Characterization of Hepatitis B Virus Surface and Core Antigens using a Baculovirus Expression System: Potential as Carriers for Foreign Epitopes.

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Abstract

Hepatitis B Virus surface (HBsAg) and core antigens (HBcAg), and a variety of derivatives, were expressed from recombinant baculoviruses in insect cells cultured in monolayer. It was initially established that HBsAg was secreted from insect cells at low efficiency.

HBsAg self-assembles into 22 nm spherical lipoprotein particles. Full-length and (four) truncated HBsAg genes were fused in-frame to segments encoding portions of the rabies virus glycoprotein (rabies G). Three different rabies G segments were utilized in various combinations with HBsAg. The products were analyzed for their ability to form chimeric 22 nm particles for potential vaccine use. No fusion proteins were secreted, and all were found to be insoluble in nonionic detergent. Expression of some fusion proteins in mammalian COS cells did not alleviate the inhibition of secretion.

To further investigate the assembly and secretion of HBsAg in insect cells, a variety of mutants and fusions were designed to test the importance of the first hydrophobic transmembrane (TM) domain (domain I) of HBsAg. Domain I appeared to increase the rate of membrane insertion at the endoplasmic reticulum (E.R.). Also, a negatively-charged residue immediately preceding domain I was important to particle secretion. It was found that domain I could be replaced by a heterologous TM domain provided the heterologous

domain was proceeded by a negatively-charged residue.

HBcAg self-assembles into 27 nm protein particles. HBcAg is released from infected insect cells by an unknown mechanism. The normally secretory HBV e antigen (HBeAg), expressed from the core plus precore gene, was not released. We found that processing of the precore signal peptide (SP) was defective in insect cells. This SP could be functionally replaced by the SP from honeybee prepromellitin. Proteolytic cleavage appeared to be occurring subsequent to mellitin SP cleavage, but its nature is unknown. Phosphorylation of HBcAg was normal, while phosphorylation of mellitin SP fusion proteins suggested restriction to fully-cleaved species. A segment of the precore domain which normally remains after SP cleavage during HBeAg synthesis was important to phosphorylation and cleavage patterns. Electron microscopy revealed no apparent mechanism for the release of HBcAg, which was present as spherical particles in the cytoplasm. Mellitin SP fusion proteins were observed as spherical particles in distended rough E.R.

A gene encoding three peptides derived from HIV-1 *env* and *gag* proteins was fused in-frame to both the N- and C-termini of HBcAg. Chimeric 27 nm particles were formed from both fusions, and were demonstrated to be cross-reactive to both HIV-1 p17 and gp120 proteins. Morphological and structural features suggested that the HIV-1 peptide was interior to the particles for the C-terminal fusion and exterior to the particle for the N-terminal fusion.

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Ian Robb performed the electron microscopy of infected cell cultures. Dr. Anthony Tam constructed the HIV-1 chimeric recombinant viruses and performed all preliminary analyses.

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List of Abbreviations

aa	amino acid
Ac	*Autographa californica*
ala	alanine
arg	arginine
asn	asparagine
C	HBV core gene/protein
ccc	covalently closed circular
CAH	chronic active hepatitis
CMI	cell mediated immunity
CPE	cytopathic effect
Da.	dalton
ds	double-stranded
DHBV	duck hepatitis B virus
DNA	deoxyribonucleic acid
DR	direct repeat
ELISA	enzyme-linked immunosorbent assay
E.M.	electron microscopy
Enh I/II	HBV enhancer I/II
env	HIV-1 envelope gene/proteins
E.R.	endoplasmic reticulum
Fig.	figure
FMDV	foot-and-mouth disease virus
gag	HIV-1 nucleocapsid gene/proteins
gln	glutamine
glu	glutamic acid
gp	glycoprotein
GSHV	ground squirrel hepatitis virus
HBcAg	hepatitis B core antigen
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HIV	human immunodeficiency virus
HBV	human hepatitis B virus
HCC	hepatocellular carcinoma
HHBV	heron hepatitis B virus
hr	hour
HSV	herpes simplex virus
IFN	interferon
Ig	immunoglobulin
k	kilo
kb	kilobases/kilobasepairs
L	HBsAg large protein
lys	lysine
mM	millimolar
M	HBsAg medium/middle protein
μCi	microcurie
μg	microgram
min	minute
ml	millilitre
m.o.i.	multiplicity of infection

NF	nuclear factor
NK	natural killer
NLS	nuclear localization signal
NPV	nuclear polyhedrosis virus
ng	nanogram
nm	nanometer
nt	nucleotide
NTP	nucleotide triphosphate
orf	open reading frame
p	protein

Introduction

I. General Introduction

The world of virology is noted for unusual biological adaptations which allow viruses to exploit their cellular hosts. Even within this setting, the human hepatitis B virus (HBV) is a unique and unusual biological entity. This is evident at many different levels, including genetic structure and viral replication, pathogenesis, and structure and function of its elaborated proteins. In addition to the importance of this virus within the world population as a pathogen, the heuristic nature of this virus has stimulated much research interest. One major stumbling block for this research has been the lack of a suitable cell culture system which supports productive HBV replication. Recent successes with *in vitro* infection of human hepatoma cell lines and studies on HBV replication proceeding from transfection of hepatoma cells with a double-stranded DNA form of the virus are rapidly expanding our understanding of the HBV life cycle. To date, however, much of our current knowledge has been inferred from the examination of human biopsies, the use of animal hepadnavirus models, and the study of the products of viral genes expressed in heterologous cells.

In this thesis, the approach of heterologous gene

expression was used to study two structural proteins of HBV: the hepatitis B surface antigen (HBsAg), and the hepatitis B core antigen (HBcAg). HBsAg is the viral envelope protein, while HBcAg is the nucleocapsid protein. Although the experimental approaches and objectives described herein for the two proteins were quite different, a common theme does unite them. This theme is the search for more affordable and efficacious vaccines using a genetic engineering approach. This encompasses not only a vaccine for HBV itself, but also involves the manipulation of these two HBV proteins as carriers for epitopes from proteins of other pathogens. This manipulation is primarily based on the ability of both of these proteins to form higher-order particulate structures.

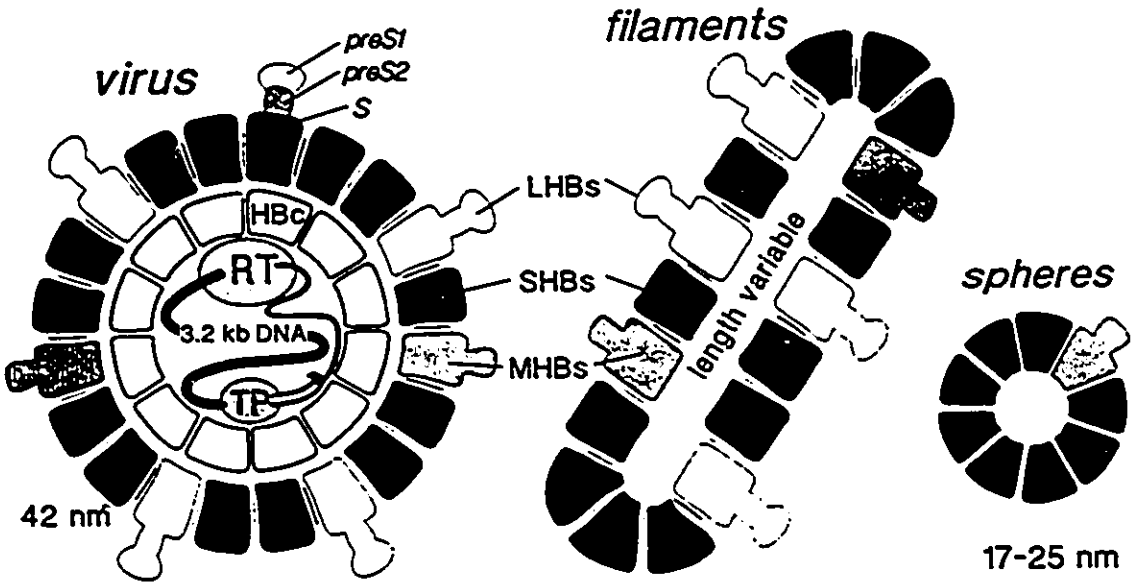
For these reasons, a better understanding of the biosynthesis and processing of HBsAg and HBcAg in a heterologous cell system was sought. It is hoped that this will be not only reflective of the viral life cycle and the role that HBsAg and HBcAg have within it, but that there will be practical implications for the future production of HBV-based vaccines. In a more general fashion, I also hope during the course of this thesis to draw attention to the many unusual features of HBV which have stimulated scientific interest. A general introduction to biological and molecular aspects of HBV infection will follow to provide a suitable context for the experimental results. This introduction will proceed to a more detailed examination of the role of HBV

structural proteins before describing the experimental approaches.

II. General Biology of HBV

The human hepatitis B virus (HBV) is the prototype virus of the Hepadnaviridae family (Summers, 1981). Other members of the family include the woodchuck hepatitis virus (WHV) (Summers *et al.*, 1978), the ground squirrel hepatitis virus (GSHV) (Marion *et al.*, 1980), the duck hepatitis B virus (DHBV) (Mason *et al.*, 1980), the tree squirrel hepatitis virus (TSHV) (Feitelson *et al.*, 1986) and the heron hepatitis B virus (HHBV) (Will *et al.*, 1987a). The different members of Hepadnaviridae display a high level of homology at genetic and biological levels; unless otherwise noted, this discussion will refer to HBV. In addition to genomic structure, members of the family Hepadnaviridae are related by their hepatotropism, narrow host specificity, and by the presence of subviral particles in the blood at high concentrations. For HBV, productive infection is seen only in humans and higher primates. Two related subviral forms are present in the blood in addition to virions (Fig. 1): a spherical lipoprotein form approximately 22 nm in diameter (designated 22 nm particles) which corresponds to an empty viral envelope, and long filamentous forms which have the same diameter. Further

Figure 1. Schematic representation of HBV viral and subviral forms. Illustrated are: (left) the HBV virion, 42 nm in diameter; (center) a subviral filamentous form, ~22 nm in diameter; (right) a spherical subviral form, also ~22 nm in diameter. The hepatitis B surface antigen family (HBsAg) is illustrated as a black segment for the major (S) protein, a lightly shaded segment for the middle (M) protein, and a clear segment for the large (L) protein. Spherical subviral particle forms contain no L protein. The viral nucleocapsid contains hepatitis B core antigen (HBcAg), an ~3.2 kb partially double-stranded DNA genome, the viral polymerase, including a reverse transcriptase activity (RT), and the polymerase-encoded terminal protein (TP) used to prime negative-strand transcription. Reprinted from Heerman and Gerlich (1991).


virus
preS1
preS2
S
HBc
RT
3.2 kb DNA
TP
42 nm
LHBs
SHBs
MHBs
filaments
length variable
spheres
17-25 nm

information on subviral particles will be presented in the section on envelope proteins. It is speculated that high concentrations of these particles protect the replicating virus from the immune response by reacting with and eliminating neutralizing antibodies.

A variety of outcomes can result from HBV infection. The host factors which influence these different outcomes are not known. Primary exposure of susceptible hosts to HBV can result in infections which range from subacute to severe primary forms, though the classic result is acute hepatitis B. This is a moderately severe disease characterized by hepatocellular injury and inflammation. The virus itself appears to be nonlytic, and injury is thought to be due to immunological consequences of infection (Chisari *et al.*, 1989).

Primary infection is usually self-limiting, and the virus cleared. However, infected individuals can develop a persistent infection. Because the virus is endemic in densely populated areas of the world, including South-East Asia and Africa, this has major consequences in terms of epidemiology and mortality. The number of chronic carriers is estimated at over 250 million world-wide (Beasley *et al.*, 1981b). The carrier rate can be 10% or greater in endemic areas. Transmission is by the parenteral route, and is primarily from the pool of chronically infected individuals. HBV can be either horizontally (e.g. by sexual transmission or intravenous drug use) or vertically (from carrier mothers to

their newborn) transmitted. (Ganem and Varmus, 1987).

Mortality is more prevalent during chronic versus acute infection. Varying degrees of hepatocellular injury can occur, and an aggressive chronic active hepatitis (CAH) can develop. In addition, chronic HBV infection is causally linked to hepatocellular carcinoma (HCC), a common cancer. The likelihood of developing this cancer is 200-fold greater for an infected vs. an uninfected individual. Together, CAH, HCC, and cirrhosis resulting from chronic infection are a major cause of premature death due to liver failure in endemic areas (Ganem and Varmus, 1987).

III. Viral Structure and Replication

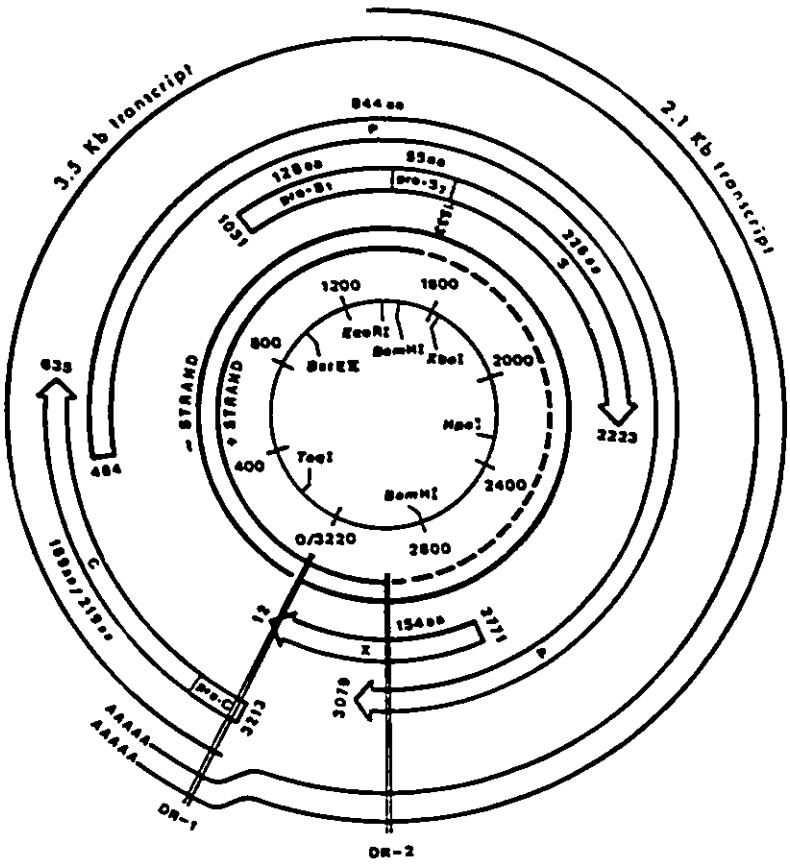
A. Virion Structure

HBV is a spherical enveloped virion approximately 42 nm in diameter, also known as the Dane particle after its discoverer (Dane *et al.*, 1970) (Fig. 1). It has a spherical nucleocapsid approximately 27 nm in diameter (Almeida *et al.*, 1971). The genetic material of HBV is highly unusual. It was characterized in a series of studies by Robinson, Summers and coworkers (Almeida *et al.*, 1971; Kaplan *et al.*, 1973; Robinson and Greenman, 1974; Robinson *et al.*, 1974; Summers *et al.*, 1975; Robinson, 1977; Hruska *et al.*, 1977; Landers *et al.*,

1977). It consists of a partially double-stranded circular DNA molecule (Summers *et al.*, 1975; Delius *et al.*, 1983) (Fig. 2). Synthesis of the positive (mRNA sense) strand from the negative strand template is not completed before packaging of the core structures into virions. Positive strand size is heterogeneous, and varies from between 40-85% of the total genome length (Summers *et al.*, 1975). The molecule is not covalently closed, and is held in a circular form by single-stranded cohesive ends (Sattler and Robinson, 1979). The total length of the negative strand is approximately 3.2 kilobases (kb). This extremely small size requires that an unusually efficient use of coding capacity be maintained to provide all the required viral functions.

Two major structural proteins are incorporated into HBV virions (Figs. 1 and 2): HBsAg in the envelope (Peterson *et al.*, 1977; Peterson, 1981) and HBcAg in the nucleocapsid (Robinson and Greenman, 1974). The HBV polymerase is also found in virions, remaining attached to the positive strand 3'-terminus at which it was active before packaging (Kaplan *et al.*, 1973). The polymerase protein also encodes a protein primer (terminal protein; TP) which initiates negative strand transcription from an RNA pregenome (Bartenschlager and Schaller, 1988). TP remains covalently attached to the 5'-terminus of the negative strand within the virion (Gerlich and Robinson, 1980). A capped oligoribonucleotide covalently attached to the 5'-terminus of the positive strand is also

Figure 2. Schematic representation of the HBV *adw* genome. Nucleotide numbering is as according to Pasek *et al.* (1978). The dotted region of the positive strand illustrates incomplete synthesis, resulting in a variable single stranded region in virions. Open reading frames are indicated by the boxed arrows, including their start points and the size of encoded proteins. The locations of the two major transcripts, the 3.5 kb genomic and the 2.1 kb subgenomic, with their common polyadenylation site, are illustrated in the outer ring. The positions of the direct repeat elements (DR1 and DR2) are indicated by the thin double lines. These elements are within a terminally redundant region central to initiation and recircularization events during replication. Reprinted from Robinson and Marion (1988).


3.5 Kb transcript
2.1 Kb transcript
844 aa
P
128 aa
pre-S1
85 aa
pre-S2
1051
1551
228 aa
S
1200
1800
EcoRI
BamHI
XbaI
800
BstEX
2000
2223
+ STRAND
- STRAND
HpaI
400
TaqI
2400
BamHI
0/3220
2600
638
484
C
1884aa/2190aa
12
154 aa
X
3171
P
3018
pre-C
3215
AAAAA
AAAAA
DR-1
DR-2

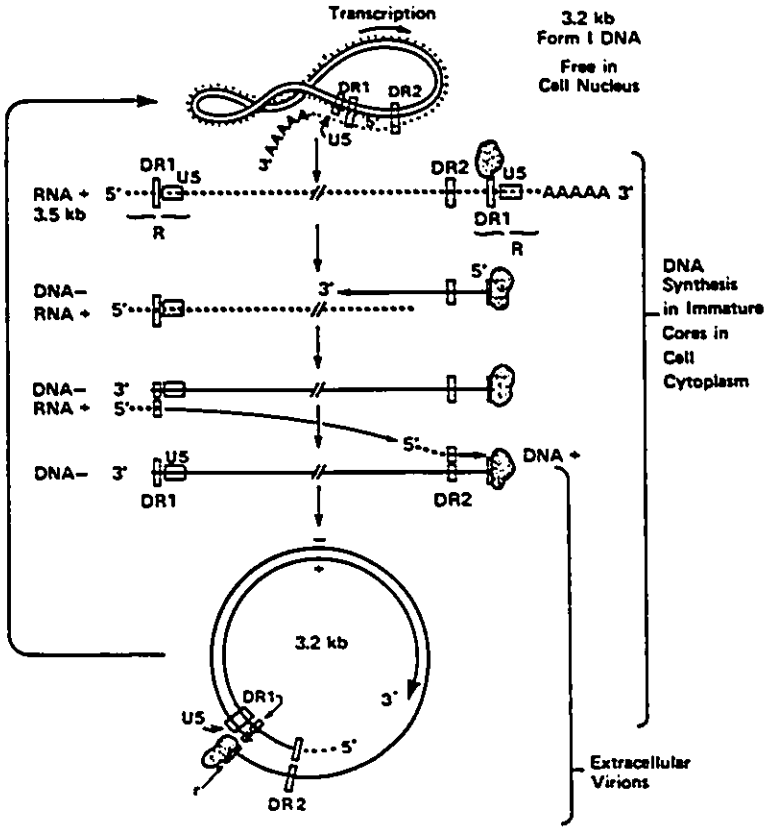
present within virions, and is believed to act as a primer for positive strand synthesis (Lien *et al.*, 1986).

B. Replication

The mechanism of hepadnaviral replication was first proposed from observations of DHBV infected duck hepatocytes (Summers and Mason, 1982; Mason *et al.*, 1982). Observations in infected human livers were in accordance (Blum *et al.*, 1984; Fowler *et al.*, 1984; Miller *et al.*, 1984a; Miller *et al.*, 1984b; Will *et al.*, 1987). The proposed mechanism is similar in overall strategy to that used by retroviruses. Because of this similarity, as well as certain nucleotide homologies (Miller and Robinson, 1986) and a similar overall coding strategy, hepadnaviruses and retroviruses are believed to share a common evolutionary ancestor. The replication process will be discussed here briefly as this subject has been extensively reviewed (Robinson and Marion, 1988; Ganem and Varmus, 1987; Seeger *et al.*, 1991a). It must be noted that much of the proposed replication strategy is inferred from indirect observations due to cell culture limitations and the inability to solubilize an active virion polymerase.

The first step of replication involves conversion of the open circular partially double-stranded virion DNA to a covalently closed circular (ccc) form (Tuttleman *et al.*, 1986) (Fig. 3). These ccc forms are found free in the nucleus where

Figure 3. The replication cycle of HBV. The cycle is shown as beginning from the covalently closed circular form (form I) present in the nucleus. RNA is represented as a dotted line, DNA by a solid line. The terminal protein (TP) primer (stippled) for negative strand synthesis is represented. DR1 and DR2 are 12 nt direct repeats. U5 represents the retroviral U5-homologous sequence. Reprinted from Robinson and Marion (1988).


