysThrLysPro -

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c:            SerAspGlyAsnCysThrCysIleProIleProSerSerTrpAlaPheGlyLysPheLeu -

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     1921 ---------+---------+---------+---------+---------+---------+ 1980
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a:
b:          MetGlyValGlyLeuSerProPheLeuLeuAlaGlnPheThrSerAlaIleCysSerVal  -
c:            TrpGluTrpAlaSerAlaArgPheSerTrpLeuSerLeuLeuValProPheValGlnTrp -

          GGTTCGTAGGGCTTTCCCCCATTGTTTGGCTTTCAGTTATATGGATGATGTGGTATTGGG
     1981 ---------+---------+---------+---------+---------+---------+ 2040
          CCAAGCATCCCGAAAGGGGGTAACAAACCGAAAGTCAATATACCTACTACACCATAACCC

a:
b:          ValArgArgAlaPheProHisCysLeuAlaPheSerTyrMetAspAspValValLeuGly  -
c:            PheValGlyLeuSerProIleValTrpLeuSerValIleTrpMetMetTrpTyrTrpGly -

          GGCCAAGTCTGTACAGCATCTTGAGTCCCTTTTTACCGCTGTTACCAATTTTCTTTTGTC
     2041 ---------+---------+---------+---------+---------+---------+ 2100
          CCGGTTCAGACATGTCGTAGAACTCAGGGAAAAATGGCGACAATGGTTAAAAGAAAACAG

a:
b:          AlaLysSerValGlnHisLeuGluSerLeuPheThrAlaValThrAsnPheLeuLeuSer  -
c:            ProSerLeuTyrSerIleLeuSerProPheLeuProLeuLeuProIlePhePheCysLeu -
```

```
          TTTGGGCATACATTTAAACCCTAACAAAACAAAAAGATGGGGTTATTCTCTAAATTTCAT
     2101 ---------+---------+---------+---------+---------+---------+ 2160
          AAACCCGTATGTAAATTTGGGATTGTTTTGTTTTTCTACCCCAATAAGAGATTTAAAGTA

a:
b:         LeuGlyIleHisLeuAsnProAsnLysThrLysArgTrpGlyTyrSerLeuAsnPheMet  -
c:          TrpAlaTyrIleEnd(surface end)

          GGGCTATGTCATTGGATGTTGGGGATCATTGCCACAAGATCACATCATACACAAAATCAA
     2161 ---------+---------+---------+---------+---------+---------+ 2220
          CCCGATACAGTAACCTACAACCCCTAGTAACGGTGTTCTAGTGTAGTATGTGTTTTAGTT

a:
b:         GlyTyrValIleGlyCysTrpGlySerLeuProGlnAspHisIleIleHisLysIleLys  -
c:

          AGAATGTTTTAGAAAACTCCCTGTTCACAGGCCTATTGATTGGAAAGTGTGTCAACGAAT
     2221 ---------+---------+---------+---------+---------+---------+ 2280
          TCTTACAAAATCTTTTGAGGGACAAGTGTCCGGATAACTAACCTTTCACACAGTTGCTTA

a:
b:         GluCysPheArgLysLeuProValHisArgProIleAspTrpLysValCysGlnArgIle  -
c:

          TGTGGGTCTTCTGGGTTTTGCTGCCCCTTTCACACAATGTGGTTATCCTGCTTTAATGCC
     2281 ---------+---------+---------+---------+---------+---------+ 2340
          ACACCCAGAAGACCCAAAACGACGGGGAAAGTGTGTTACACCAATAGGACGAAATTACGG

a:
b:         ValGlyLeuLeuGlyPheAlaAlaProPheThrGlnCysGlyTyrProAlaLeuMetPro  -
c:

          CTTGTATGCATGTATTCAATCTAAGCAGGCTTTCACTTTTTCGCCAACTTACAAGGCCTT
     2341 ---------+---------+---------+---------+---------+---------+ 2400
          GAACATACGTACATAAGTTAGATTCGTCCGAAAGTGAAAAAGCGGTTGAATGTTCCGGAA

a:
b:         LeuTyrAlaCysIleGlnSerLysGlnAlaPheThrPheSerProThrTyrLysAlaPhe  -
c:

          TCTGTGTAAACAATACCTGAACCTTTACCCCGTTGCCAGGCAACGGCCAGGTCTGTGCCA
     2401 ---------+---------+---------+---------+---------+---------+ 2460
          AGACACATTTGTTATGGACTTGGAAATGGGGCAACGGTCCGTTGCCGGTCCAGACACGGT

a:
b:         LeuCysLysGlnTyrLeuAsnLeuTyrProValAlaArgGlnArgProGlyLeuCysGln  -
c:

          AGTGTTTGCTGATGCAACCCCCACTGGCTGGGGCTTGGTCATGGGCCATCAGCGCATGCG
     2461 ---------+---------+---------+---------+---------+---------+ 2520
          TCACAAACGACTACGTTGGGGGTGACCGACCCCGAACCAGTACCCGGTAGTCGCGTACGC

a:
b:         ValPheAlaAspAlaThrProThrGlyTrpGlyLeuValMetGlyHisGlnArgMetArg  -
c:

          TGGAACCTTTCTGGCTCCTCTGCCGATCCATACTGCGGAACTCCTAGCCGCTTGTTTTGC
     2521 ---------+---------+---------+---------+---------+---------+ 2580
          ACCTTGGAAAGACCGAGGAGACGGCTAGGTATGACGCCTTGAGGATCGGCGAACAAAACG

a:
b:         GlyThrPheLeuAlaProLeuProIleHisThrAlaGluLeuLeuAlaAlaCysPheAla  -
c:

          TCGCAGCAGGTCTGGAGCAAACATTCTCGGAACTGACAACTCTGTTGTCCTCTCCCGCAA
     2581 ---------+---------+---------+---------+---------+---------+ 2640
          AGCGTCGTCCAGACCTCGTTTGTAAGAGCCTTGACTGTTGAGACAACAGGAGAGGGCGTT

a:
b:         ArgSerArgSerGlyAlaAsnIleLeuGlyThrAspAsnSerValValLeuSerArgLys  -
c:
```

```
          ATATACATCGTTTCCATGGCTGCTAGGCTGTGCTGCCAACTGGATCCTGCGCGGGACGTC
     2641 ---------+---------+---------+---------+---------+---------+ 2700
          TATATGTAGCAAAGGTACCGACGATCCGACACGACGGTTGACCTAGGACGCGCCCTGCAG

a:                      X:MetAlaAlaArgLeuCysCysGlnLeuAspProAlaArgAspVal   -
b:         TyrThrSerPheProTrpLeuLeuGlyCysAlaAlaAsnTrpIleLeuArgGlyThrSer  -
c:

          CTTTGTTTACGTCCCGTCGGCGCTGAATCCTGCGGACGACCCTTCTCGGGGCCGCTTGGG
     2701 ---------+---------+---------+---------+---------+---------+ 2760
          GAAACAAATGCAGGGCAGCCGCGACTTAGGACGCCTGCTGGGAAGAGCCCCGGCGAACCC

a:         LeuCysLeuArgProValGlyAlaGluSerCysGlyArgProPheSerGlyProLeuGly   -
b:          PheValTyrValProSerAlaLeuAsnProAlaAspAspProSerArgGlyArgLeuGly  -
c:

          GCCCTGTCGTCCTCTTCTCTGCCTGCCGTTCCGGCCGCCCACGGGGCGCACCTCTCTTTA
     2761 ---------+---------+---------+---------+---------+---------+ 2820
          CGGGACAGCAGGAGAAGAGACGGACGGCAAGGCCGGCGGGTGCCCCGCGTGGAGAGAAAT

a:         AlaLeuSerSerSerSerLeuProAlaValProAlaAlaHisGlyAlaHisLeuSerLeu   -
b:          ProCysArgProLeuLeuCysLeuProPheArgProProThrGlyArgThrSerLeuTyr  -
c:

          CGCGGCCTCCCCGTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTCACCTCT
     2821 ---------+---------+---------+---------+---------+---------+ 2880
          GCGCCGGAGGGGCAGACACGGAAGAGTAGACGGCCTGGCACACGTGAAGCGAAGTGGAGA

a:         ArgGlyLeuProValCysAlaPheSerSerAlaGlyProCysAlaLeuArgPheThrSer   -
b:          AlaAlaSerProSerValProSerHisLeuProAspArgValHisPheAlaSerProLeu  -
c:

          GCACGTCGCATGGAGACCACCGTGAACGCCCACCAAATCTTGCCCAAGGTCTTACATAAG
     2881 ---------+---------+---------+---------+---------+---------+ 2940
          CGTGCAGCGTACCTCTGGTGGCACTTGCGGGTGGTTTAGAACGGGTTCCAGAATGTATTC

a:         AlaArgArgMetGluThrThrValAsnAlaHisGlnIleLeuProLysValLeuHisLys   -
b:          HisValAlaTrpArgProProEnd(polymerase end)
c:

          AGGACTCTTGGACTCTCTGCAATGTCAACGACCGACCTTGAGGCATACTTCAAAGACTGT
     2941 ---------+---------+---------+---------+---------+---------+ 3000
          TCCTGAGAACCTGAGAGACGTTACAGTTGCTGGCTGGAACTCCGTATGAAGTTTCTGACA

a:         ArgThrLeuGlyLeuSerAlaMetSerThrThrAspLeuGluAlaTyrPheLysAspCys   -
b:
c:

          TTGTTTAAAGACTGGGAGGAGTTGGGGGAGGAGATTAGATTAAAGGTCTTTGTACTAGGA
     3001 ---------+---------+---------+---------+---------+---------+ 3060
          AACAAATTTCTGACCCTCCTCAACCCCCTCCTCTAATCTAATTTCCAGAAACATGATCCT

a:         LeuPheLysAspTrpGluGluLeuGlyGluGluIleArgLeuLysValPheValLeuGly   -
b:
c:

          GGCTGTAGGCATAAATTGGTCTGCGCACCAGCACCATGCAACTTTTTCACCTCTGCCTAA
     3061 ---------+---------+---------+---------+---------+---------+ 3120
          CCGACATCCGTATTTAACCAGACGCGTGGTCGTGGTACGTTGAAAAAGTGGAGACGGATT

a:         GlyCysArgHisLysLeuValCysAlaProAlaProCysAsnPhePheThrSerAlaEnd(X end)
b:
c:                                  pre-core:MetGlnLeuPheHisLeuCysLeuIle -
```

```
      TCATCTCTTGTTCATGTCCTACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGG
3121  ---------+---------+---------+---------+---------+---------+ 3180
      AGTAGAGAACAAGTACAGGATGACAAGTTCGGAGGTTCGACACGGAACCCACCGAAACCC

a:
b:
c:          IleSerCysSerCysProThrValGlnAlaSerLysLeuCysLeuGlyTrpLeuTrpGly -

      GC
3181  -- 3182
      CG

a:
b:
c:              -
```