Transcription
3.2 kb
Form I DNA
Free in
Cell Nucleus
DR1
DR2
U5
AAAAA
RNA +
3.5 kb
5'
3'
R
DNA–
RNA +
DNA–
DNA +
3.2 kb
DNA
Synthesis
in Immature
Cores in
Cell
Cytoplasm
Extracellular
Virions

the conversion is believed to occur. This process firstly requires completion of the positive strand by the polymerase already present at the 3' end. Next, strand ligation covalently closes the circular genome. This requires removal of the negative strand protein primer and of the positive strand oligonucleotide primer (see below). This is followed by supercoiling of the genome. The exact molecular events and cellular factors required for this conversion are not clear at present.

The ccc form is believed to act as a template for transcription of several capped species (as described below) (Figs. 2 and 3) (Miller and Robinson, 1984; Tagawa *et al.*, 1986). The largest is 3.5 kb, which is larger than genome-size. It acts as both a template for core and polymerase protein translation, and as a proviral template for negative strand transcription. Its size is a consequence of a transcription event which proceeds around the circular template and through its original initiation site before terminating. This creates a 100-300 bp terminally redundant region on the pregenomic RNA which is central to replication (Buscher *et al.*, 1985; Enders *et al.*, 1985; Will *et al.*, 1987b). Note that this event requires that the transcript proceeds once through a polyadenylation signal, which is only utilized for termination upon the second encounter. The genomic region which is duplicated contains a pair of 11 bp direct repeats known as DR1 and DR2 (Figs. 2 and 3) (Buscher

et al., 1985; Enders *et al.*, 1985). The pregenomic transcript is initiated just upstream of the DR1 sequence, and after proceeding through the initiation site, terminates downstream of the same DR1 sequence. This species thus has DR1 present at its 5′ end, and DR2/DR1 sequences present at its 3′ end. At this stage, the pregenome is reminiscent of retroviral RNA genomic structure. The HBV proviral RNA species is larger than its ds DNA form, while the opposite is true for retroviruses. In both, cases, however, terminal redundancies allow for priming and recircularization events.

The pregenome is transported to the cytoplasm where it is packaged into immature core particles (Enders *et al.*, 1987; Hirsch *et al.*, 1990). Here it acts as a template for synthesis of the negative strand by a reverse transcriptase activity of the viral-specific polymerase (Summers and Mason, 1982). The negative strand is initiated within the DR1 sequence present at the 3′ end of the pregenome (Seeger *et al.*, 1986; Lien *et al.*, 1987; Will *et al.*, 1987). As referred to above, TP is covalently attached to the negative strand 5′ end and is believed to act as a primer (Molnar-Kimber *et al.*, 1983). The 3′ end of the negative strand is specified by "run-off" of the pregenome transcript 5′ end (Seeger *et al.*, 1986; Lien *et al.*, 1987; Will *et al.*, 1987).

Transcription is believed to be accompanied by an RNase H activity of the polymerase, which digests the RNA template from the RNA-DNA hybrid. Digestion is not complete; it leaves

a small capped RNA structure from the pregenome 5' end with its DR1 sequence intact (Seeger and Maragos, 1989). This RNA species then acts as a primer for synthesis of positive strand DNA by dissociating from the negative strand 3' end and reassociating with the DR2 sequence present at its 5' end (Lien *et al.*, 1986; Seeger *et al.*, 1986). Transcription can only proceed a short distance to the negative strand 5' end, where TP is bound. Recircularization of the negative strand template via its terminal DR1 redundancies allows access to the remainder of the template and positive strand transcription to proceed.

Packaging of the core structures into virions occurs prior to completion of positive strand synthesis. The most abundant intracellular species are incomplete negative strand DNAs, while the most abundant extracellular species are open circle ds DNA. This suggests that completion of the negative strand DNA and conversion to the circular form of DNA are coordinated with export. However, packaging can likely occur earlier after nucleocapsid formation, as viral particles containing RNA-DNA hybrids can be found in the blood (Miller *et al.*, 1984).

C. Open Reading Frames of the Viral Genome

Four open reading frames have been identified in hepadnaviruses (Fig. 2) (Galibert *et al.*, 1979; Valenzuela *et al.*, 1980). In a clockwise order, the C open reading frame

(orf) encodes the nucleocapsid protein, which is 184 amino acids (aa) for HBV. Serologically, this protein is recognized as HBcAg for HBV. It will often be referred to as core or C protein in this thesis for convenience. It shares a region of significant homology with retroviral *gag* proteins (Miller and Robinson, 1986). In addition, the core gene is preceded by an inframe extension which encodes 29 aa known as the preC region (Ou *et al.*, 1986). This peptide contains a signal sequence which targets the core plus preC polypeptide (abbreviated as preC protein) to the secretory membranes, resulting in the secretion of the related e antigen, as described below.

The S orf encodes the viral envelope proteins. They are composed of three related species synthesized from three in-frame initiation codons. Collectively, they are recognized serologically as HBsAg due to their common reactivity (for HBV). The S gene encodes the smallest species, a 226 aa transmembrane protein (termed major, or S protein). The major protein is common to the C-terminal region of the other two preS proteins. Upstream initiation at the second methionine adds a 55 aa extension known as the preS2 region to create the S plus preS2 (middle, or M) protein (Stibbe and Gerlich, 1983; Machida *et al.*, 1984). Initiation at the third methionine adds the preS1 region, which varies in size depending on subtype. This creates the S plus preS2 plus preS1 (large, or L) protein (Heerman *et al.*, 1984). In contrast to the preC region, the functions of the preS regions are not clear (see below).

The X orf has the smallest coding potential of all four orfs at 154 aa. Although it is conserved in all mammalian hepadnaviruses, its role has remained obscure. Initial speculations were based in part on the presence of a transactivating protein in an analogous position in human retroviruses (Miller and Robinson, 1986). It was subsequently verified that X protein can transactivate its own and other target promoter elements, both in heterologous cell systems and in HBV-infected hepatoma cells (Spandau and Lee, 1988; Zahm *et al.*, 1988; Twu and Robinson, 1989; Colgrove *et al.*, 1989). It is speculated that X protein is an important regulator of the pregenome/core promoter in combination with the HBV major enhancer, as will be described below. It may exert its effects through the NF-κB system (Lucito and Schneider, 1992). Also, X protein appears to be a protein serine/threonine kinase, and this activity may be important to its transactivation function (Wu *et al.*, 1990). This latter report also demonstrated the presence of X protein in HBV virions. X protein can be immunohistochemically detected in chronically infected liver samples (Katayama *et al.*, 1989; Zentgraf *et al.*, 1990) and antibody reactivity to X protein is present in patients' sera (Kay *et al.*, 1985; Moriarty *et al.*, 1985). These data strongly suggest an important role for the X protein in the HBV life cycle. However, the X gene varies most widely in size among the members of Hepadnaviridae, and a functional copy of the X gene is not present in DHBV

(Robinson and Marion, 1988). While one set of studies has found reduced replication and transcription for X-negative mutant genomes in hepatoma cells (Yaginuma *et al.*, 1987; Koike *et al.*, 1989), another study found normal levels of virions produced (Blum *et al.*, 1992). Thus the role of X remains to be fully explained. While the codon preference of the other three genes is similar to that of other viral genes, the codon preference of the X gene resembles that of eukaryotes (Miller and Robinson, 1986). This suggests that it may be a transduced cellular gene.

The P orf encodes the viral polymerase (Schlict *et al.*, 1989a; Chang *et al.*, 1989a). This orf spans about 80% of the viral genome, and therefore overlaps the other orfs, including all of the S orf. The HBV polymerase is required to carry out a complex sequence of enzymatic events during replication (Radziwill *et al.*, 1990; Chang *et al.*, 1990; Wang and Seeger, 1992). It also primes the initiation of negative strand replication (Bartenschlager *et al.*, 1988; Seeger and Maragos, 1991; Wang and Seeger, 1992) and is thought to be central to the packaging of the pregenome in core structures (Bartenschlager *et al.*, 1990; Hirsch *et al.*, 1990). This complexity is reflected at the molecular level by a multiple-domain structure. Based on homology with retroviral *pol* proteins (Toh *et al.*, 1983) and mutagenic analysis, HBV P protein is composed of (from N-terminal to C-terminal): terminal protein (negative-strand primer), non-essential

spacer, reverse transcriptase/DNA polymerase, and RNAse H domains (Radziwill *et al.*, 1990).

Reverse transcription of pregenomic RNA *in vivo* is correlated with packaging into core particles. Genomes expressing mutant P proteins which are defective for replication have been identified which show an accompanying deficit in the ability to package pregenomic RNA (Roychoudhury *et al.*, 1991). Thus, P is either directly or indirectly required to create a replication competent immature core particle. A *cis*-acting encapsidation site at the 5' end of the pregenomic RNA has been recently identified (Junker-Niepmann *et al.*, 1990; Hirsch *et al.*, 1991). Packaging-competent mutants which are defective for replication have also been identified, which demonstrates that the two functions are separable (Chang *et al.*, 1990). Surprisingly, although P can function in *trans*, it preferentially acts in *cis* to package RNA species which are competent for its translation (Hirsch *et al.*, 1990; Bartenschlager *et al.*, 1990). Thus pregenomic packaging is coupled to P protein translation. The use of a protein primer for polymerase activity is fairly unique (also used by adenoviruses and possibly picornaviruses). This function is based on mutagenic analysis of P (Seeger and Maragos, 1990; Seeger and Maragos, 1991) and the presence of nascent negative strands in core particles which are covalently bound to protein (Molnar-Kimber *et al.*, 1983). Note that while the tight coupling of P protein translation,

pregenome encapsidation, and polymerase activity initially suggests that protein priming might be the central event to these processes, the encapsidation signal is at the opposite end of the pregenome (at the 5' end) from the initiation site (at the 3' end).

It has been assumed that HBcAg is a necessary requirement for replication competent core structures. However, a recent report has demonstrated priming and reverse transcriptase activities of P protein expressed from an *in vitro* translation reaction in the absence of HBcAg. HBcAg interaction may therefore occur after of the initiation of negative strand synthesis (Wang and Seeger, 1992).

D. Transcription

Infected tissue samples or hepatoma cell lines (which can support HBV replication) produce two main families of RNA generally known as genomic and subgenomic species (referenced below) (Fig. 2). The genomic family includes several species of about 3.5 kb (actually larger than genome-size), while the subgenomic family is composed primarily of a 2.5 kb species with a minor 2.1 kb species. A minor 0.8 kb species can also be identified in some tissues. These different species initiate at different places in the HBV genome, but all species terminate at the common polyadenylation signal which is located within the precore region just downstream of the

genomic RNA family initiation site (Cattaneo *et al.*, 1983; Cattaneo *et al.*, 1984).

It is now clear that the genomic species initiate in a heterogeneous fashion and can be separated into two functional transcriptional units. The majority of the transcripts initiate just downstream of the precore initiation codon, while a minority initiate upstream (Enders *et al.*, 1985; Yaginuma *et al.*, 1987). The latter act as mRNA for translation of the precore protein, resulting in HBeAg expression (Ou *et al.*, 1986; Roossinck *et al.*, 1986). The former functions as the pregenomic RNA and also as the mRNA template for core and P gene translation. It has been conclusively demonstrated that this latter species is both selectively encapsidated into cytoplasmic cores and translated to produce the P protein (Ou *et al.*, 1990). P translation thus initiates internally from the RNA, and may occur via a cap independent mechanism.

The 2.5 and 2.1 kb subgenomic transcripts are responsible for the translation of the HBsAg proteins (Cattaneo *et al.*, 1983; Standring *et al.*, 1984). A unique 2.5 kb transcript initiates just upstream of the first initiation codon, that of preS1 (Will *et al.*, 1987b). The 2.1 kb family initiates on both sides of the preS2 initiation codon in a heterogeneous fashion, such that both M and S proteins can be produced (Cattaneo *et al.*, 1983). The 2.5 kb species appears to be regulated independently of the 2.1 kb species (Will *et al.*, 1987b; Antonucci and Rutter, 1989; Raney *et al.*, 1990). The

0.8 kb species initiates just upstream of the X orf, and is capable of supporting X protein translation. This species appears to be extremely repressed under conditions of viral replication, and has only been seen from unconventional templates (Gough *et al.*, 1983; Saito *et al.*, 1985; Trenin and Laub, 1987; Araki *et al.*, 1989).

Promoters have been identified which support the transcription of all four major RNA species. The core promoter specifies transcription of the 3.5 kb family (Yaginuma and Koike, 1989; Yee, 1989), the preS1 promoter (SP1) specifies the 2.4 kb RNA (Siddiqui *et al.*, 1986), the S promoter (SP2) the 2.1 kb RNA family (Cattaneo *et al.*, 1983), and the X promoter the 0.8 kb RNA (Trenin and Laub, 1987). For the S gene specific transcripts, the two promoters involved have an important functional distinction (Bulla and Siddiqui, 1988). SP1 contains a ubiquitous TATA element, and specifies precise initiation of the 2.4 kb species. SP2 depends upon an SV40-like CCAAT element, consistent with heterogeneous cap sites, which interacts with different regions about the cap sites containing potential initiation signals (Cattaneo *et al.*, 1983; Raney *et al.*, 1989). These features potentially allow for differential regulation of the expression of HBsAg proteins, depending upon the presence of promoter-binding factors. Not as much is known about the composition of the core promoter, but it also appears to specify heterogenous cap sites and to have multiple promoter factor binding regions

(Yaginuma and Koike, 1990; Yuh *et al.*, 1992).

HBV contains two functional enhancer elements. One (EnhI) is located just upstream of the X gene promoter (Shaul *et al.*, 1985; Tognoni *et al.*, 1985). As it appears to be genetically linked to the X promoter, and is responsive to X protein, it may also be a constitutive part of the X promoter (Spandau and Lee, 1988; Colgrove *et al.*, 1989). The second enhancer (EnhII) is located upstream of the core promoter (Yee, 1989). Both can enhance the activity of distally situated promoters (Bulla and Siddiqui, 1988; Antonucci and Rutter, 1989; Honigwachs *et al.*, 1989). Both elements can bind to, and are stimulated by, a diverse combination of regulatory factors (or enhancer binding proteins) in a combinatorial fashion (Schaller and Fisher, 1992). Both positive and negative regulation have been demonstrated (Gerlach and Schloemer, 1992). Further consideration to this will be given below.

IV. Serology and Immune Response

A. Markers of Infection

Consideration of the immune response during HBV infection becomes exceptionally important because of the diversity of clinical states which occur. HBV appears to be non-cytolytic during infection, as chronic productive infections often occur

in the absence of liver disease. It is believed that the host immune response is responsible for the cytopathic effect seen in the liver. It may also strongly influence the different possible outcomes (Feitelson, 1989; Thomas, 1990a). During infection, antibodies against four major antigen systems are observed: HBsAg, pre-S antigens, HBcAg, and HBeAg.

HBsAg is the specific serological marker of infection (Milich, 1987). It has a common group-specific marker, designated a, and two subtype-specific marker groups, *d/y* and *w/r* (creating four major serological subtypes *adw*, *ayw*, *adr* and *ayr*) (LeBouvier and Williams, 1975). There is no correlation between the course or severity of disease and the subtype involved. HBsAg appears in the blood at approximately 6 weeks after infection. It is usually cleared from the blood by approximately three months. Persistence of HBsAg for longer than 6 months defines a chronic state of infection (Sherlock, 1985).

HBcAg is not detected in the blood despite the fact that it is highly immunogenic. The related secretory HBeAg is detected at approximately the same time as HBsAg, and is associated with ongoing viral replication. It is usually cleared during recovery, while continued maintenance implies persistent infection. Recently, however, HBV variants with defects in the precore region which ablate HBeAg synthesis have been detected during chronic infection (Brunetto *et al.*, 1989; Carman *et al.*, 1989; Okamoto *et al.*, 1990).

HBV DNA in the blood is the most reliable marker for ongoing viral replication (Sherlock and Thomas, 1983). It can be detected by hybridization or PCR-related techniques. Polymerase activity can also be detected by supplying exogenous nucleotides to samples containing virions (allowing continuation of plus strand synthesis) (Summers and Mason, 1982).

Antibody to HBcAg is typically the first humoral immune marker of infection. High titres of IgM anti-HBcAg appear at about the time of HBsAg decline, and mark acute infection (Hoofnagle *et al.*, 1973; Chau *et al.*, 1983); persistent levels mark chronic disease (Hoofnagle *et al.*, 1973). Anti-HBe occurs later, and clearance of HBeAg with concurrent anti-HBeAg appearance is often associated with a resolution of infection and termination of viral replication (Realdi *et al.*, 1980; Hoofnagle *et al.*, 1981).

Anti-HBsAg appears at approximately three months after infection and persists for an extended period (Sherlock, 1985). A minority of cases do not exhibit this response. Antibodies to HBsAg are neutralising while antibodies to HBcAg and HBeAg are not (Iwarson *et al.*, 1985a), although HBcAg does elicit some protective immunity in chimps (Murray *et al.*, 1984; Iwarson *et al.*, 1985b). Current vaccines involve subviral (22 nm) particles composed of S proteins (as described below) isolated from human serum or produced by recombinant gene expression in yeast. Efficacy of the vaccine

is good, but not absolute (≥90%) after a full course of immunization (Hoofnagle *et al.*, 1978; Szmuness *et al.*, 1980).

Antibodies to the preS regions appear earlier than those for S protein (Neurath *et al.*, 1985; Budkowska *et al.*, 1986). Pre-S2 peptides are also protective in chimps, and inclusion in future vaccine designs has been proposed (Itoh *et al.*, 1986; Neurath and Kent, 1986). Related to this, preS regions (Milich *et al.*, 1985, 1986), as well as HBcAg (Milich *et al.*, 1987b) may provide important helper T cell (Th) help for production of HBsAg-specific antibodies.

Anti-P activity can also be detected during acute infection (Stemler *et al.*, 1988; Chang *et al.*, 1989b). More recently, antibodies to X protein have been detected in both acute and chronic patients (Kay *et al.*, 1985; Myers *et al.*, 1986; Abraham *et al.*, 1989). The relationship of this response to other viral markers is not yet clear.

B. Clearance of Infection

One of the abiding mysteries regarding HBV infection is how the virus is cleared from infected tissue. HBV does not appear to be directly cytopathic for hepatocytes (Barker *et al.*, 1973). Hepatocellular necrosis has long been believed to be a result of cell-mediated immunity (CMI) against viral products expressed on the infected cell surface (Dudley *et al.*, 1972; Mondelli and Eddleston, 1984). However, little information is

available about the progression of CMI in the acutely infected liver for the obvious reasons. Specificities seen for peripheral blood mononuclear cells (PBMC) may reflect a different repertoire than for those cells sequestered in the liver. An accumulation of cytotoxic T (Tc) and Th cells, and an increase in natural killer cell (NK) activity in the chronically infected liver has been demonstrated, but this is circumstantial (Egginck *et al.*, 1982). Also, as an immune defect may underlie the progression to chronic disease, conclusions derived from observations of chronic carriers are not likely relevant to normal viral clearance. Recent data from DHBV-infected ducks confirm the long-held suspicion that up to 100% of hepatocytes may be infected, and yet the majority suffer no consequences during resolution of the infection (Jilbert *et al.*, 1992). This strongly suggests that other mechanisms besides infected cell elimination contribute to clearance.

HBcAg and HBeAg have been strongly implicated as important targets for clearance during acute and chronic disease. The highly immunogenic nature of HBcAg confirms its accessibility to the immune system (Hoofnagle *et al.*, 1973). Specific proliferation of PBMC T cells from acute patients (Ferrari *et al.*, 1990) to HBcAg, and the presence of Tc directed against HBcAg in the PBMC of chronically infected patients have been demonstrated (Mondelli *et al.*, 1982; Pignatelli *et al.*, 1987). Analysis of infiltrates from areas of necrosis in the liver

show CD8+ and NK cells predominate (Egginck *et al.*, 1982). Cloning of infiltrate cell lines has confirmed the presence of $CD4^+$ cells which could provide HBcAg-specific help to autologous B cells (Ferrari *et al.*, 1987a); cytotoxic potential has also been demonstrated (Ramadori *et al.*, 1987; Ferrari *et al.*, 1987b). It has been proposed that HBeAg is expressed on the infected cell surface in addition to being secreted, and could therefore also be an important target (Schlict and Schaller, 1989). The HBeAg-minus variants referred to above are associated with rapidly progressive disease, suggesting impaired viral clearance.

The earliest detectable peripheral immune response during acute disease is a T-cell response to pre-S antigens (Vento *et al.*, 1987), which is followed by humoral and cellular HBcAg responses (Eddleston, 1988). A later intense T-cell response to HBsAg is most closely correlated in time with the onset of liver damage in acute disease (Vento *et al.*, 1987). This reaction was not seen during chronic disease. These results suggest that anti-HBsAg CMI may be essential to successful resolution of acute disease.

V. Pathogenesis

A. Tropism

HBV DNA and viral products are primarily found in the liver. Replicative viral DNA forms have also been found in extrahepatic tissues including bone marrow, peripheral blood lymphocytes (PBL), and kidneys, suggesting a broader tropism than initially thought (Blum *et al.*, 1983; Dejean *et al.*, 1984a; Elfassi *et al.*, 1984). Factors which specify this tropism are only now being elaborated.

Initial interaction with the target cell are specified by the viral envelope proteins. As referred to above, HBV is unusual in that it produces three distinct species of its envelope protein. A receptor activity on liver cell plasma membranes for the L protein has been reported (Pontisso *et al.*, 1989). Candidate proteins from liver cell membranes and soluble fractions which bind to a specific region of pre-S1 are currently being characterized as putative viral receptors (Petit *et al.*, 1992). A recent report has proposed that the relative presence of the preS1 region (which is easily degraded) is correlated with the infectivity of HBV preparations in human hepatoma cells (Bchini *et al.*, 1990).

It was earlier observed that the preS2 region could bind to human serum albumin when polymerized with glutaraldehyde (pHSA) (Machida *et al.*, 1983). pHSA can also mediate an

interaction of M protein-containing structures with liver membranes (Pontisso *et al.*, 1989). However, the *in vivo* relevance of pHSA is questionable, especially as HSA can block the interaction of preS2 with pHSA (Yu *et al.*, 1985; Ishihara *et al.*, 1987).

A receptor activity for the S protein was detected in primate kidney cell lines, but not in liver-derived lines (Peeples *et al.*, 1987). This may suggest an alternate route of access for extrahepatic tissues not involving preS regions. Infection of PBL has been invoked as one mechanism of lowering immune responsiveness to HBV during infection.

Replication of HBV can proceed after transfection of human hepatoma cells, and virions are produced (Sureau *et al.*, 1986; Chang *et al.*, 1987; Sells *et al.*, 1987; Tsurimoto *et al.*, 1987; Yaginuma *et al.*, 1987). Transfection of other non-liver cell lines has not been successful, demonstrating that there are intracellular events which specify tropism in addition to binding (Honigwachs *et al.*, 1989). More recently, infection of human hepatoma cells with HBV virions has been reported (Bchini *et al.*, 1990), suggesting that hepatoma cells can also support penetration and uncoating.

The enhancer elements which transcriptionally regulate HBV gene expression (detailed above) can bind multiple protein species including both ubiquitous factors (e.g. jun/AP-1, EF-C, NF-1) and transcriptional factors found primarily in the liver (e.g. C/EBP, HNF-1) (Ben-Levy *et al.*, 1989; Patel *et*

al., 1989; Faktor *et al.*, 1990; Lopez-Cabrera *et al.*, 1991). Tissue specificity for liver-derived cells has been demonstrated for the EnhI element, especially when in combination with the HBV core promoter (Shaul *et al.*, 1985; Honigwachs *et al.*, 1989), and for EnhII (Yee, 1989). The SP1 and core promoters also show a strong preference for liver-derived cells (Antonucci and Rutter, 1989; Chang and Ting, 1989). In addition to these elements, a glucocorticoid-responsive element within the S orf has been identified which can modulate HBV replication (Tur-Kaspa *et al.*, 1986; Tur-Kaspa *et al.*, 1988), and a proposed β-interferon responsive element may also contribute to tissue specificity (Thomas *et al.*, 1986).

Products of HBV are found in the liver of mice transgenic for the complete HBV genome (for review, see Chisari, 1991). Again, variable expression in a spectrum of extrahepatic tissues similar to that reported for HBV-infected humans is also seen, although viral products have been notably absent from lymphoid tissue. Replicative DNA species are more limited to liver, kidney and heart in these animals, and are correlated with the presence of 3.5 kb RNA species (which includes pregenome). Further study will provide more information on the host factors necessary for tissue-specific expression. However, the transgenic approach likely bypasses at least part of the tissue requirements for replication in

human tissue, and conclusions about tropism must be made with caution.

B. Chronic Infection and Hepatocellular Carcinoma

Generally, resolution of acute hepatitis is accompanied by the clearance of viral DNA and protein products, and normalization of liver function tests. Chronic hepatitis is accompanied by a persistent low level of viral replication, and HBsAg positivity. The factors which promote a chronic carrier state for HBV are not well understood. Children born to carriers become infected peri- or post-natally with high frequency (>90%), and invariably become carriers themselves (Stevens *et al.*, 1975; Beasley *et al.*, 1981a). In contrast, the rate of chronic infection is 5-10% for adult-acquired infection (Aldershvile *et al.*, 1980). This strongly suggests that the immune state of the individual is central to the development of the carrier state. Infants are believed to be immature with respect to immune components thought necessary for clearance of infected cells e.g. γ-IFN production, Tc activity, and NK activity (Thomas, 1990a; Thomas, 1991). In addition, they may become tolerant to viral proteins to which they were exposed in utero. HBeAg has been proposed as an important tolerogen, and is thought to be capable of crossing the placental barrier (Milich *et al.*, 1990). In the adult, numerous mechanisms for the failure to adequately resolve

infection have been proposed. These include a failure to induce neutralising anti-HBsAg responses, a failure to adequately present viral products for immune-mediated lysis, or a defect in immunomodulatory molecules and/or cells (e.g. IFN or T suppressor cells) promoting a tolerant or non-responsive state (Feitelson, 1989; Barnaba and Balsano, 1992). It remains problematic whether such observed correlations between immune responsiveness and disease outcome are causally related.

Chronic infection is associated with a high risk for HCC (Szmuness, 1978; Beasley, 1981b). However, HBV has not been shown to be directly oncogenic. An extended period of time is required before the appearance of neoplasms in chronically infected patients. One proposal is that transformation is a result of persistent liver cell injury causing inflammation and regeneration (Chisari *et al.*, 1987). Increased turnover would cause the accumulation of genetic rearrangements over time resulting in a precancerous state (Chisari *et al.*, 1989).

Chronic persistence is associated with the integration of viral DNA into the genome of host cells (e.g. Summers *et al.*, 1980). However, unlike retroviruses, this integration is not nearly as specific or central to the viral life cycle. Integration near oncogenes consistent with aberrant activation has been seen for HBV, but these are rare, random events (Dejean *et al.*, 1986; Hsu *et al.*, 1988). Integration usually occurs within the preC regulatory region of the genome (Dejean

et al., 1984b; Nagaya *et al.*, 1987), and expression of S proteins from integrated genomes at varying levels is often retained during chronic infection (Chisari *et al.*, 1989). Overexpression of viral proteins is one possible cause of chronic cellular injury; the L protein is a particularly good candidate, as it is specifically retained intracellularly (Chisari *et al.*, 1989). X protein is another possibility in light of its demonstrated *trans*-activation potential (Hohne *et al.*, 1990; Kim *et al.*, 1991). Integration may also promote chromosomal deletions or rearrangements predisposing to transformation (Rogler *et al.*, 1985; Hino *et al.*, 1986).

Future observations from the WHV model may shed important light on this process, as infected woodchucks develop HCC very quickly, often within a year of infection (Popper *et al.*, 1987). Comparisons with the oncogenicity of GSHV have suggested that the determinants for this lie within the virus, and not the host cell (Seeger *et al.*, 1991b). Differences in structural components between WHV and other hepadnaviruses may therefore lead to an identification of determinants for carcinogenesis.

VI. The Role of HBV Structural Proteins

A. Envelope Proteins

HBsAg is initially synthesized as a transmembrane species at the endoplasmic reticulum (E.R.) (Eble *et al.*, 1986). HBV virion assembly is believed to occur at this site, by budding of the nucleocapsid through the membrane-bound surface proteins into the E.R. lumen (Kamimura *et al.*, 1981; Patzer *et al.*, 1986). Therefore, unlike typical viral glycoproteins, HBsAg is not present at the E.R. prior to transport to some other membrane site of virion assembly. Residence at the E.R. is reflected by the physical structure of S protein. It is very hydrophobic, and three to four alpha-helical coils capable of spanning a membrane are predicted (see Fig. 12) (Howard *et al.*, 1988).

As mentioned, HBsAg can assemble into subviral particles which are composed of envelopes without nucleocapsids. This process is unique to HBV. These are seen in infected blood, but can also be produced by expression of S (Dubois *et al.*, 1980; Laub *et al.*, 1983; Liu *et al.*, 1982; Moriarty *et al.*, 1981) or M protein (Michel *et al.*, 1984; Persing *et al.*, 1985; Cheng and Moss, 1987) in heterologous systems. Only the S protein is required, and it can therefore be viewed independently of M and L proteins for 22 nm particle assembly.

The exact nature of the assembly process at the E.R.

membrane is not known. After insertion, subunits presumably associate with each other to form a membrane bound lattice. Subunit contacts are known to involve disulfide bonds, and probably also ionic bonds (Vyas *et al.*, 1972; Guerrero *et al.*, 1988). The half-life of 22 nm particle secretion from mammalian cells is very long (~5 hrs), and no HBsAg can be detected intracellularly apart from the E.R. (Patzer *et al.*, 1984). It is believed this reflects a lengthy, rate-limiting reorganization process within the E.R. membrane. Lipid content of subviral particles is lower than that of conventional cellular membranes, suggesting some selectivity of host lipid inclusion (Gavilanes *et al.*, 1982). Host proteins are excluded from particles. Following subunit association, it is postulated that a spherical particle structure is formed by invagination of the lipoprotein lattice. Since a nucleocapsid trigger is not involved, S protein clearly retains all the necessary information to direct this process.

All three forms of HBsAg are variably glycosylated. The major site of glycosylation colocalizes with the HBsAg a determinant (Bhatnager *et al.*, 1982; Peterson *et al.*, 1982). As glycosylation only occurs within the E.R., models of transmembrane HBsAg necessarily include a lumenal loop at this position. The antigenicity of this domain also requires that it face the particle exterior after particle formation. Glycosylation of S protein gives rise to a p24/gp27 pair (Peterson, 1981). Glycosylation of the L protein at the same

site produces a p39/gp42 pair (Heerman *et al.*, 1984). Curiously, the preS2 peptide when present in the M protein is also invariably glycosylated at a novel site to give a gp33/gp36 pair (Stibbe and Gerlich, 1983). This site within preS2 is silent within L protein, indicating it is revealed by a conformation unique to M protein (Heerman *et al.*, 1984; Imamura *et al.*, 1987). The ramifications of this, if any, are not known. L protein is also myristylated at its preS1 N-terminal glycine residue (Persing *et al.*, 1987).

Recently, important information has been derived concerning the distinction between viral and subviral particle formation. L protein is not secreted when expressed alone, and it will prevent the secretion of M and S proteins when coexpressed (Cheng *et al.*, 1986; Persing *et al.*, 1986; Standring *et al.*, 1986; Chisari *et al.*, 1986). A retention signal within the preS1 region N-terminus believed to be responsible for this has been identified (Kuroki *et al.*, 1989). This signal may be a consequence of myristylation in this region. L protein appears selectively in virions and filamentous forms (Heerman *et al.*, 1984) (Fig. 1). Thus, L protein may act more like a conventional viral glycoprotein, retaining associated HBsAg at the E.R. membrane until nucleocapsid interaction occurs (Bruss and Ganem, 1991a). How interaction occurs and how it frees nascent virions to the lumen is not known. Filamentous forms, which also do not contain nucleocapsids, may be an alternate outcome where high

localized L protein concentrations in the membrane are present. The role of M protein in these events is under debate (Bruss and Ganem, 1991a; Ueda *et al.*, 1991). Note that this role of L protein in HBsAg retention, and possibly also its role as a membrane receptor binding protein, may be reflected by its transcriptional regulation independent of M and S proteins.

B. Nucleocapsid proteins

HBV nucleocapsids are primarily composed of core protein (HBcAg) (Hruska and Robinson, 1977) (Fig. 1). They are icosahedral, with a T=3 number, and contain 180 subunits (Onodera *et al.*, 1982; Birnbaum and Nassal, 1990). Based on sequence similarity with the mengo virus nucleocapsid protein, models suggest HBcAg subunits have a central domain folded into a β-sheet barrel structure, with the termini disposed to the exterior (Argos and Fuller, 1988). HBcAg can self-assemble into particles (known as 27 nm particles) when expressed in a variety of heterologous systems (Stahl *et al.*, 1982; Miyanohara *et al.*, 1986; Ou *et al.*, 1986; Roosinck *et al.*, 1986; Takehara *et al.*, 1988; Standring *et al.*, 1988), which are morphologically and antigenically identical to HBV nucleocapsids (Cohen and Richmond, 1982). This implies that the pregenome is not required for initiation of core particle assembly.

The C-terminus of HBcAg is highly basic, being especially rich in arginine residues. It has a resemblance to protamine, which suggests it may nonspecifically bind nucleic acids. This has been supported using various binding assays (Petit and Pillot, 1985; Gallina *et al.*, 1989). Removal of the C-terminal domain abolished nucleic acid binding activity (Gallina *et al.*, 1989), although this has been debated (Matsuda *et al.*, 1988; Schlict *et al.*, 1989b). No tracts consistent with specific recognition of genomic DNA/RNA have been identified, although a role in selective genomic packaging can not be ruled out.

Like other viral nucleocapsids, HBcAg is a phosphoprotein. A kinase activity can be demonstrated in purified nucleocapsids (Albin and Robinson, 1980; Gerlich *et al.*, 1982) or in core particles produced by expression of HBcAg (Roosinck and Siddiqui, 1987) by supplying exogenous phosphate. HBcAg expressed in *E. coli* is not phosphorylated, suggesting this activity is eukaryotic host specific. Phosphorylation occurs at C-terminal serine residues for DHBV C protein (Schlict *et al.*, 1989b). Although the role of phosphorylation is not known, it may be involved in virion formation. DHBV virions are underphosphorylated compared to cytoplasmic cores, suggesting that removal of phosphates signal cores for maturation (Pugh *et al.*, 1989).

The subcellular localization of core protein has been the subject of some debate. It can be detected in the cytoplasm

and/or the nucleus of infected liver cells by immunofluorescence (see Israel and London, 1991). Reports differ as to the preferential localization of core protein to nucleus or cytoplasm in expressing cultured cells (Ou *et al.*, 1986; Ou *et al.*, 1989; McLachlan *et al.*, 1987; Roosinck *et al.*, 1987). These analyses are confused by the cross-reactivity of core and e protein species (see below). Precore cleavage products appear to be resorted from the E.R. membrane to the nucleus, confusing the identity of HBcAg-reactive species present in the nucleus *in vivo* (Ou *et al.*, 1989; Yeh *et al.*, 1990). Nuclear localization signals (NLS) appear to be located within the basic C-terminal region, but their function may vary from cell to cell (Yeh *et al.*, 1990; Eckhardt *et al.*, 1991). Note that the localization of nucleic acid binding potential, modification by phosphorylation, and NLSs all at the C-terminus of HBcAg make it extremely difficult to separate their putative biological roles.

Localization of core protein has been proposed as a mechanism for the replenishment of nuclear ccc DNA for the long-term maintenance of viral replication during persistent infection (Tuttleman *et al.*, 1986; Ou *et al.*, 1989). This could be provided for by pregenomic RNA which becomes associated with HBcAg-related species which sort to the nucleus. This RNA could then become converted to ccc DNA which acts as a template for further transcription. However, no direct evidence for this hypothesis exists.

One of the most intriguing aspects of HBV biology is the function of the precore region. This region is conserved in all hepadnaviruses. It encodes 29 aa which contain a signal sequence, and can confer a secretory phenotype on core protein when expressed in-frame (Ou *et al.*, 1986; McLachlan *et al.*, 1987; Standring *et al.*, 1988). This ultimately results in the translocation and secretion of HBeAg. Cleavage occurs after the first 19 aa (the distal precore region), which leaves an additional 10 aa (proximal precore) at the N-terminus of the core polypeptide (Standring *et al.*, 1988; Bruss and Gerlich, 1988). Secretory HBeAg also undergoes secondary proteolytic cleavage event(s), which remove C-terminal regions (Takahishi *et al.*, 1983). The result is a somewhat heterogenous population of secreted monomeric polypeptides, depending upon the HBV subtype. The sequence specificity or identity of the responsible protease is not known, although a recent report has localized this activity to a post E.R. compartment (Wang *et al.*, 1991).

The translocation process has a surprising element of inefficiency. Signal cleavage is not complete in some systems (Lanford *et al.*, 1990; Yang *et al.*, 1992). Even after signal cleavage, translocation can be aborted and the cleaved chains resorted back to the cytoplasm (Garcia *et al.*, 1988). Nontranslocated and cleaved precore species can also be found in the nucleus (Ou *et al.*, 1989). This resorting to the nucleus occurs under the influence of the proximal precore

region which appears to cooperate with C-terminal NLSs (Yeh *et al.*, 1990).

The role of HBeAg in the viral life cycle is unknown. The precore region of DHBV can be deleted without affecting any phase of the viral life cycle (Chang *et al.*, 1987; Schlict *et al.*, 1987). The maintenance of naturally occurring HBV precore mutants during chronic infection has already been mentioned. Therefore, apart from the suggested involvement in the establishment of a chronic carrier state in infants, the role of HBeAg is still unknown.

HBeAg is serologically distinct from HBcAg (Magnius and Espmark, 1972), although the two share the majority of their primary sequences. Although crossreactive, both antigens have private specificities that are not shared (see Schlict and Wasenauer, 1991). Generally, antisera to HBcAg are directed at conformational epitopes, while HBeAg antisera recognize linear epitopes (Salfeld *et al.*, 1989). Denaturation or partial proteolysis of HBcAg reveals HBeAg antigenicity (McKay *et al.*, 1981; Takahishi *et al.*, 1980). It must be noted that particulate formation, while restricted to HBcAg in a normal setting, can be adopted by molecules that display full HBeAg antigenicity. Core polypeptides with C-terminal deletions expressed in *E. coli* could assemble into particles (Milich *et al.*, 1988; Gallina *et al.*, 1989), albeit with different stability and hydrodynamic properties. This demonstrates that only the central region of core protein is necessary for

particle formation, but that the entire primary sequence is necessary to confer the distinctive conformation characteristic of HBcAg reactivity.

C. HBV Structural Proteins as Carriers of Foreign Epitopes

The unusual ability of both HBcAg and HBsAg to form spherical particulate structures in the absence of other viral components has prompted attempts to utilize both molecules as high molecular weight carriers of foreign epitopes by genetic fusion. Despite this apparent similarity in quaternary structure, these molecules have distinctly different pathways of assembly, and these distinctions must be taken into consideration during recombinant protein design.

The first use of HBsAg as a carrier of a foreign peptide sequence involved the entire ectodomain (portion facing the exterior) of the mature herpes simplex virus glycoprotein D (HSV gD) fused within the preS2 region of HBsAg and expressed in yeast cells (Valenzuela *et al.*, 1985a). Although HBsAg is not secreted from yeast cells, it had been previously demonstrated that both S and M proteins form authentic 22 nm particles released in yeast lysates (Valenzuela *et al.*, 1982; Valenzuela *et al.*, 1985b; Imamura *et al.*, 1987). The HSV gD fusion could also form intracellular particles which had the appearance and properties of authentic particles, and were

cross-reactive to both HBsAg and HSV gD.

In a more significant study, peptides were inserted into either of the two major hydrophilic domains of the major protein, and the fusions expressed in mouse L cells (efficient for HBsAg secretion). It was found that small peptides (less than 15 aa) had no effect on particle assembly or secretion, while larger ones ablated secretion (Delpeyroux *et al.*, 1987). RIA reactivity of lysates from non-secretory fusions could still be demonstrated, and in one case, particles were isolated from lysates. The second hydrophilic domain (the site of glycosylation and the a determinant) was found to be more sensitive in terms of both particle secretion and HBsAg-reactivity of the fusion. Further studies with a poliovirus VP1 epitope inserted in the first hydrophilic domain have shown that hybrid particles can assemble in a mixed fashion with wild-type S chains, and that neutralizing antibodies against poliovirus can be elicited (Delpeyroux *et al.*, 1986; Delpeyroux *et al.*, 1988).

Other studies initiated after the first stages of the work herein came to similar conclusions using short peptides from HIV-1 gp120 (Michel *et al.*, 1988) and an epitope from the *Plasmoidium falciparum* circumzoite protein (Rutgers *et al.*, 1988), inserted into the preS2 domain. Generally, these studies demonstrated that while insertions in the preS2 domain or in the first hydrophilic domain are less disruptive, insertions near the antigenic region are more immunogenic

(Delpeyroux *et al.*, 1990). As preS domains are not required for particle formation, and do not interfere in the case of preS2, N-terminal additions should be less likely to disrupt conformationally-based interactions. Conversely, insertions in the second domain would be in a position competent for presentation to and stimulation of the immune system.

While HBsAg proved to be a feasible carrier, HBcAg quickly proved to be much more amenable to this type of manipulation. The first use of HBcAg involved a peptide from the foot and mouth disease virus (FMDV) VP1 protein fused within the precore sequence near the HBcAg N-terminus (Clarke *et al.*, 1987). Since then, peptides originating from a variety of pathogens have been fused within the precore sequences, at a truncated C-terminus, and even internally, at the site of a predicted outer loop (Borisova *et al.*, 1989; Stahl and Murray, 1989; Schodel *et al.*, 1990; Clarke *et al.*, 1990; Moriarty *et al.*, 1990; Schodel *et al.*, 1992). In all cases, hybrid 27 nm particles were formed. Fused peptides were good immunogens in many cases (Clarke *et al.*, 1987; Stahl and Murray, 1989), and immunogenicity depended upon the particulate nature of the chimeric particles (Clarke *et al.*, 1990). In sum the data demonstrate that HBcAg is not highly sensitive to peptide additions for its normal biosynthesis, although insertion at an internal site did disrupt HBcAg antigenicity (Schodel *et al.*, 1992). While HBcAg is not normally secreted, its high level of expression in many systems, its ease of isolation,

and its high immunogenicity have made it a carrier system of choice.

VII. Objectives of Research

Our initial experimental designs were aimed at the expression and secretion of chimeric 22 nm particles from a baculovirus expression system. In accordance with the information above, I hoped to eventually demonstrate the utility of such particles as cheap and efficacious bivalent vaccines. The rabies virus glycoprotein (rabies G) was used as a source of foreign peptides for fusion. This protein has been well characterized at the structural and antigenic levels (Cox *et al.*, 1977; Dietzschold *et al.*, 1982; Dietzschold *et al.*, 1983; Macfarlan *et al.*, 1984). In addition, there is a significant prevalence of rabies in regions of Southeast Asia, which is also endemic for HBV. For both of these pathogens, the cost of modern subunit vaccines is a significant problem; a bivalent vaccine would be a considerable advantage in this regard.

The expression of both S and M HBsAg using a baculovirus system has been characterized previously (Kang *et al.*, 1987; Scully and Kang *et al.*, 1988). Extracellular antigen could be detected in both cases, and 22 nm S particles were purified.

Generally, the baculovirus system provides very high levels of expression for heterologous proteins, and is capable of most eukaryotic post-translational modifications.

Difficulties were experienced in obtaining secretion of the proposed recombinant HBsAg particles. Therefore, our secondary goal became one of further characterization of HBsAg synthesis in insect cells. It was hoped that further understanding of the requirements for 22 nm particle assembly might allow better manipulation in the future. I concentrated on the function of one transmembrane domain and its possible role in particle assembly. This was investigated by mutagenesis of the genetic region encoding this domain and by its replacement with heterologous coding segments. The effects were assayed by the detection of extracellular HBsAg reactivity and by biochemical characterization of the mutant proteins.

In order to complete a more comprehensive investigation of HBV structural proteins, a second project involving HBcAg was initiated. I investigated the expression of both core and precore proteins using a baculovirus system. The competency of the precore signal to direct HBeAg synthesis was assessed and compared with that of a heterologous signal. This study was not directly concerned with the use of HBcAg as a carrier of foreign peptides. However, a full understanding of the competency for secretion of various HBcAg-related peptides from insect cells may allow the design of better chimeric particles in the future. In addition, contributions were made

to a study in which peptides from HIV-1 were successfully used to create chimeric HBcAg particles. This will also be reported here.

Materials and Methods

I. Materials

All oligonucleotides were synthesized using an Applied Biosytems 380B DNA synthesizer by Nancy Delcellier in our laboratory. Goat anti-HBsAg, alkaline phosphatase-conjugated rabbit anti-goat IgG and alkaline phosphatase-conjugated rabbit anti-sheep IgG were purchased from Accurate Chemical, Westbury, New York, U.S.A. Sheep anti-rabies G was a gift from M. Ferguson, WHO International Lab for Biological Standards, South Mimms, England. Rabbit anti-gp120ΔS was prepared by Yan Li in our laboratory. Rabbit anti-HIVHBcAg and anti-HBcHIV recombinant fusion proteins were prepared by Anthony Tam in our laboratory. Alkaline phosphatase-coupled goat anti-rabbit IgG and alkaline phosphatase-coupled rabbit anti-sheep IgG were from BioRad (Mississauga, Canada)

II. Plasmids and Viruses

Plasmid pAcYM1 was provided by David Bishop, NERC Virus Research Institute, Oxford, U.K. pVT-Bac was from David Thomas, NRC Biotechnology Research Institute, Montreal, Canada. HBV-SphII was a gift from M. Sherman, Toronto General

Hospital, Toronto, Canada. pVGR-2 was a gift from Robert Lazzarini, NIH, Bethesda, Maryland, U.S.A. pNEO and pSV1 were purchased from Pharmacia Inc., Baie D'urfe, Canada. The Prospect Hill Virus G2 subclone M5' was constructed by Mark Parrington in our laboratory (Parrington *et al.*, 1991).

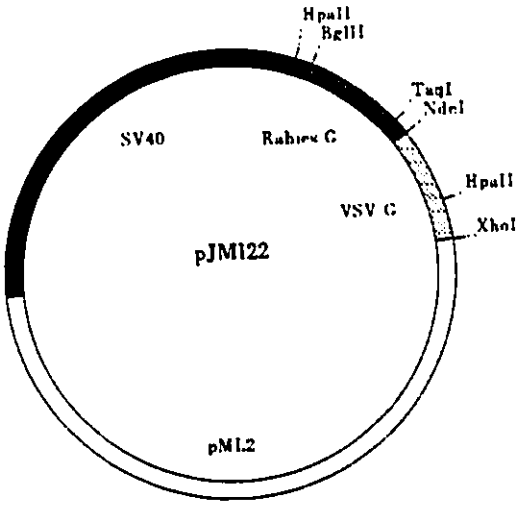
A 1.4 kb *Bam*HI fragment from HBV adw2 (which encodes all of HBsAg and part of the preS2 region) subcloned into pUC8 to create pUC8-HBs was the original source of HBsAg used here (Ono *et al.*, 1983). This fragment was subsequently modified by the addition of synthetic oligonucleotides to both termini which recreated the translation termination codon, added restriction enzyme sites for further subcloning, and recreated the complete preS2 coding region. This was subcloned as a *Xho*I fragment into the SV40-based expression vector pJC119 (Sprague *et al.*, 1983), as modified to pJM119 by John McCullough in our laboratory, to create pJM119-PsYK25 (abbreviated to pJM119). For a more convenient source of HBsAg, the *Xho*I fragment of pJM119 was blunt end subcloned at the *Bam*HI site of pUC18 (recreating the *Xho*I site and destroying the pUC18 *Bam*HI site) to create pUCHBs (Fig. 4b).

The fusion subclone pJM122, constructed by John McCullough in our laboratory, was used as a source for 5'-terminal fragments of rabies G (Fig. 4a). This subclone contained a 497 bp. *Bgl*II/*Nde*I fragment from pVGR-2 subcloned as an in-frame fusion with a C-terminal fragment from the VSV G gene.

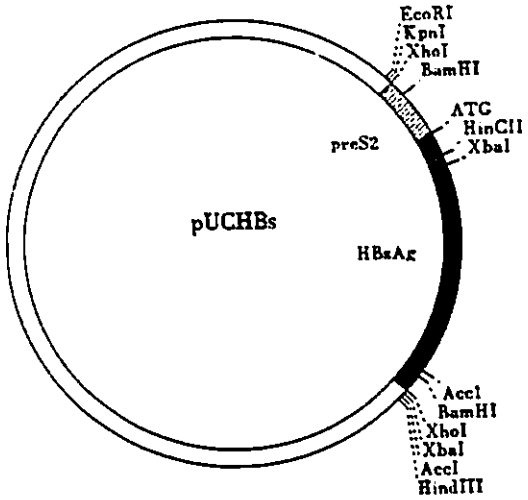
The HBsAg subclone N7, constructed by N. Delcellier in our

Figure 4. Schematic representation of plasmids used during the construction of HBsAg/rabies G recombinant fusions. (A) Plasmid pJM122. Contains an N-terminal segment of the rabies G gene (shaded region) fused to a segment of the VSV G gene (stippled region). Derived from pJC119, an expression vector containing regulatory sequences from about the SV40 origin (black region) in plasmid vector pML2. Restriction sites used for further subcloning are indicated (and below). (B) Plasmid pUCHBs. Contains complete M protein gene as derived from pJM119-PsSYK25 (Scully and Kang, 1988), inserted into pUC18. The S gene (denoted HBsAg, black region) and the preS2 region (lined region) are indicated. The position of the S gene start codon is indicated as ATG. (C) Plasmid pHBsN7. Contains a small N-terminal S gene segment (black) in pUC18. The start codon is indicated. Both termini were modified by cross-over linker-directed mutagenesis (see text). (D) Plasmid pHIVHBsA. Contains a synthetic coding region derived from structural proteins of HIV-1 (lined region) fused to a *Hin*cII site near the N-terminus of the S gene (black region).

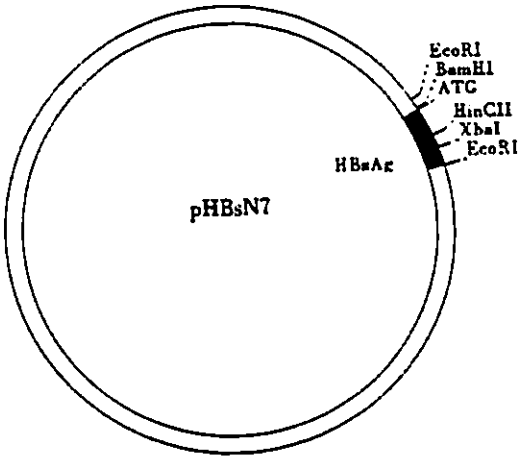
A


HpaII
BglII
TaqI
NdeI
SV40
Rabies G
HpaII
VSV G
XhoI
pJM122
pML2

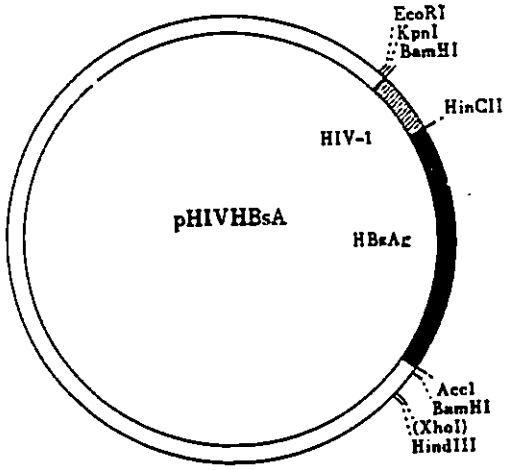
B


EcoRI
KpnI
XhoI
BamHI
ATG
HinCII
XbaI
preS2
pUCHBs
HBsAg
AccI
BamHI
XhoI
XbaI
AccI
HindIII

C


EcoRI
BamHI
ATG
HinCII
XbaI
EcoRI
HBsAg
pHBsN7

D


EcoRI
KpnI
BamHI
HinCII
HIV-1
pHIVHBsA
HBsAg
AccI
BamHI
(XhoI)
HindIII

laboratory, was used as a source of a 5'-terminal fragment of HBsAg (Fig. 4c). This contained a fragment of HBsAg from the initiation codon to the *Xba*I site at nt 92 subcloned between the *Bam*HI and *Xba*I sites of pUC18. This fragment had been modified at both termini by crossover linker mutagenesis (Sung *et al.*, 1986; Garson *et al.*, 1990): the 5' terminus had a *Bam*HI site and the consensus translation initiation sequence ACC placed immediately upstream of the initiation codon; the 3' terminus had *Pst*I, *Sst*I, and *Bam*HI restriction enzyme sites placed after the *Xba*I site (only the utilized *Xba*I site is shown).

The HBsAg/HIV-1 fusion subclone pHIVHBs-A, constructed by Anthony Tam in our laboratory, was used a source of a 3'-terminal fragment of HBsAg in the construction of HBsR2 (Fig. 4d). This contained a fragment of HBsAg (from pJM119) from the *Hin*CII site at nt 64 to the 3' *Xho*I site fused at the *Hin*CII site to a synthetic oligonucleotide encoding fragments of HIV-1 structural proteins. This insert was subcloned in pUC18 between the *Bam*HI site (to a complementary site contributed by the HIV segment) and the *Hin*cII site (blunt end ligated to the HBsAg-contributed *Xho*I site, which destroys both sites).

The wild type baculovirus *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV) and the HBcAg recombinant virus AcNPV-YM1KTc were from David Bishop, NERC Institute of Virology, Oxford, U.K. The pAcYM1-based HBsAg recombinant virus AcNPV-HBsYK14A7 (abbreviated to 14A7) was derived from

the pAcRP6-based virus AcNPV-HBsYK14 (Kang *et al.*, 1987) by John McCullough in this laboratory. The gp120 signal-deletion mutant recombinant virus AcNPV-gp120ΔS was from Yan Li in this laboratory.

III. Recombinant DNA constructions

Recombinant DNA construction was performed using standard techniques (Maniatis *et al.*, 1982). In addition to purification of plasmid DNA by CsCl gradient centrifugation, Qiagen affinity column (Qiagen Inc., Chatsworth, California, U.S.A.) purification was carried out according to the manufacturers specifications. DNA fragments were isolated using either a GeneClean system (BIO/Can Scientific, Mississauga, Canada) or a UEA unidirectional electroelution apparatus (IBI, Markham, Canada).

Polymerase chain reaction (PCR) amplification of DNA was done in a Perkin-Elmer Cetus (Rexdale, Canada) thermocycler using *Taq*I polymerase (Pharmacia or Perkin-Elmer Cetus) or Vent polymerase (New England Biolabs, Beverly, Massachussetts, U.S.A.) in the supplied reaction buffers. Amplification was typically done using thirty cycles of 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C, followed by a 10 min extension segment at 72°C. For specific amplifications the annealing temperature was sometimes dropped as low as 37°C.

Crossover linker-directed mutagenesis was performed as described using ds oligonucleotides (Sung *et al.*, 1986; Garson *et al.*, 1990). In order to lower the recovery of background vector clones to the minimum, the vector to be mutagenized was typically cleaved outside of the target insert with two non-compatible restriction enzymes prior to crossover linker ligation to eliminate vector religation. After linker ligation, the resultant linear vector was also purified from any recircularized vector forms after electrophoresis and prior to transformation for the same reason.

All pUC18 subclone fusion junctions except for that of pUCHBsR2 were sequenced to confirm they were in-frame with the aid of a Genesis 2000 Automated DNA Analysis System (DuPont, Mississauga, Canada). To reach internal junctions, pUCHBsR1 was primed with oligonucleotide RGSeq2 homologous to nt 389 to 400 of the rabies G sequence, while the C-terminal fusions were primed with oligonucleotide RGSeq1, complementary to nt 30 to 13. A *Hin*dIII/*Xba*I fragment from pUCHBsR2 which spanned the fusion junction was sequenced by the method of Maxam and Gilbert (1980).

Figs. 6 and 11 illustrate the various segments utilized in the construction of HBsAg/rabies G fusions. Figs. 13 and 20 illustrate various mutations done on the HBsAg S gene. Fig. 22 illustrates mutations done on the C/precore gene. For HBsAg, numbering of nucleotides and amino acids refers to the S gene. For rabies G, nucleotide numbers refer to the complete coding

region of the gene, while amino acid numbers refer to the mature protein sequence.

A. HBsR1

The large *Kpn*I/*Hinc*II fragment from pHIVHBsA was ligated stepwise with oligonucleotides JW5/6 and JW7/8 (below). The resultant plasmid, pUCHBsTaq, contains a reconstructed HBsAg 5′ region and a *Taq*I site 5′ to the normal initiator methionine codon.

JW5/6

```
5'-     CTCGAATGGAGAACATC
3'- CATGGAGCTTACCTCTTG***
    KpnI TaqI
```

JW7/8

```
***ACATCAGGATTCCTAGGACCCCTGCTCGTGTTACAGGCGGGGTTTTTCTTGTT
TAGTGTAGTCCTAAGGATCCTGGGGACGAGCACAATGTCCGCCCCAAAAAGAACAA
            ......HBsAg coding sequence.....    HincII
```

The asterisks indicate compatible 5′-overhanging termini used to ligate JW5/6 with JW7/8. The initiation codon of HBsAg is in bold type. Restriction sites are underlined (here and below). Note that in many cases, this refers to compatible restriction site termini and not the entire recognition sequence (e.g. the *Kpn*I and *Hinc*II sites above).

The small rabies G *Bgl*II/*Taq*I fragment from pJM122 was ligated in frame with the *Taq*I/*Bam*HI HBsAg fragment from pUCHBsTaq between the *Bgl*II and *Bam*HI sites of pNEO. The result was pJWHBsR1. The *Bam*HI/*Bgl*II fragment from pJWHBsR1

was subcloned at the *Bam*HI site of pAcYM1 to create pAcHBsR1.

B. HBsR2

The *Xho*I/*Hin*cII HBsAg fragment from pJM119 was blunt-end was blunt-end subcloned at the *Hin*cII of pUC18 to create pUCHBsHinc. The *Bgl*II/*Taq*I rabies G fragment from pJM122 was blunt-end subcloned at the *Hin*cII of pUCHBsHinc to create pJWHBsR2. The *Bam*HI fragment from pJWHBsR2 was subcloned at the *Bam*HI site of pAcYM1 to create pAcHBsR2.

C. HBsR3

The *Eco*RI/*Xba*I HBsAg fragment from N7 was blunt-end subcloned in-frame at the *Bgl*II site of pJM122 creating pJWHBsR3Nde. pJWHBsR3Nde was cleaved with *Nde*I and ligated with the palindromic linker JW3A (which places a stop codon and a *Bam*HI site after the rabies fragment) creating pJWHBsR3. The *Bam*HI insert from pJWHBsR3 was subcloned into pAcYM1 to create pAcHBsR3.

```
JW3A        5'- TAAGGATCCT   -3'
JW3A        3'-   TCCTAGGAAT -5'
                   BamHI
```

The termination codon is in bold type.

D. HBsR4

The large *Hin*dIII/*Acc*I fragment from pUCHBs was ligated to JW9/10, which can promote a deletion of the 3′ end of HBsAg and place a *Bam*HI site after the sequence encoding amino acid residue 102. Linear fragment (after ligation) was used to transfect *E. coli*, and the crossover linker-directed truncation pUCHBsR4 was isolated. The *Bam*HI fragment from pUCHBsR4 was subcloned in-frame at the *Bgl*II site of pJM122 to create pJMHBsR4Nde. JW3A was ligated at the *Nde*I site of pJMHBsR4Nde (as above) to create pJMHBsR4. A *Hin*cII/*Hpa*I HBsAg/RG fragment from pJMHBsR4 was blunt-end subcloned at the *Hin*cII of N7 to created pJWHBsR4, replacing the 5′ end of the HBsAg fragment with that from the N7 subclone (making it identical to that of HBsR3). The *Bam*HI fragment from pJWHBsR4 was subcloned into pAcYM1 to create pAcHBsR4.

```
JW9       5′-  CTTCTGGATTATCAAGGTGGATCC     -3′
JW10      3′-     GACCTAATAGTTCCACCTAGGTCGA -5′
                                 BamHI HindIII
```

The sequence prior to the *Bam*HI site is identical to nt 288 to 305 of the HBsAg sequence.

E. HBsR5

A fragment was PCR amplified from pUCHBs which encoded HBsAg up to Ser(151). The 5′ primer was originally used in the

crossover linker-directed modification of HBsAg for the N7 subclone (N. Delcellier in our laboratory). The 3' primer was 5'-AGCTTGGATCCAGAGAAACGGAC-3' and contained a *Bam*HI site just after codon 151. The product was subcloned at the *Bam*HI site of pUC18 to create pUCHBsR5. The *Bam*HI insert from pUCHBsR5 was subcloned at the *Bgl*II site of pJM122 to create pJMHBsR5Nde. This was modified with JW3A (as above) to create pJMHBsR5. A *Hin*cII/*Hpa*I HBsAg/RG fragment blunt-end subcloned at the *Hin*cII site of N7 to create pJWHBsR5 (to reconstruct the appropriate 5' terminus, as above). The *Bam*HI fragment from pJWHBsR5 was subcloned into pAcYM1 to created pAcHBsR5.

F. HBsR6

pUCHBs was cleaved with *Acc*I and ligated with excess annealed linkers JW1/2 (below), which places a *Bam*HI site prior to the termination codon of HBsAg.

```
JW1      5'- ATACATTG      -3'
JW2      3'-   TGTAACCTAG -5'
              AccI   BamHI
```

The product was cloned at the *Bam*HI site of pUC18 to create pUCHBsAcc. The *Bam*HI fragment from pUCHBsAcc was subcloned in-frame at the *Bgl*II site of pJM122 to create pJWHBsR6Nde. The fusion fragment was modified and subcloned into N7 as above to create pJWHBsR6. The *Bam*HI fragment of pJWHBsR6 was subcloned into pAcYM1 to create pAcHBsR6.

G. Fusions to other rabies G fragments

Three different rabies G fragments were PCR amplified from pVGR-2: RGm with JW19 and JW20 (nts 57-450); RGs with JW21 and JW22 (nts 1005-1374); RGl with JW19 and JW22 (nts 57- 1374). RGs encoded amino acids Thr(316) to Lys(439); RGm encoded Lys(1) to Ser(131); RGl encoded Lys(1) to Lys(429).

JW19 5'- TTCGAATTCAGATCTAAATTCCCTATTTACACG -3'
*Eco*RI *Bgl*II

JW20 3'- CATTTTTGGTGGTTCCTCAGAATTCCTAGGAGATCTAGA -5'
***BamHI *XbaI*

JW21 5'- TTCGAATTCAGATCTACCATATTCAACAAGACCTTG -3'
*Eco*RI *Bgl*II

JW22 3'- GAGGGCTTGACCCCCTTCATTCCTAGGAGATCTAGA -5'
***BamHI *XbaI*

The 3' primers (JW20/22) contain a translation termination codon (asterisks).

The amplified PCR fragments were subcloned between the *Eco*RI and *Xba*I sites of pUC18. This created pUCRGs, pUCRGm, and pUCRGl, respectively. HBsAg BamHI fragments were isolated from pUCHBsR4, pUCHBsR5 and pUCHBsAcc, and subcloned at the *Bgl*II site of pUCRGm to create pUCR4RGm, pUCR5RGm and pUCR6RGm. The *Bam*HI fragment from pUCHBsR5 was subcloned at the *Bgl*II site of pUCRGs and pUCRGl to create pUCR5RGs and pUCR5RGl. *Hinc*II fragments from all 5 fusions were blunt-end subcloned at the *Hinc*II site of N7 to create pJWR4RGm, pJWR5RGm, pJWR6RGm, pJWR5RGs, pJWR5Rgl. *Bam*HI fragments from

these plasmids were subcloned at the *Bam*HI site of both pAcYM1 and pSV1.

H. Mutagenesis of HBsAg

pUCHBs was PCR amplified with: JWK2 or JWA2 with JW23, which create point mutations at amino acid 2 of HBsAg; JWΔN or JWMel and JW23, which create N-terminal deletions of HBsAg. The products were subcloned at the *Bam*HI site of either of pAcYM1 (to create pAcHBsK2, pAcHBsA2, and pAcHBsΔN) or pVT-Bac (to create pAcHBsΔNMel).

JWK2 5'- CCGGATCCACCATGAAGAACATCACATCA -3'
*Bam*HI

JWA2 5'-CCCGGATCCACCATGGCGAACATCACATCA -3'
*Bam*HI

JWΔN 5'- CCGGATCCACCATGCAGAGTCTAGACTCGTGG -3'
*Bam*HI

JWMel 5'- CCGGATCCACAGAGTCTAGACTCGTGG -3'
*Bam*HI

JW23 3'- GAGACCCATATGTAAATTCCTAGGCC -5'
******Bam*HI**

The 5' primers, with the exception of JWMel, contained the consensus translation initiation sequence ACC preceding an initiation codon. JWMel had a *Bam*HI site for fusion in-frame with the mellitin signal peptide of pVT-Bac (Tessier *et al.*, 1991). The 3' primer JW23 included the normal HBsAg termination codon (asterisks).

pVGR-2 was PCR amplified with primers RG1 and RG2 (nts 1369

to 1456 of rabies G). The product was subcloned between the *Bam*HI/*Eco*RI sites of pUC18 to create pUCRG. The *Bam*HI/*Eco*RI fragment of pUCHBsΔN was subcloned between the *Bgl*II/*Eco*RI sites of pUCRG to create pJWHBsΔNRG. M5' (Parrington *et al.*, 1991), a cDNA clone from the Prospect Hill Virus, was PCR amplified with PHV1 and PHV2 (nts 3344 to 3460 of the M genomic segment), and subcloned to create pUCPHV. The *Bam*HI/*Eco*RI fragment of pUCHBsΔN was subcloned between the *Bgl*II/*Eco*RI sites of pUCHBV to create pJWHBsΔNPHV. The *Bam*HI fragments of both fusion subclones were subcloned into pAcYM1 to create pAcHBsΔNRG and pAcHBsΔNPHV.

RG1 5'- CCCGGATCCACCATGTGGGGGAAGTATGTATTA -3'
*Bam*HI

RG2 3'- ACATATTCTCAGTTAGCTCTAGACTTAAGGGG -5'
*Bgl*II *Eco*RI

PHV1 5'- CCCGGATCCACCATGTCTGGGGAATGGTTAATG -3'
*Bam*HI

PHV2 3'- ACGGGAGCTTCCCAACAGTTTCTAGACTTAAGGGG -5'
*Bgl*II *Eco*RI

I. Mutagenesis of HBcAg

pHBV-SphII was PCR amplified as follows: with JWPC and JW24 (the complete core plus precore gene); with JWP22 and JW24 (nt 57 of precore to the end of core); with JWCoreM and JW24 (the core gene); with JWP22M and JW24 (the core gene. The products were subcloned at the *Bam*HI site of either pAcYM1 (for JWPC and JW22) or pVT-Bac (for JWCoreM and JWP22M).

JWPC 5'- CCGGATCCACCATGCAACTTTTTCACCTC -3'
BamHI

JWP22 5'- CCGGATCCACCATGTCCAAGCTGTGCCTTGGG -3'
BamHI

JWCoreM 5'- CCGGATCCAATGGACATTGACCCTTAT -3'
BamHI

JWP22M 5'- CCGGATCCATCCAAGCTGTGCCTTGGG -3'
BamHI

JW24 3'- GCCCTTAGAGTTACAATCCCTAGGCC -5'
***BamHI

The 5' primers JWPC and JWP22 contain the consensus translation initiation sequence ACC preceding the initiation codon. JWCoreM and JWP22M contain a *BamHI* site allowing fusion in-frame with the mellitin signal peptide coding region in pVT-Bac. The 3' primer JW24 contains the HBcAg translation termination signal.

IV. Cell culture and recombinant viruses

Two cell lines from *Spodoptera frugiperda* (fall armyworm) were used to propagate baculovirus: IPLB-SF-21 (SF21) was from David Bishop and *S. frugiperda* clone 9 (SF9) was from Max Summers, Texas A&M University, College Station, Texas, U.S.A. These lines were used interchangeably, although SF9 was preferred for routine virus propagation and recombinant virus isolation because of its faster growth rate, and SF21 was preferred for metabolic labelling experiments because of its

greater adherence in monolayer. We will refer to insect cells generically as SF cells. Cells were maintained at 28°C in TMN-FH medium supplemented (supplements from Gibco BRL, Burlington, Canada) with 10% heat-inactivated fetal bovine serum (FBS), 2mM L-glutamine, penicillin-streptomycin (50 units/ml penicillin G sodium, 50 μg/ml streptomycin sulfate) and kanamycin sulfate (50 μg/ml). All tissue culture vessels were from Corning (Corning, U.S.A.) or Nunc (Nunc, Kamstrup, Denmark). Cells were routinely maintained in reciprocating culture using 80 cm^2 tissue culture flasks. All experimental infections were done in monolayer culture in 25 cm^2 flasks, or in 30 mm or 50 mm polystyrene dishes.

COS-1 cells were originally from the American Type Culture Collection (Rockville, Maryland, U.S.A.). They were grown as monolayers in MEM supplemented with 10% FBS, 2mM L-glutamine, penicillin-streptomycin (50 units/ml penicillin G sodium, 50 μg/ml streptomycin sulfate) and kanamycin sulfate (50 μg/ml). Cells were maintained at 37°C in a humidified Shel-lab incubator (John's Scientific Inc., Toronto, Canada) in 100 mm polystyrene dishes. They were passaged by dispersing cells with 1.25% trypsin in phosphate-buffered saline (PBS; 137 mM NaCl, 8.1mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.9mM $CaCl_2$, 0.5 mM $MgCl_2$). Cells were pelleted at 200xg for 5 min in a Sorvall GLC-2B clinical centrifuge to remove the trypsin, washed once in fresh medium, and resuspended in medium for plating.

Recombinant baculoviruses were isolated and handled

according to Summers and Smith (1987). Transfection was as described, except in some cases a CellPhect transfection kit (Pharmacia) was used for the $CaCl_2$ buffer system. Both visual inspection of occlusion-negative plaques and plaque hybridization techniques were used to select recombinant virus. Plaque hybridisation was performed by transferring virus from the agarose overlay to nitrocellulose filters as described. Hybridization was with plasmid segments labelled with [^{32}P]-dNTP (NEN or Amersham) by nick-translation. Viruses were plaque purified at least twice.

Recombinant viral plaques picked through an agarose overlay were resuspended in 1 ml of media to allow diffusion of the virus from the overlay. An initial passage was performed by infecting a confluent T25 flask (monolayer) with 0.5 ml of the viral suspension in a total volume of 5 mls. Further passages for stock virus were done by infecting cells at an m.o.i. of 0.1 pfu/cell. All virus passages were incubated for 5 days to allow confluent infection. For experimental infections an m.o.i. of ≥5 pfu/cell was used.

COS-1 cells were transfected with recombinant pSV1 plasmids using a DEAE-dextran method as specified by a CellPhect transfection kit. Cells were incubated for 3 days following transfection before testing the media for secreted products.

V. Metabolic labelling of infected cells

Recombinant baculovirus-infected cells were removed from growth media at 1-3 days p.i., and starved for 1 hr in Graces medium minus the appropriate substrate (either methionine or phosphate). The cells were labelled with [^{35}S]-methionine (0.1-1.0 mCi/ml) or ^{32}Pi (50 μCi/ml) in Grace's medium. In some cases, after the appropriate period labelling medium was removed, cells were washed once with Grace's medium and incubated in either Grace's medium supplemented with 0.5% fetal bovine serum or in complete TMN-FH for the appropriate chase period.

VI. Analysis of infected cell protein

Infected cells were harvested, washed once with PBS, dissociated by boiling for 5 min in sample buffer (10% SDS, 10% β-mercaptoethanol, 30% glycerol, bromophenol blue, 10 mM Tris-HCl; pH 6.9), and loaded on a discontinuous SDS-polyacrylamide gel (SDS-PAGE; 12-15% resolving gel) system for electrophoresis using a BioRad mini Protean II slab cell system.

Some cell samples were first extracted with NP40 buffer (1% NP40, 150 mM NaCl, 10 mM Tris-HCl; pH 7.5) on ice for 20 min and microfuged to pellet insoluble material. The pellet was

boiled with sample buffer to dissociate protein for an NP40 buffer insoluble fraction sample. The NP40 soluble fraction was diluted 1:1 with sample buffer and the NP40 insoluble fraction was diluted 1:1 with NP40 buffer to create similar buffer conditions. Some samples were extracted similarly with RIPA buffer (1% Triton-X100, 1% Sodium deoxycholate, 0.1% SDS, 50 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl; pH 8.0) supplemented with 10mM $MgCl_2$, and protein was analyzed from both the RIPA buffer soluble and insoluble samples.

Proteins in polyacrylamide gels were stained in 30% methanol/10% acetic acid/0.25% Coomassie Blue and destained in 30% methanol/10% acetic acid. For fluorography, polyacrylamide gels were fixed in 25% isopropanal/10% methanol/65% H20 and soaked in Amplify (Amersham Canada Limited, Oakville, Canada), according to the manufacturer's instructions, vacuum-dried, and exposed to Cronex X-ray film (DuPont) at -70°C.

For immunoblotting, polyacrylamide gels were transferred to nitrocellulose using a BioRad mini Trans-Blot cell according to the manufacturer's instructions. Membranes were stained by an alkaline phosphatase color reaction using a BioRad Immun-Blot assay kit according to the manufacturer's instructions. The blotted membrane was incubated with the appropriate first antibody at a concentration of 1/3,000-1/10,000, and with second antibody at 1/3,000.

For immunoprecipitation, labelled cells were extracted with NP40 buffer as above and the supernatant fraction was diluted

to 1 ml with RIPA buffer. In some cases the insoluble pellet was extracted with sample buffer as above, microfuged to remove insoluble debris, and the supernatant diluted to 1 ml with RIPA buffer minus SDS. Samples were incubated with first antibody diluted 1/100-1/200 at 4°C overnight while rotating. Samples were then microfuged for 15 min to remove aggregated material and the supernatant incubated with protein-A sepharose (5-10 μg/ml) for 1 hr while rotating to recover immunoprecipitated complex. Complex was washed twice in RIPA buffer and once in 0.1% NP40/10mM Tris-HCl (pH 8.0), and boiled in sample buffer to dissociate the labelled protein.

VII. Proteinase K assay

Infected, labelled cells were disrupted by 40 strokes with a ground glass pestle. Proteinase K (1.5 μg/ml; Boehringer Mannheim) was added and the samples were incubated for 3 hrs on ice. Control digestion samples were adjusted to 1% Triton X-100. Reactions were stopped by adding PMSF to 2mM and boiling the samples for 3 min. The samples were diluted to 1 ml with RIPA buffer for immunoprecipitation.

VIII. Carbonate Extraction

Infected, labelled cells were disrupted by 40 strokes with a ground glass pestle. 0.45 ml aliquots were adjusted to 100mM sodium carbonate (pH 11.5) and incubated on ice for 30 min. Samples were centrifuged at 100,000 x g in a SW50 rotor (Beckman) for 30 min. The supernatant was neutralized to an approximation with acetic acid before being adjusted to pH 7.5 with Tris-HCl. A 10x RIPA buffer concentrate was added and the volume adjusted to 1 ml.

The pellet was recovered in RIPA buffer, boiled to dissociate protein, and microfuged to remove insoluble material. The final volume was adjusted to 1 ml with RIPA buffer.

IX. Radioimmunoassay

HBsAg and related antigens were assayed using the Abbott AusRIA II kit according to the manufacturers instructions. HBcAg and related antigens were assayed for using the Abbott HBe (rDNA) kit according to the manufacturers instructions. Media samples were used directly. Cells were first disrupted by sonication for 2 min.

X. Electron Microscopy

Infected cells were fixed 2 days p.i. in 2.5% glutaraldehyde in 0.1M sodium cacodylate (pH 7.4). They were then postfixed in 2.0% osmium tetroxide (in cacodylate buffer). Following dehydration in graded ethanol, samples were embedded in Spurr epoxy resin and sectioned on a Reichert OMUS ultramicrotome with a diamond knife. Sections were stained with methanolic uranyl acetate and Reynold's lead citrate and examined using a JEOL model JEM1010 electron microscope.

Results

I. HBV Surface Antigen Studies

Our initial investigations were concerned with the use of HBsAg as a generic carrier for foreign epitopes in a heterologous expression system. A successful chimeric HBsAg fusion polypeptide should be capable of forming 22 nm particles which can be secreted from the heterologous cell, and are immunogenic for the foreign peptide epitope(s). A preliminary requisite is that HBsAg alone is capable of secreting 22 nm particles from the heterologous expression system used.

Our laboratory has demonstrated that recombinant baculovirus-infected insect cells (SF cells) are capable of secreting HBsAg S and M protein-derived 22 nm particles as assayed by a commercial RIA kit (Abbott AusRIA II) (Kang *et al.*, 1987; Scully and Kang, 1988). Another study has reported the absence of HBsAg from the medium of HBsAg-expressing SF cells as assayed by Western Blot (Lanford *et al.*, 1989). The authors suggested that the use of spinner culture for infected insect cells caused lytic release of 22 nm particle forms in our study. I therefore decided to investigate the capacity of SF cells infected in monolayer to secrete HBsAg.

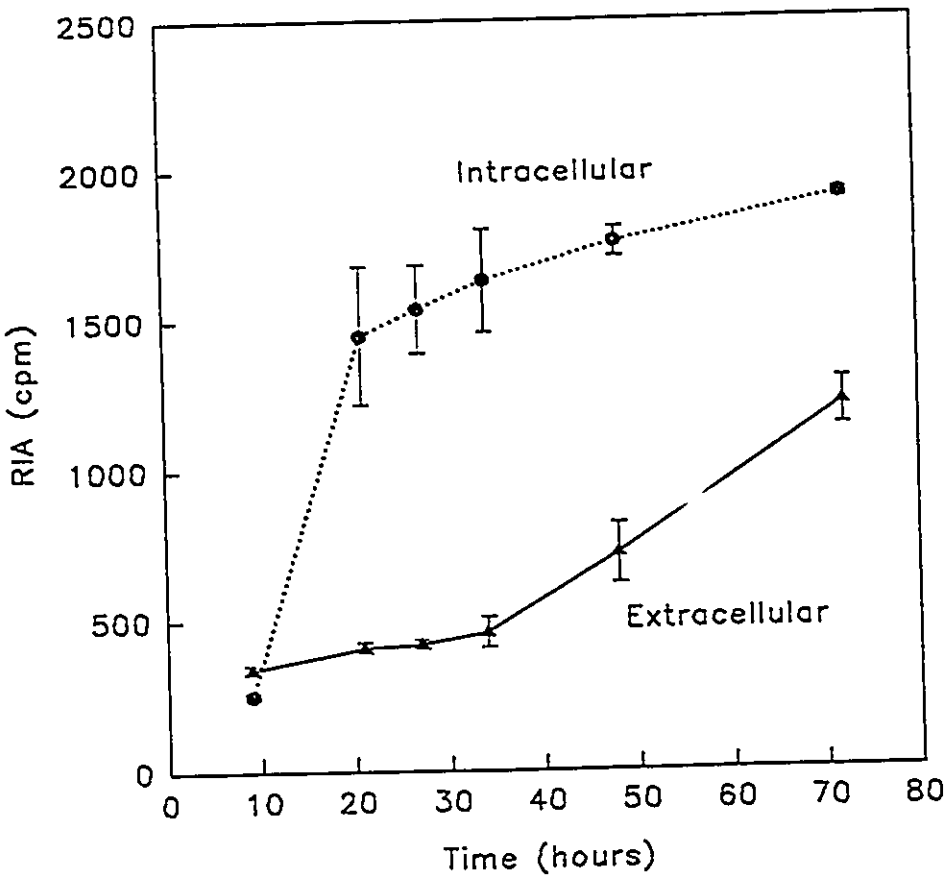
The recombinant baculovirus AcNPV-HBsYK14A7 (14A7) was used

to express the S protein utilizing the transfer vector pAcYM1. Cells were infected in monolayer with 14A7, and replicate samples were taken from both the media and cells after various times for testing by RIA (Fig. 5). Cells were first disrupted by sonication. Equal fractions of both samples were tested for HBsAg in parallel. HBsAg was detected in the medium at early times p.i. (between 20-40 hrs). This does not differ significantly from results obtained previously for cells maintained in spinner culture (Kang *et al.*, 1987). At the same time, a steady rate of accumulation was seen intracellularly. The ratio of extracellular to intracellular (E/I) counts was low, indicating an inefficient secretion process. HBsAg accumulation in the medium and the E/I ratio increased significantly after 40 hrs p.i. Cellular lysis due to the viral infection is likely contributing at this time, but can not account for the entirety of extracellular HBsAg. Cells were >90% viable by Trypan Blue exclusion at the times shown here. A semi-quantitative analysis by RIA estimated the yield in the supernatant at ~120 ng/ml/10^6 cells at 3 days p.i., similar to that already estimated for reciprocating cells.

I attempted to estimate the rate of release8 of HBsAg using a pulse-chase analysis of [^{35}S]-methionine-labelled polypeptides (data not shown). Little or no accumulation of HBsAg in the medium was seen, even after long chase times. No reduction in the amount of intracellular HBsAg was seen. Significant levels did accumulate in the medium only after

Figure 5. Determination of intracellular and extracellular HBsAg by RIA. SF cell monolayers were infected in parallel with virus 14A7 and then harvested in triplicate at various times p.i. Supernatant medium was recovered from pelleted cells, which were then lysed by sonication. Equal fractions of both samples were analyzed using the AusRIA II RIA kit.

HBsAg Secretion (RIA)



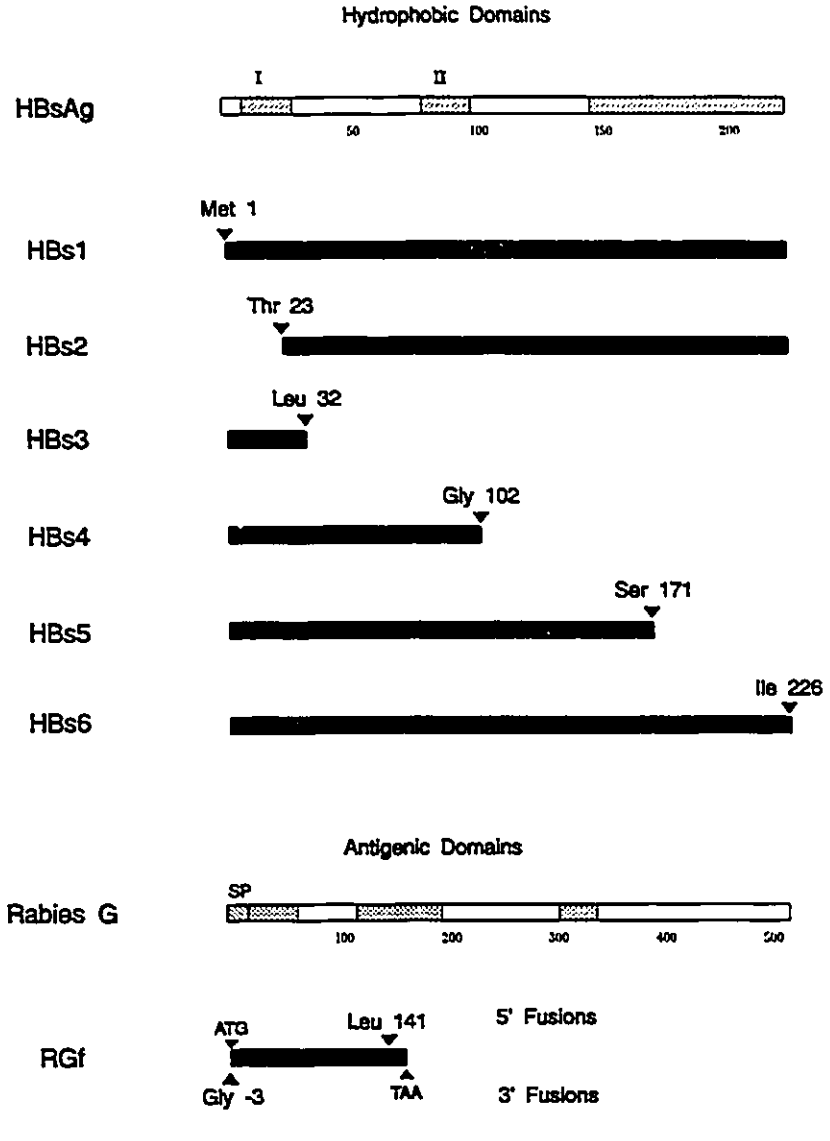
long periods of continuous labelling. Because temperature can affect the accuracy of protein folding, and subsequently their efficiency of secretion (see discussion), labelling periods plus or minus chase periods at 37°C were attempted (the normal temperature for HBsAg biosynthesis), but this resulted in no enhancement of extracellular HBsAg levels. This is not surprising considering the low viability of insect cells at 37°C.

Despite an apparently inefficient secretion process, the RIA assay used allowed for easy detection of extracellular HBsAg produced from monolayer cells. HBsAg contains one highly conformational epitope (the a determinant), and the RIA assay is specific for assembled, particulate HBsAg (Vyas *et al.*, 1972; Hollinger *et al.*, 1978). The structural competency of various HBsAg derivatives for 22 nm particle formation and secretion could therefore be assessed in further studies.

I next attempted to utilize HBsAg as a carrier for a foreign peptide. For our fusion partner the rabies virus glycoprotein (RG) was used. This protein has been well characterized (Cox *et al.*, 1977; Dietzschold *et al.*, 1982; Dietzschold *et al.*, 1983; Macfarlan *et al.*, 1984), and is of sufficient interest for vaccine study.

A fusion utilizing a convenient restriction fragment from the N-terminus of the RG gene (encoding 164 amino acids), which could be fused in-frame to the unique *Hinc*II of HBsAg (Fig. 6; see Materials and Methods for all constructions) was

Figure 6. Schematic representation of HBsAg/Rabies G recombinant fusions. The HBsAg S gene is represented on the top line. Predicted transmembrane hydrophobic domains are indicated by hatched regions. The first two are labelled Signal I and Signal II (important to membrane insertion) according to the nomenclature of *Eble et al.*, 1987. Segments fused to a rabies G fragment are indicated by the black boxes (HBs1-HBs6) on the lines below. The codons at the point of fusion are indicated. The rabies G gene is represented on the penultimate line. Major antigenic domains are indicated by stippled regions. The N-terminal signal peptide is indicated by the hatched region. The fragment used for fusion is represented on the last line (RGf). Above the fragment, the normal start codon and the site of fusion are indicated for N-terminal fusion to HBs1 and HBs2. Below the line the site of fusion and a termination codon placed using a synthetic oligonucleotide are indicated for C-terminal fusion to HBs3-HBs6. The designation Gly -3 reflects the addition of amino acids by the in-frame ligation of restriction sites. Amino acid numbering is displayed below both genes.


Hydrophobic Domains
HBsAg
I
II
50
100
150
200
Met 1
HBs1
Thr 23
HBs2
Leu 32
HBs3
Gly 102
HBs4
Ser 171
HBs5
Ile 226
HBs6
Antigenic Domains
Rabies G
SP
100
200
300
400
500
RGf
ATG
Leu 141
5' Fusions
Gly -3
TAA
3' Fusions

designed. The RG segment included the secretory signal peptide, and contained a major antigenic domain(s) which would allow detection by polyclonal antisera (Dietzschold *et al.*, 1982). This fusion (designated HBsR2) had a deletion of the first 29 aa of HBsAg, which encompasses the first hydrophobic domain. This region is not cleaved from the mature polypeptide and therefore does not act as a signal peptide. It was hoped that it would be dispensable to 22 nm particle formation.

After isolation, the corresponding recombinant baculovirus (AcHBsR2) was used to infect insect cells in monolayer. Samples of the cells and the medium were taken after 3 days of infection for RIA. The medium sample was negative despite significant intracellular counts (data not shown). Therefore some assembly of HBsAg was occurring intracellularly, but secretion of this structure was blocked.

Two likely explanations exist for this result: either the N-terminal domain of HBsAg deleted from the fusion was essential for 22 nm particle secretion, or the RG peptide was interfering with secretion. It had recently been demonstrated that both the first and second hydrophobic domains were essential *in vitro* to insertion in microsomal membranes, a necessary prerequisite to particle formation *in vivo* at the E.R. membrane (Eble *et al.*, 1987). The first possibility was therefore tested. A second fusion (designated HBsR1) was constructed which fused the same RG fragment to the N-terminal methionine of HBsAg (Fig. 6). The corresponding virus,

AcHBsR1, was tested as previously described, and was also negative for secretion. These results suggested that the RG peptide was inhibitory to particle secretion.

A number of successful fusions at or near the N-terminus had by this time been published (Valenzuela *et al.*, 1985a; Delpeyroux *et al.*, 1986; Michel *et al.*, 1988); the main difference from our constructs was the short length of the foreign peptides used. I wished to continue the approach of utilizing longer fusion peptides because of the increased chance of eliciting neutralising antisera against the corresponding virus. Therefore, the feasibility of various C-terminal fusions was investigated. Preliminary information suggested that a portion of the C-terminus was dispensable to particle formation (Bruss *et al.*, 1989). The resultant deletion mutants were not secreted, but could be cosecreted with wild-type S protein.

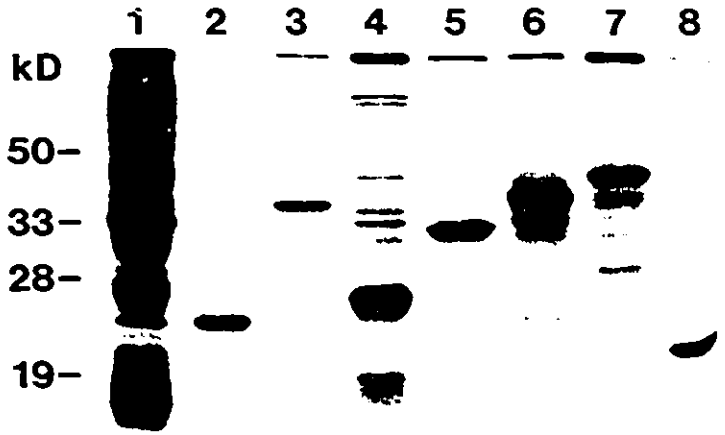
Recombinant viruses AcHBsR3-AcHBsR6 (Fig. 6) were isolated and tested. These constructions contained a slightly shorter N-terminal peptide of RG (still including the signal peptide). HBsR3 included the first hydrophobic domain, plus most of the first hydrophilic domain of HBsAg, while HBsR4 also included all of the second hydrophobic domain. These two polypeptides lacked the major HBsAg determinants (as well as the normal site of glycosylation) present in the second hydrophilic domain. HBsR5 contained in addition the second hydrophilic domain plus part of the hydrophobic C-terminus. HBsR6 has the

RG peptide fused to the C-terminus. A control virus encoding only the fusion peptide from RG (AcRGf) was also constructed.

These new viruses were tested by RIA for secretion, and again all were negative (data not shown). The results for intracellular material was somewhat variable, but generally the products produced by recombinant viruses AcHBsR1, -R2, -R5 and -R6 were clearly reactive. This again suggests some structuring of the fusion polypeptides into 22 nm particle-like forms. Coinfection with 14A7 did not result in elevation of extracellular counts for any fusion construct.

These fusion polypeptides were further characterized in an attempt to determine a biochemical basis for the inhibition of secretion by the RG peptide. Recombinant virus-infected cells were analyzed by SDS-PAGE. All fusion polypeptides, as well as HBsAg and RGf, could be distinguished by SDS-PAGE when stained by Coomassie Blue (data not shown). The level of expression could be characterized as low to medial; generally, HBsR3 and HBsR4 were most easily visible. Infected cells were labelled with [^{35}S]-methionine and total protein analyzed by SDS-PAGE (Fig. 7). HBsR1 was not available at the time of this analysis. Prominent protein species were present in all samples which had mobilities consistent with the sizes expected. It is not clear whether the rabies G signal peptide is cleaved from RGf, HBsR1 or HBsR2. HBsAg was present as both non-glycosylated (p24) and glycosylated (gp27) forms, as expected (lane 1). Higher molecular weight glycosylated

Figure 7. Analysis of total infected cell protein for HBsAg/RGf fusions. Plated cells were infected with recombinant baculovirus and then labelled with [^{35}S]-methionine (~250 μCi/ml) for 30 min at day 2 p.i. Cells were infected with wild type AcNPV (lane 1), 14A7 (lane 2), AcHBsR2 (lane 3), AcHBsR3 (lane 4), AcHBsR4 (lane 5), AcHBsR5 (lane 6), AcHBsR6 (lane 7), AcRGf (lane 8). The positions of molecular weight standards in kilodaltons are indicated (and in figures below). Infected cells were harvested, dissociated in lysis buffer, and separated by SDS-PAGE before fluorography.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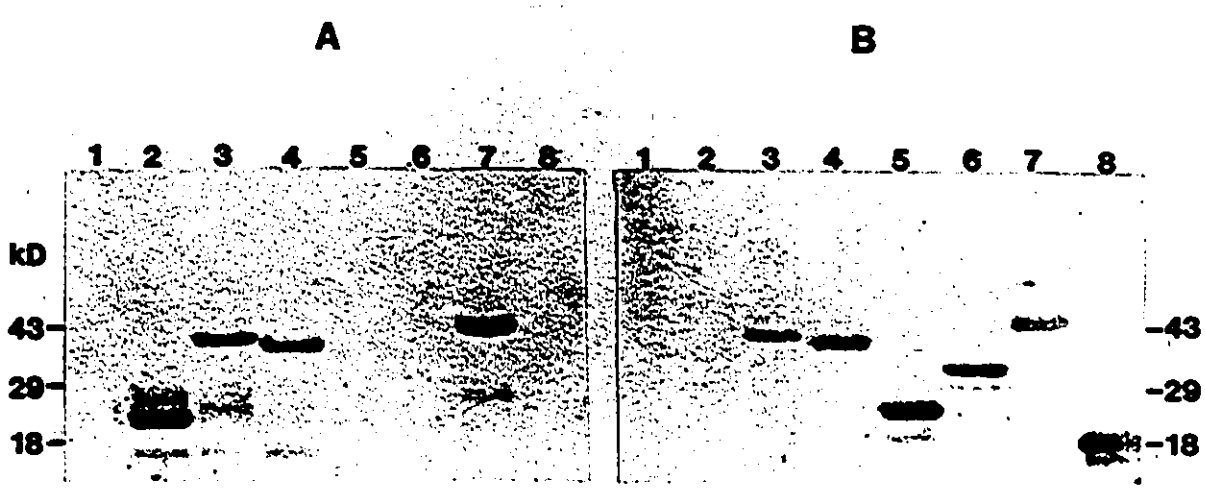
7
8
kD
50–
33–
28–
19–

species may be present for HBsR5 and HBsR6 as minor components, but not for HBsR1 and HBsR2 (data not shown for HBsR1). HBsR3 and HBsR4 do not have the S protein glycosylation site. The RG peptide contains a glycosylation site normally utilized during RG synthesis. Therefore, RGf or any of the fusions may also be invariably glycosylated at a site distinct from that of HBsAg. No further attempt to clarify this was made.

Fusion polypeptides were immunologically characterized by Western Blot analysis using both polyclonal anti-HBsAg sera (Fig. 8a) and anti-RG sera (Fig. 8b). NB. AcHBsR5 was not available for this analysis. Anti-HBsAg serum detected the products of viruses 14A7, AcHBsR1, R2, and R6, as expected. Anti-RG serum detected the products of viruses AcR1-R4, R6, and RGf, consistent with the presence of the RGf peptide. This confirms that the anti-RG serum can recognize epitopes present on the (much shorter) RGf peptide, even when fused to HBsAg. Again, glycosylated species were not clearly discernible for fusion polypeptides (see below). In other experiments, the HBsR1, HBsR2, HBsR5, and HBsR6 polypeptides were very unstable compared to wild-type HBsAg (data not shown). It was found empirically that heating harvested cells in medium prior to washing in PBS circumvented this problem (Fig. 8 and below). This suggests that these polypeptides are susceptible to proteolytic breakdown by a (heat-sensitive) protease(s).

Another secretory protein expressed in insect cells, tissue

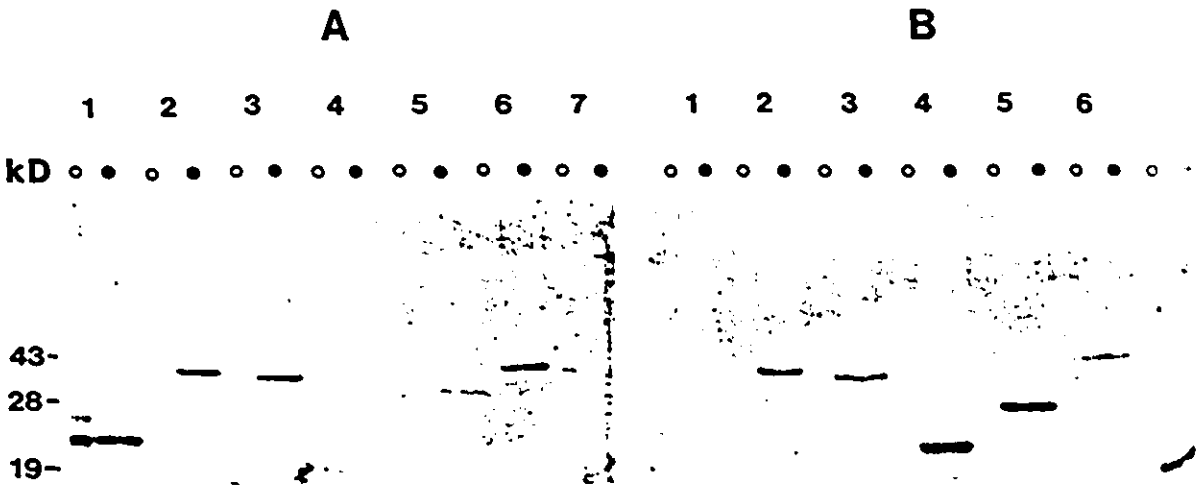
Figure 8. Western Blot analysis of infected cells for HBsAg/RGf fusions. Infected cells were harvested 3 days p.i. Proteins were separated by SDS-PAGE, and transferred to nitrocellulose by electroblotting. Cells were infected with wild type AcNPV (lane 1), 14A7 (lane 2), AcHBsR1 (lane 3), AcHBsR2 (lane 4), AcHBsR3 (lane 5), AcHBsR4 (lane 6), AcHBsR6 (lane 7), AcRGf (lane 8). After transfer, membranes were incubated with either (A) goat anti-HBsAg sera or (B) sheep anti-rabies G sera before being incubated with the appropriate second antibody.


A
B
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
kD
43
29
18
43
29
18

plasminogen activator (TPA), was found in the soluble fraction of cell lysates prepared using 1% NP40-containing buffers. When secretion was blocked by inhibition of glycosylation after treatment of the cells with tunicamycin, much of the protein was found in the NP40 insoluble fraction (Jarvis and Summers, 1989). Also, analysis of HBsAg S protein expressed in insect cells after preparation of lysates by sonication showed that p24 and gp27 were evenly distributed between supernatant and pellet fractions after centrifugation (Lanford *et al.*, 1989). The L protein, which is completely inhibited from secretion by the preS1 region, was found in the pellet. A correlation between intracellular solubility and secretion potential had therefore been established. Therefore, the solubility of our fusion polypeptides was examined for a similar correlation. It was also important to resolve the degree to which our fusion polypeptides were glycosylated, a measure of their competence for E.R. membrane insertion.

Infected cells were solubilized using 1% NP40-containing buffer, and the soluble fraction was recovered after centrifugation. The pellet was treated with SDS-containing sample buffer to recover insoluble protein. Equal fractions of both samples were analyzed by Western Blot using anti-HBsAg (Fig. 9a) or anti-rabies G serum (Fig. 9b). Only HBsAg shows partial solubility in 1% NP40 (Fig. 9a, lane 1). All fusion polypeptides and the RGf peptide were found in the insoluble compartment.

Figure 9. 1% NP40 solubility of HBsAg/Rgf fusions by Western Blot analysis. Cells were infected with recombinant baculovirus 14A7 (lane 1), AcHBsR1 (lane 2), AcHBsR2 (lane 3), AcHBsR3 (lane 4), AcHBsR4 (lane 5), AcHBsR5 (lane 6), AcHBsR6 (lane 7), and RGf (lane 8). Cells were harvested at 3 days p.i., solubilized in 1% NP40 buffer, and centrifuged to pellet insoluble material. Equal aliquots from both fractions were separated by SDS-PAGE and blotted to nitrocellulose. Filters were incubated with (A) goat anti-HBsAg sera, or (B) sheep anti-rabies G sera. For each infected sample, the open symbols correspond to the soluble fraction, closed symbols to the insoluble fraction.


A
B
1 2 3 4 5 6 7
1 2 3 4 5 6
kD
43-
28-
19-

In Fig. 10, a similar experiment is shown in which cells were solubilized in RIPA buffer (which contains both ionic and nonionic detergents), and analyzed by Western Blot using anti-rabies G sera. Using anti-HBsAg sera, HBsAg had a distribution identical to that seen using NP40 buffer (data not shown). In addition to HBsAg, only RGf shows partial solubility in RIPA buffer (Fig. 10, lane 8). These results suggest that the addition of the RGf segment promotes an irreversible association or aggregation of fusion polypeptide. This experiment had better resolution for the various fusion proteins, and glycosylated species could clearly be seen for HBsR5 and HBsR6, but not for HBsR1 and HBsR2. The ratio of glycosylated to nonglycosylated species appears to be similar to that for HBsAg. This result demonstrates that HBsR5 and HBsR6 are still capable of membrane insertion, and therefore any aggregation likely occurs subsequent to this. This is consistent with the suggestion that there is some assembly of fusion polypeptide similar to that of HBsAg, based on RIA reactivity. This may not be the case for HBsR1 and HBsR2.

Considered in isolation, several features of a peptide could conceivably be primary to inhibition of normal particle secretion, including size, overall hydrophobicity, or a conformation which is retained by elements of the secretory mechanism as aberrant (Lodish, 1988; Rose and Doms, 1988; Hurtley and Helenius, 1989). I hypothesized that the latter two possibilities were at least partially responsible for the

Figure 10. RIPA buffer solubility of HBsAg/Rgf fusions by Western Blot analysis. Cells were infected with recombinant baculovirus 14A7 (lane 1), AcHBsR1 (lane 2), AcHBsR2 (lane 3), HBsR3 (lane 4), AcHBsR4 (lane 5), AcHBsR5 (lane 6), AcHBsR6 (lane 7), AcRGf (lane 8). Cells were harvested at day 3 p.i., solubilized with RIPA buffer, and centrifuged to pellet insoluble material. Equal aliquots from both fractions were separated by SDS-PAGE and blotted to nitrocellulose. Filters were incubated with sheep anti-rabies G sera. Open and closed symbols correspond to soluble and insoluble fractions, respectively.


1
2
3
4
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6
7
8
kD
43–
28–
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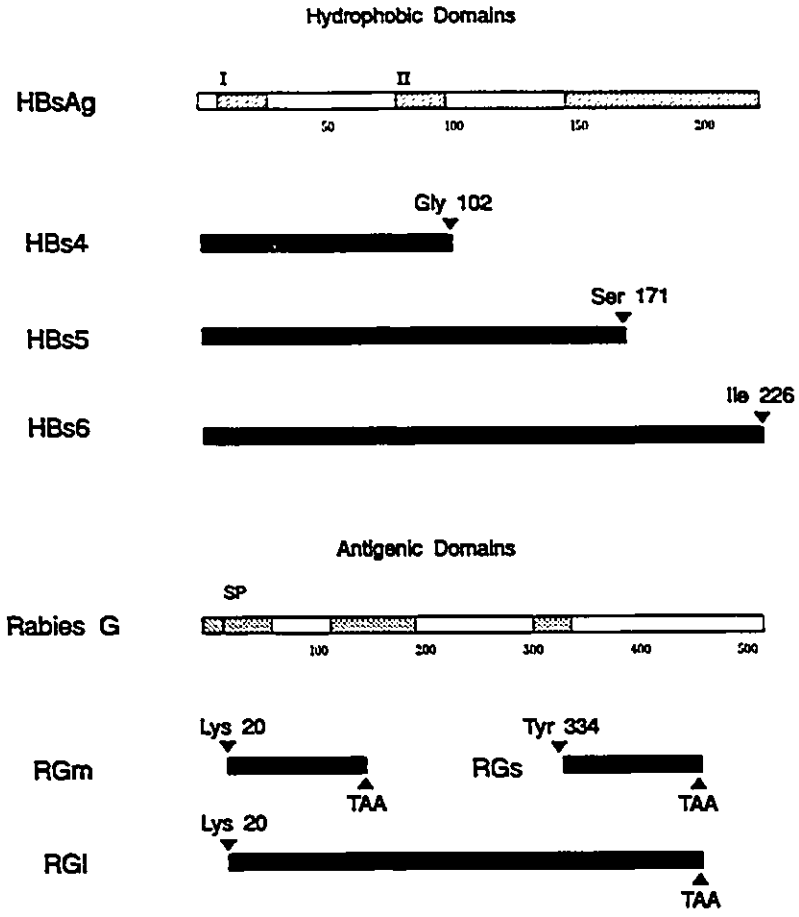
results observed, and designed three novel RG protein segments for fusion with this in mind (Fig. 11). The first, RGm (medium size) was similar to RGf, except that it was shortened at both the N- and C-termini, eliminating the signal peptide and greatly reducing its hydrophobicity. The second, RGs (small) was derived from the C-terminal region of rabies G, just N-terminal to the transmembrane anchor. This peptide was also more hydrophilic, and included an antigenic region to allow immunological detection (Dietzschold *et al.*, 1982). This peptide would likely adopt a conformation dissimilar to the N-terminal peptides. The last peptide, RGl (large), included the entire ectodomain of rabies G. This peptide should contain all of the structural information present during folding of the corresponding domain in the native protein (providing it can fold without becoming involved in aberrant contacts with HBsAg). HBsAg segments HBs4, HBs5, and HBs6 were selected for fusion with these novel peptides. The C-terminus still appeared to be the best candidate for peptide addition, as it was the only region demonstrated to be dispensable to particle formation.

One further possible reason for the failure of chimeric particle secretion was the use of baculovirus-infected insect cells. It had been demonstrated that the efficiency of secretion for TPA decreases greatly after two days of infection (Jarvis and Summers, 1989). Furthermore, mammalian cell systems are much more efficient for HBsAg secretion than

Figure 11. Schematic representation of further HBsAg/Rabies G fusions. The S gene, rabies G gene, and segments HBs4-HBs6 are as indicated in Fig. 6. The segments RGm, RGs, and RGl are indicated below the Rabies G gene. The points of C-terminal fusion to HBs4-HBs6 are indicated above each fragment, and the positions of a termination codon placed using a synthetic oligonucleotide are indicated below the fragments.


Hydrophobic Domains
HBsAg
I
II
50
100
150
200
Gly 102
HBs4
Ser 171
HBs5
Ile 226
HBs6
Antigenic Domains
SP
Rabies G
100
200
300
400
500
Lys 20
Tyr 334
RGm
TAA
RGs
TAA
Lys 20
RGI
TAA

insect cells or the amphibian *Xenopus laevis* oocytes (Simon *et al.*, 1988a; Simon *et al.*, 1988b; Lanford *et al.*, 1989). It might be that the secretion of HBsAg from infected insect cells is already constrained, and can tolerate no further alterations. This possibility was tested by expressing the novel chimeric polypeptides in mammalian COS-1 cells.

Recombinant fusion inserts HBsR4RGm-R6RGm, HBsR6RGs, and HBsR6RGl (Fig. 11) were constructed and inserted into the SV40 late expression vector pSV1. The same *BamHI* cassettes were inserted into pAcYM1 for isolation of the corresponding recombinant baculoviruses. Recombinant pSV1 plasmids were isolated and used for transient expression in COS-1 cells by standard transfection procedure (after optimization of the procedure as monitored by the secretion of RIA-reactive material from pSVHBsAg). Again, all plasmids expressing fusion polypeptides were negative for secretion despite positive results for HBsAg alone (data not shown).

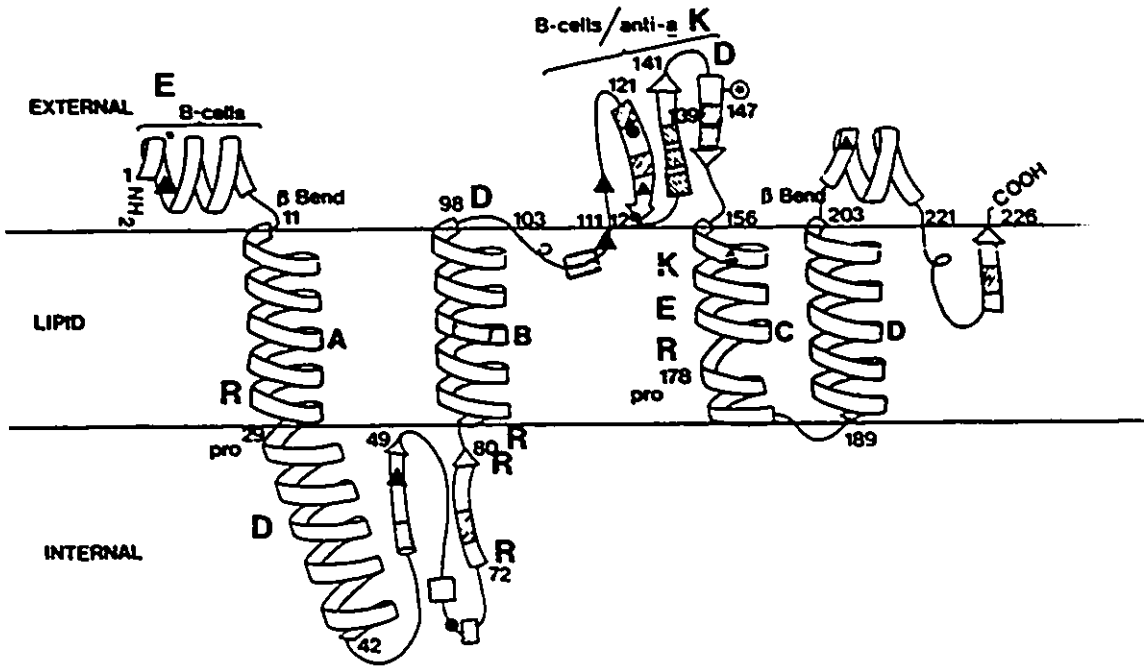
These results suggested that it was unlikely that the inhibition of secretion could be avoided by the use of alternate rabies G peptides. They also demonstrated that expression in virus-infected insect cells was not responsible for the inhibition observed. It still remained possible that fusion of some of these peptides to another point within HBsAg would not inhibit particle secretion. However, the most likely conclusion was that peptides of this size simply can not be incorporated into chimeric 22 nm particles.

At this point in time, this investigation of HBsAg as a carrier of foreign peptides was abandoned. These first studies clearly pointed out that too little was known about the process of 22 nm particle formation and secretion to allow a consistent manipulation of the protein as a carrier. I decided instead to investigate this process more directly, focusing on specific aspects of the protein structure-function relationship.

One aspect of HBsAg directly related to its ability to form 22 nm particles is its retention at the E.R. membrane. Sequence elements which specify entry, orientation and retention within the E.R. membrane must therefore be central to this process. More specifically, this refers to hydrophobic membrane spanning domains. Two domains have been implicated in this process (Eble *et al.*, 1987; Bruss and Ganem, 1991b). The first spans approximately residues 8-28 of the HBsAg S protein sequence, while the second spans residues 80-100 (Fig. 12). The first domain (referred to as domain I), which emerges first from the ribosome during translation, may specify the initial entry and topology of the polypeptide in the membrane. Domain I is not cleaved from mature HBsAg (Peterson, 1981). I therefore decided to investigate features of this domain which might be important to these functions.

During the course of these studies, the "positive-inside" rule was proposed, which states that the distribution of charged residues about a transmembrane (TM) domain specifies

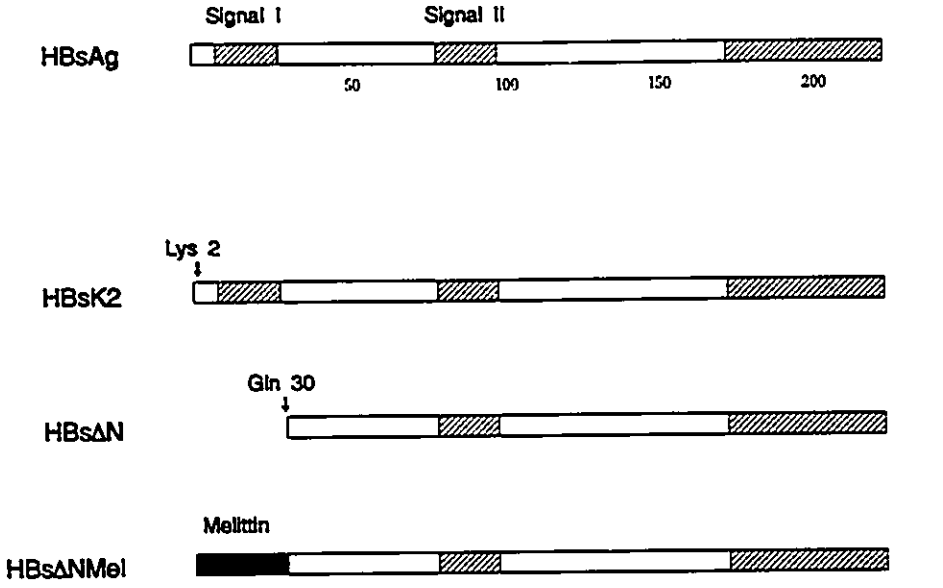
Figure 12. Schematic representation of HBsAg secondary structure. Predicted α-helical coils which span the bilayer are labelled A, B, C and D. β-sheet structures are indicated by arrows. The amino and carboxyl termini are identified, and landmark amino acid numbering is given. Cysteine residues are shown as shaded regions. The positions of B-cell determinants for man (including the major a determinant) are shown. There is a single site for N-linked glycosylation (Asn 146). Variable residues associated with w/r (triangles) or d/y (circles) subtypes are shown. Reprinted from Howard *et al.* (1988). Charged residues are superimposed on the original in the one-letter code at their approximate positions.


B-cells/anti-a K
D
141
121
139
147
EXTERNAL
E
B-cells
1
NH2
β Bend
11
98 D
103
111
125
156
β Bend
203
221
COOH
226
LIPID
A
B
K
E
R
C
D
R
178
pro
pro
25
49
80
R
R
D
R
72
INTERNAL
42
189

it membrane topology (von Heijne, 1986; von Heijne and Gavel, 1988). This rule specifies that cytoplasmic domains which flank TM domains are enriched in positively charged amino acids. These regions appear to be specifically retained within the cytoplasm by unknown mechanisms. The topology of both cleaved signal peptides and internal signal/anchor domains is specified in this manner. This rule has been shown to apply to numerous membrane-spanning proteins, both prokaryotic and eukaryotic, including viral glycoproteins (Parks and Lamb, 1991). I speculated that this rule might also apply to specify HBsAg topology, although it is unlike typical viral glycoproteins. In addition, there does not appear to be a strong topological signal about domain I for HBsAg (see below). I planned to test whether the positive inside rule still applied in this more unusual situation.

We designed a series of mutants to test the function and importance of domain I of HBsAg (Fig. 13). All mutants from this point on were constructed using PCR-directed mutagenesis (see Materials and Methods). Current models propose a C-terminal cytoplasmic orientation for domain I (Howard *et al.*, 1988). The first charged amino acid of HBsAg is a glutamic acid residue at position 2, preceding domain I (Fig. 12). One positively charged amino acid (Arg 24) occurs near the C-terminal side of domain I (Fig. 12). These residues together do not constitute a strong topological signal, but are not inconsistent with the positive-inside rule if the proposed

Figure 13. Schematic representation of HBsAg mutants. The HBsAg S gene is as represented in Fig. 3. The point mutation changing Glu 2 to Lys 2 in HBsK2, the deletion up to codon Gln 30 in HBsΔN, and the deletion accompanied by fusion to the mellitin signal peptide in HBsΔNMel, are all indicated at the appropriate position. On the lines below, the amino acid sequence at the N-terminus of HBsAg about the first predicted transmembrane domain is compared with the sequence of the mellitin signal peptide.



HBsAg: M E N I T S G {F L G P L L V L Q A G F F L L T R I L T I} P Q S L D

Melittin: M K F L V N V A L V F M V V Y I S Y I Y A

model in Fig. 12 is correct (Howard *et al.*, 1988). A point mutation changing Glu 2 to a positively-charged lysine (mutant HBsK2) was constructed to test the importance of charged residue distribution about domain I. Other studies have concluded that domain I was essential to, or facilitated the formation of 22 nm particles (Streeck *et al.*, 1991; Araki *et al.*, 1990, Bruss and Ganem, 1991b). This domain (the first 30 amino acids) was deleted from mutant HBsΔN to test this. Finally, I wished to observe the effect of replacing this domain with a heterologous TM domain. The signal peptide from honeybee prepromellitin, an insect neurotoxin, has proven effective in promoting efficient secretion from insect cells by replacing the native signal peptides of an inefficiently secreted protein (Tessier *et al.*, 1991; Li *et al.*, 1993). The mellitin signal peptide can be conveniently fused to a recombinant protein using the baculovirus transfer vector pVT-Bac. Unlike HBsAg, the mellitin signal peptide has a positively-charged amino acid N-terminal to the TM domain (Fig. 13), consistent with the N-terminal cytoplasmic orientation specified by signal peptides prior to their cleavage (Boyd and Beckwith, 1990). This is the opposite topology to that proposed for domain I (Fig. 12). I tested whether addition of this domain to the HBsΔN mutant (creating HBsΔNMel) would result in any further observable behaviour over the deletion alone.

The corresponding recombinant baculoviruses were

constructed and used to infect insect cells in monolayer. Cells were labelled with [^{35}S]-methionine 2 days p.i. and total infected cell protein was analyzed by SDS-PAGE (Fig. 14). All three mutants exhibited prominent protein species which had mobilities consistent with that predicted. The deletion from HBsΔN was evident from its increased mobility over HBsAg. The mobility of HBsΔNMel clearly demonstrates that the mellitin signal peptide was not cleaved. In contrast to the appearance of the rabies G fusion polypeptides (Fig. 7), all three mutants in addition to HBsAg clearly displayed higher molecular weight glycosylated species. The fraction of the total does not appear to differ greatly from that of HBsAg. When infected cell lysates were immunoprecipitated with anti-HBsAg, HBsΔNMel occasionally displayed a different profile. In this case, no glycosylated species, and very little full-length non-glycosylated species, could be seen. This was accompanied by the appearance of a prominent species with a lower molecular weight, suggesting that this polypeptide was susceptible to proteolytic degradation. It was found that heat treatment at the end of the labelling protocol was not effective in preventing this.

Medium from infected cell monolayers were analyzed by RIA at three days p.i. for the presence of extracellular HBsAg (Fig. 15). The level of extracellular HBsK2 was greatly reduced compared to HBsAg. Removal of domain I in mutant HBsΔN resulted in a greater reduction, although reactivity was still

Figure 14. Analysis of total infected cell proteins for HBsAg mutants. Cells were infected with recombinant baculovirus (see headings above lanes; virus 14A7 for HBsAg), labelled with [^{35}S]-methionine (250 μCi/ml), and analyzed by SDS-PAGE as in Fig. 7. The Ac prefix for recombinant baculovirus designations has been omitted from the lane headings here and in succeeding figures for convenience. PH shows the position of the polyhedrin protein in wild type-infected cells. Filled arrowheads show the position of HBsAg p24 and gp27 species (and of related species for HBsK2 and HBsΔNM). Open arrowheads show the positions of truncated protein and glycoprotein for HBsΔN.

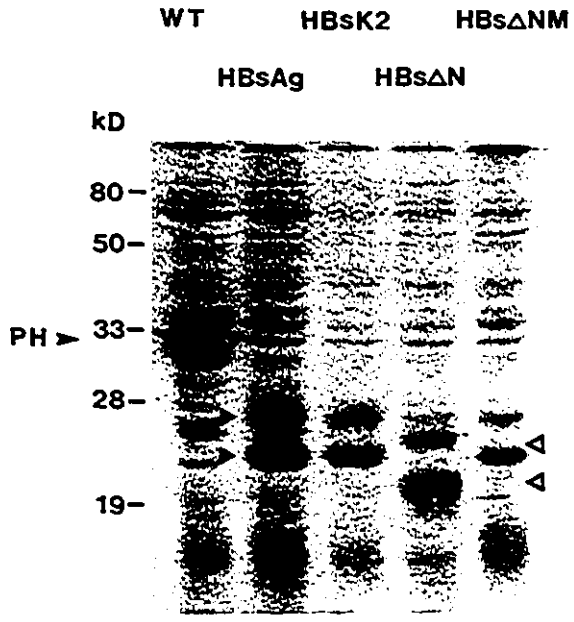
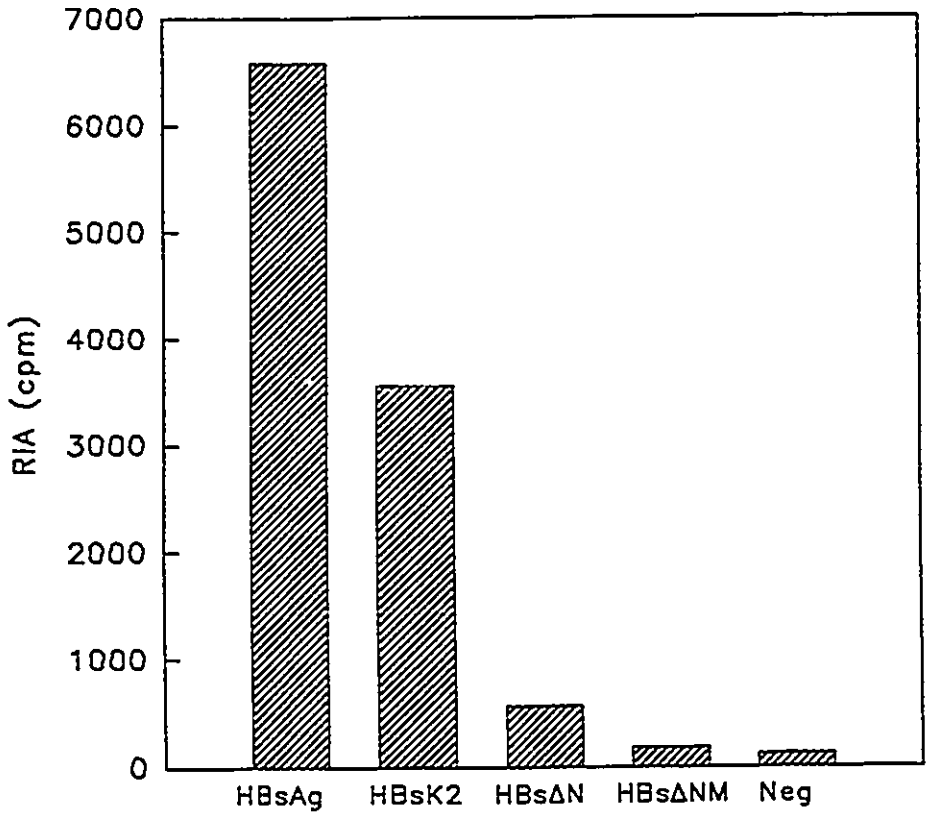

WT
HBsK2
HBsΔNM
HBsAg
HBsΔN
kD
80
50
PH
33
28
19

Figure 15. Determination of extracellular HBsAg and HBsAg mutants by RIA. Cells were infected with recombinant baculovirus. Media was harvested at day 2 p.i. and equal fractions were analyzed using the AusRIA II RIA kit. These results were reproducible over three independent determinations. Neg refers to the kit negative control.

Radioimmunoassay: HBsAg Mutants

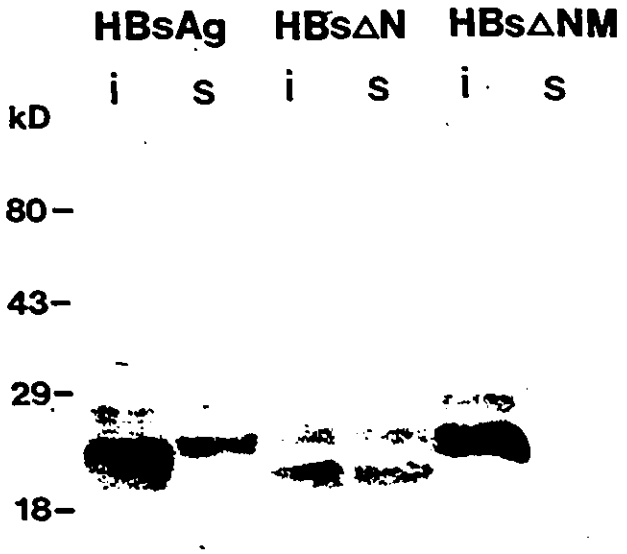


observable. Somewhat surprisingly, fusion of the mellitin signal peptide to HBsΔN resulted in an even further reduction, to background level.

These results suggest that the charge at residue 2 is important for 22 nm particle formation. Alternatively, it is possible that lysine at this position has some other deleterious effect not related to charge. Domain I clearly facilitates particle formation. The results for HBsΔNMel demonstrate that addition of the mellitin signal peptide is more deleterious to particle formation than the effect of the HBsK2 mutation, despite the similarity of their N-terminal positive charge. This is likely due to some primary or secondary sequence characteristics of the mellitin peptide. Note, however, that HBsΔNMel does not have a charged residue corresponding to Arg 24 (present in HBsK2).

I attempted to identify at which point these alterations were exerting their effects. Infected cells were extracted with 1% NP40 buffer to investigate the solubility of the recombinant proteins. Soluble and insoluble fractions were resolved by SDS-PAGE and analyzed by Western Blotting using anti-HBsAg sera (Fig. 16). HBsAg, and mutants HBsK2 and HBsΔN displayed similar solubility (~30-50% found in the soluble fraction; not shown for HBsΔN). In contrast to the RGf fusions, this demonstrates that secretion can be greatly inhibited without an accompanying redistribution. HBsΔMel was found entirely in the insoluble fraction, duplicating the

Figure 16. 1% NP40 solubility of HBsAg mutants by Western Blot. Infected cells (see lane headings for the appropriate virus) were solubilized at day 2 p.i. in 1% NP40 buffer. Fractions were separated and analyzed as in Fig. 9 using goat anti-HBsAg sera. Insoluble and soluble fractions are designated by i and s, respectively, over the appropriate lanes.


HBsAg
HBsΔN
HBsΔNM
i
s
i
s
i
s
kD
80–
43–
29–
18–

behaviour of the RGf fusions (Figs. 9 and 10). Therefore, two stages or modes of inhibition of secretion are possible: a decrease in the levels of secretion as seen for HBsK2 and HBsΔN, and a further decrease caused or accompanied by the retention of fusion polypeptide in the insoluble fraction. Again, the presence of a normal ratio for the glycosylated species supports an inhibition that occurs subsequent to membrane insertion. This strengthens the conclusion that the presence of a heterologous peptide with an incompatible structure leads to some form of irreversible aggregation. HBsΔNMel was not found to be sensitive to degradation during harvesting and washing prior to Western Blot analysis, only during metabolic labelling. This situation is the opposite to that seen for the RGf fusions, and suggests a fundamental difference in the respective proteolytic processes.

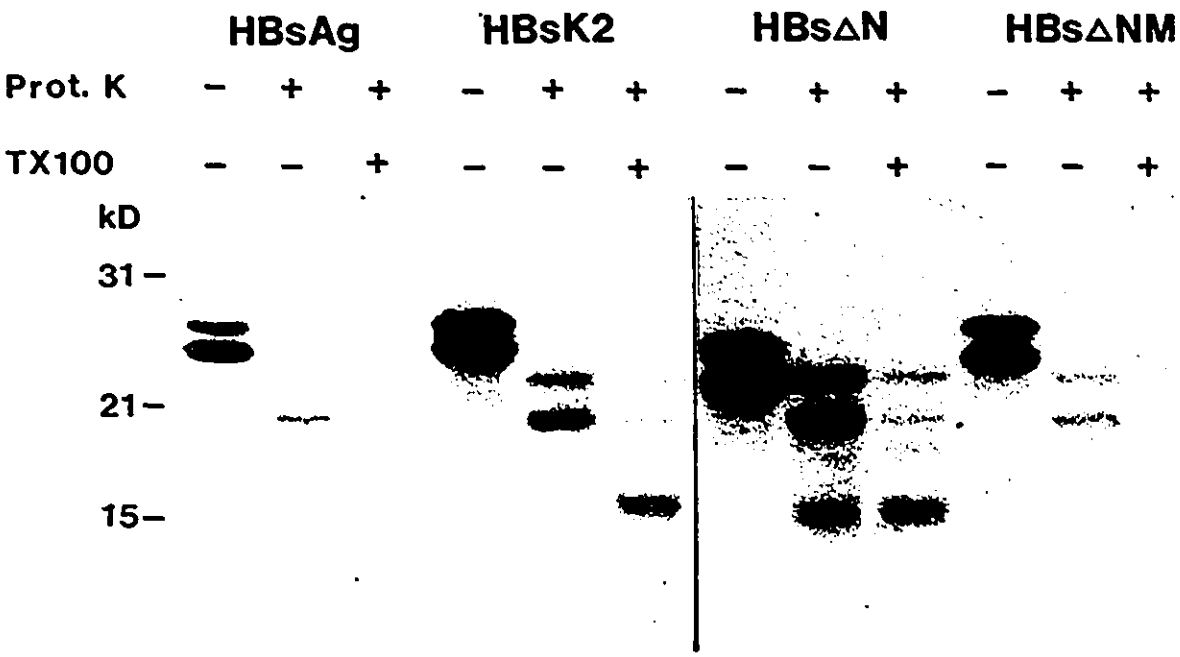
The topology of these polypeptides within membranes using a proteinase K sensitivity assay was investigated. If the importance of charge at residue 2 is in the determination of TM topology for domain I, mutants HBsK2 and HBsΔNMel may differ in this from HBsAg. When used to treat microsomal material, proteinase K in the appropriate concentration will digest accessible protein not protected by microsomal membranes. Any change in the tracts exterior to the microsomal membrane would be reflected by a different pattern of protected fragments. Infected cells were labelled with [^{35}S]-methionine for 10 min at two days p.i., homogenized to release

microsomal material and then treated with 1.5 μg/ml proteinase K (Fig. 17). A control digestion with 1% Triton X-100 added was performed to remove the protecting membranes. Proteins were recovered by immunoprecipitation and analyzed by SDS-PAGE. This procedure will detect fragments which contain the HBsAg a determinant.

For HBsAg alone, two protected fragments are seen after digestion, corresponding to truncated protein and glycoprotein. The sizes of the protected fragments are consistent with digestion occurring within the first hydrophilic domain. A cytoplasmic localization for this domain is in agreement with current structural models for HBsAg (see Fig. 12). All other mutants showed the same pattern of protected fragments. The difference in size between HBsΔN and HBsAg is eliminated after proteinase digestion, once again consistent with cleavage within the first hydrophilic domain. These results demonstrate that the HBsK2 and HBsΔNMe1 mutants did not have any significant change in their TM topology. More specifically, there was no observed inversion of domain I to a N-terminal cytoplasmic orientation. Such an inversion would have placed the second hydrophilic domain cytoplasmic instead of the first, and should have resulted in the disappearance of protected fragments.

If the mutant polypeptides are capable of entering the E.R. membrane with a topology similar to that of HBsAg, then the inhibition of secretion must be occurring either prior to or

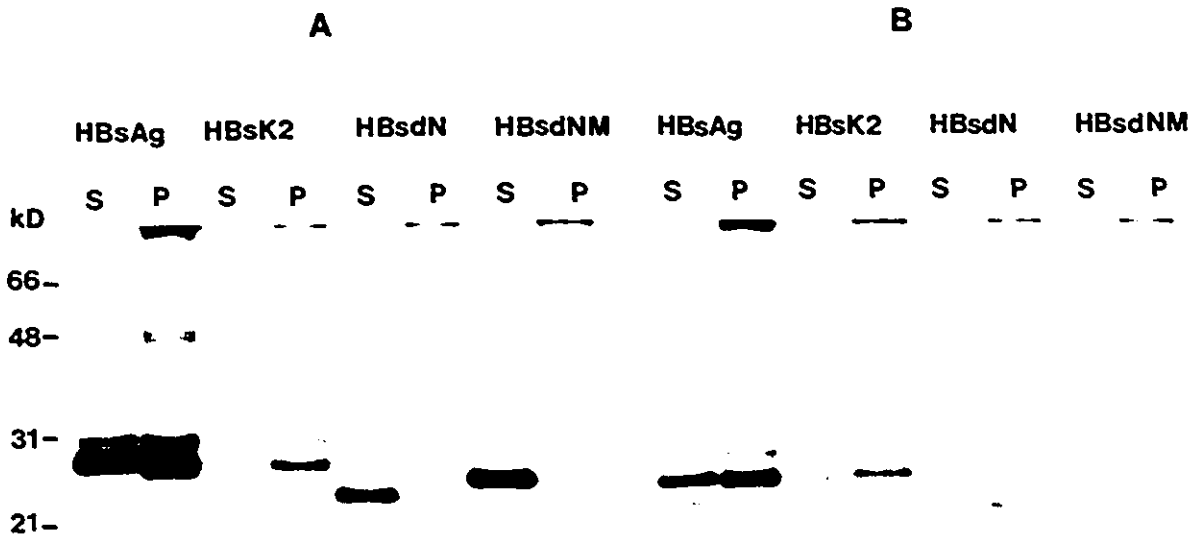
Figure 17. Proteinase K susceptibility for HBsAg mutants. Infected cells (see lane headings) were labelled at day 2 p.i. with [^{35}S]-methionine (1.0 mCi/ml) for 10 min. Cells were harvested, disrupted by Dounce homogenization, and separated into three equal fractions. Fractions were incubated alone as control, digested with proteinase K, or digested with proteinase K in the presence of 1% Triton X-100 (total volumes were equalized) as indicated under the lane headings for each virus. Samples were adjusted to 1 x RIPA buffer and protein was recovered by immunoprecipitation. Recovered protein was separated by SDS-PAGE before fluorography.


HBsAg
HBsK2
HBsΔN
HBsΔNM
Prot. K
TX100
kD
31–
21–
15–

subsequent to this process. If it occurs before, it would likely be by a reduction of the rate of entry in the membrane. This is especially relevant to HBsK2 and HBsΔN, where no aggregation is indicated. This possibility was investigated using a carbonate extraction procedure (Figs. 18 and 19, Table I). The high pH of the carbonate buffer used converts all membrane structures to sheet forms. After centrifugation, protein can be separated into membrane bound (pelleted) and non-membrane bound (supernatant) forms. The latter should include cytoplasmic polypeptide, as well as that found interior to any membrane bound compartments, including the E.R. lumen. The degree to which these two compartments contribute to the soluble fraction can be distinguished by the fraction of glycosylated polypeptide, which occurs only after E.R. entry.

Cells were labelled with [^{35}S]-methionine at two days p.i., extracted with 0.1 M Na_2CO_3, and centrifuged to separate membrane-bound forms from non membrane-bound forms. Protein was recovered by immunoprecipitation and analyzed by SDS-PAGE. A preliminary experiment was performed by extracting parallel plates after a 30 min pulse label, and after a 6 hr chase period (Figure 18a and b). For HBsAg and HBsK2, the majority of the recovered polypeptide was found in the insoluble fraction (P) after the pulse, while the opposite was true for HBsΔN and HBsΔNMel (Figure 18a). HBsAg, and possibly HBsK2 appear to contain glycosylated polypeptide in the soluble

Figure 18. Sodium carbonate solubility for HBsAg mutants. Infected cells in parallel (see lane headings for the appropriate virus) were labelled at day 2 p.i. with [^{35}S]-methionine (1.0 mCi/ml) for 30 min. Cells were harvested immediately (A) or chased for 6 hrs. before harvesting (B). Cells were disrupted by Dounce homogenization and solubilized in 0.1 M Na_2CO_3. Soluble fractions (indicated by an S lane heading under each virus) were separated from insoluble, pelleted material (indicated by P) by ultracentrifugation. Samples were adjusted to 1X RIPA buffer in equal volumes, dissociated by boiling, and protein recovered by immunoprecipitation using goat anti-HBsAg sera. Recovered protein was separated by SDS-PAGE before fluorography.


A
B
HBsAg
HBsK2
HBsdN
HBsdNM
HBsAg
HBsK2
HBsdN
HBsdNM
S
P
kD
66–
48–
31–
21–

fraction, although the situation is not clear because of the presence of background bands. This suggests a contribution to the soluble fraction from the E.R. lumen. In contrast, HBsΔN and HBsΔNMel clearly show no glycosylated polypeptide in the soluble fraction (Figure 18a).

After a 6 hr chase period, little change in the distribution of polypeptide was seen for HBsAg and HBsK2 (Fig. 18b). A small decrease in the overall amount was seen, but the meaning of this is not known. It may indicate release through the secretory pathway, although this was not seen in the pulse-chase experiments. There was a decrease in the levels of soluble HBsΔN and HBsΔNMel recovered. For HBsΔN, this appears to be at least partially compensated for by an increase seen in the membrane bound insoluble fraction. No glycosylated species were seen for HBsΔNMel in the insoluble fraction after either time point. A minor amount could be seen upon extended exposure. Instead, a lower molecular weight species can be seen. As mentioned, this pattern appears to be due to degradation.

These results suggest that HBsAg and HBsK2 insert into the E.R. membrane with a grossly similar rate. In contrast, efficient entry of HBsΔN and HBsΔNMel is not seen as evidenced by the high ratio of non membrane-bound to membrane-bound polypeptide. It is not clear to what extent the possible degradation of HBsΔNMel is contributing to the results seen. HBsΔN appears to be capable of a delayed progression to the

E.R. membrane, as seen by the change in distribution after the chase period. NB. A delay is based on the assumption that the membrane-bound fractions for HBsAg and HBsK2 are already saturated after the pulse period (and can therefore show no further increase over time).

In order to make a closer comparison of HBsAg and HBsΔNMel, carbonate solubility over a time course was examined. PMSF was included in the extraction buffer to avoid degradation of HBsΔNMel. After extraction and recovery of the products by immunoprecipitation, the results were quantitated by fluorography followed by laser densitometry (Fig. 19 and Table I). Again, the presence of gp27 in the soluble fraction is uncertain (Fig. 19a, open symbols). Low levels of this species are not surprising, if release of reorganized mature particles to the E.R. lumen is the rate-limiting step (Simon *et al.*, 1988b). HBsΔNMel shows no glycosylated species at detectable levels in the soluble fraction. However, prominent levels of glycosylated species in the insoluble fraction can now be seen (Fig. 19, solid symbols). Minor levels of the putative degraded species could be seen late in the chase period upon longer exposure (data not shown). The results of this experiment were compared after quantitation (Table I). NB. glycosylated species in the soluble fraction were not measured for this analysis. For HBsAg, peak levels were found for both fractions after 45 min of chase. The lower levels seen in the 90 min insoluble sample appear to be due to sampling error.

Figure 19. Sodium carbonate solubility over time for HBsAg and HBsΔNMe1. Cells were infected with recombinant baculovirus 14A7 (A) or AcHBsΔNME1 (B) and labelled at day 2 p.i. with [^{35}S]-Met. Cells were harvested immediately (lane 1), or chased for 45 min (lane 2), 90 min (lane 3), 180 min (lane 4), or 360 min (lane 5). Cells were solubilized in 0.1M Na_2CO_3, separated into fractions, and analyzed as in Fig. 18. In this case, open symbols designate the soluble supernatant and closed symbols the insoluble pellet for each virus.

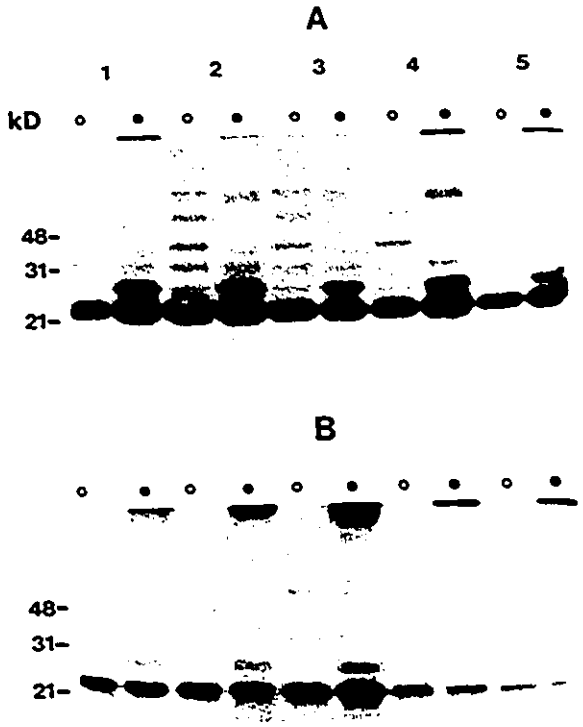

A
1
2
3
4
5
kD
48-
31-
21-
B
48-
31-
21-

Table I

Carbonate Solubility[a]

Time[b] (min)	HBsAg			HBsΔNMel		
	Soluble p24	Insoluble gp27		Soluble p23	Insoluble gp25	
Pulse	1.9	4.3	1.1	0.63	0.72	0.02
45	4.3	4.5	1.3	0.75	0.93	0.10
90	2.4	2.8	0.58	1.0	1.6	0.30
180	2.3	4.2	0.90	0.41	0.41	0.04
360	1.4	3.1	0.50	0.29	0.20	0.04

[a] Values given in arbitrary units as determined by laser densitometry of fluorographed film from preceding experiment.

[b] Pulse refers to sample taken after initial labelling period. Other values are chase times.

There is a significant decline by 6 hrs for soluble material, but only a modest decline in insoluble material. For HBsΔNMel, the levels peak after 90 min of chase, followed by a decline to low levels in both fractions concurrent with the appearance of the putative degraded species. Note that this apparent continued accumulation of membrane-bound material occurs although no decline in intracellular HBsAg was seen by pulse-chase analysis. HBsAg shows little variation in the percentage of insoluble polypeptide which is glycosylated (14-22%). The levels of glycosylated HBsΔNMel were too low to allow significant comparisons.

Thus, HBsAg and HBsΔNMel behave more similarly than suggested by the previous experiment. The rate of E.R. entry for all polypeptides except HBsΔN appears to be similar. Also, HBsΔNMel is clearly capable of forming membrane bound glycosylated polypeptide early after biosynthesis, and the membrane bound fraction is not in a significantly lower amount than for the soluble fraction. Therefore, degradation of HBsΔNMel likely accounted for the apparent preferential distribution of HBsΔNMel to the soluble fraction in the previous experiment. This is significant, however, as only this mutant was seen to be susceptible to proteolytic degradation. Degradation appears late in the chase period at a time of significant decline in HBsΔNMel levels in the second experiment.

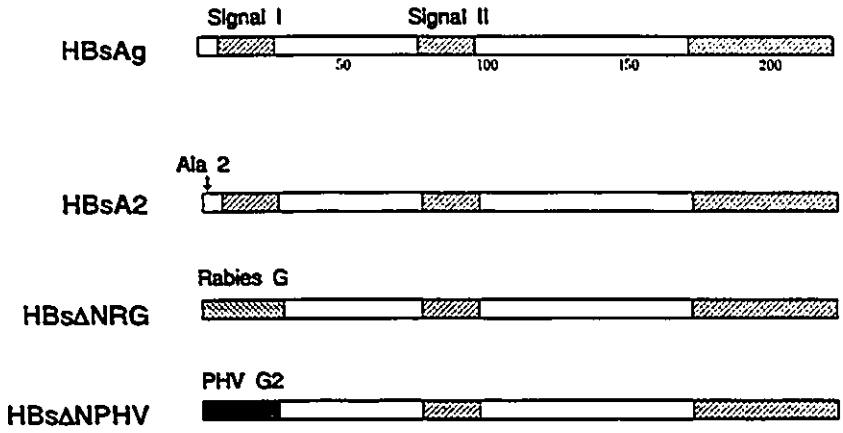
In summary, these results suggest that: 1) within the

limits of sensitivity of this assay, HBsAg and HBsK2 appear to be released to the E.R. lumen, while HBsΔN and HBsΔNMel do not; 2) a delay in entry to the E.R. membrane is apparent only for HBsΔN. This suggests that the mellitin signal peptide can promote rapid entry in the E.R., but that it causes an inhibition of secretion subsequent to entry; 3) that for HBsΔNMel, there appears to be a specific susceptibility to proteolytic breakdown, perhaps related to aggregation (Lippincott-Schwartz *et al.*, 1988; Klausner and Sitia, 1990).

To this point, the results suggest an important role for the negatively charged amino acid at position 2. However, it is not apparent whether this residue does function in the specification of TM topology as proposed. The results of the proteinase K protection and carbonate solubility assays put this in some doubt, as no distinction could be made between HBsAg and HBsK2.

To further investigate the importance of charged amino acids about domain I, a second set of mutations were designed (Fig. 20). HBsA2 was again a point mutation at residue 2, this time to the neutral alanine. Two other fusions were also designed, using the HBsΔN insert fragment fused to heterologous TM regions derived from other viral glycoproteins. However, instead of using an N-terminal signal peptide, internal anchor domains were tested. Most viral glycoproteins belong to the class of integral membrane proteins termed class I (von Heijne, 1988). These proteins have a signal peptide followed

Figure 20. Schematic representation of other HBsAg mutants. The point mutation changing Glu 2 to Ala 2 in HBsA2, the N-terminal deletion accompanied by fusion to a coding segment derived from the rabies G protein in HBsΔNRG, and a similar deletion/fusion event using a coding segment derived from the Prospect Hill virus G2 (PHV G2) protein in HBsΔNPHV, are all indicated at the appropriate position. On the lines below, the sequence about the first predicted transmembrane domain of HBsAg is compared with the transmembrane anchor domains derived from RG and PHV G2 used in the recombinant fusions.



HBsAg: M E N I T S G (F L G P L L V L Q A G F F L L T R I L T I) P Q S L D

PHV G2: F S E (W L M G I L S G N W M V V A V L V V L L I L S I F L F S L C C) P R R V V H

RG: W T K (Y V L L S A G A L T A L M L I I F L M T C C) R R V N R

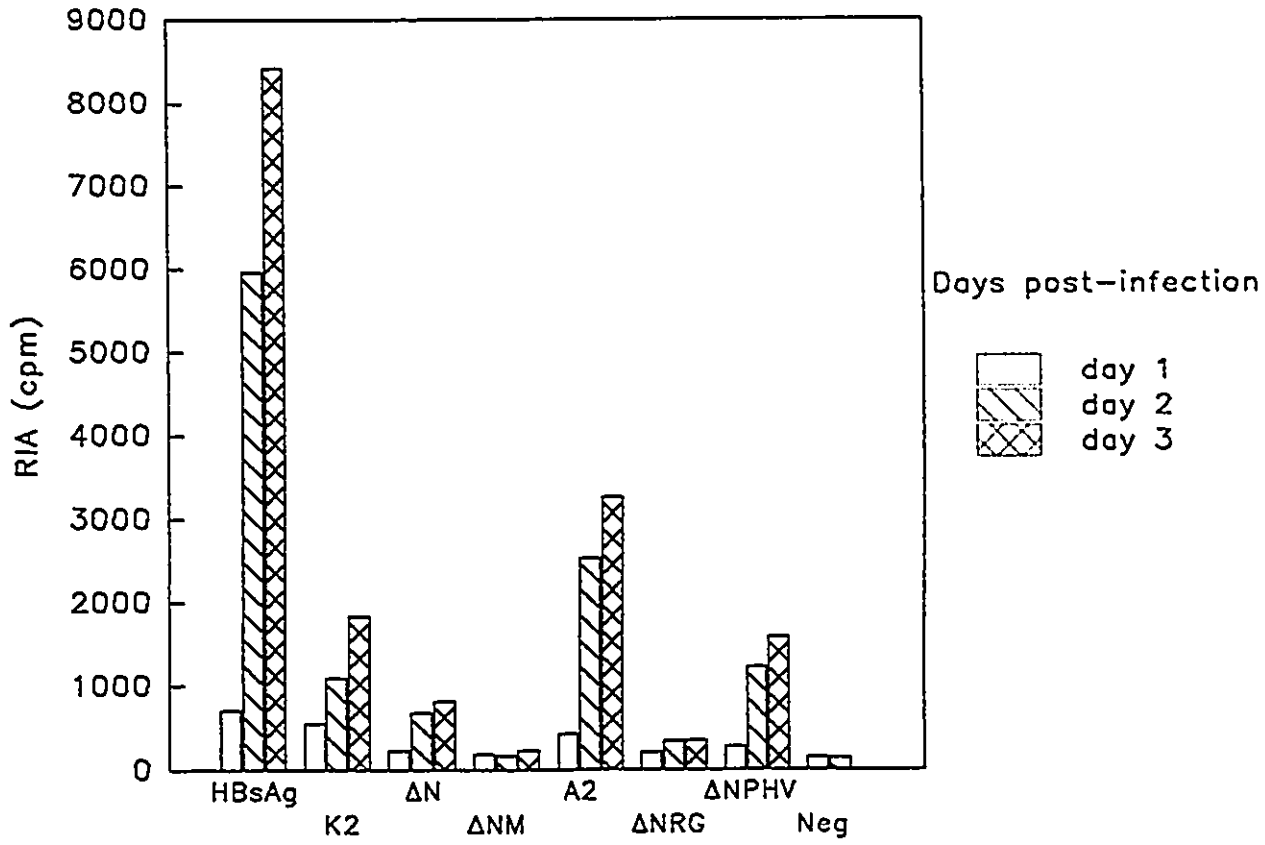
by a second TM domain, typically found near the C-terminus. These domains anchor the protein in the membrane prior to transport, and have a C-terminal cytoplasmic orientation. Note that this is the same orientation proposed for domain I of HBsAg. If a heterologous domain including flanking amino acids is able to correctly specify a topology compatible with that of HBsAg, it should restore a secretory phenotype to the HBsΔN deletion mutant. Two glycoproteins for which we had cDNA clones on hand in the laboratory were tested: rabies G, and the Prospect Hill virus (PHV; a member of the hantavirus genus of the Bunyaviridae family) G2 glycoprotein (Parrington *et al.*, 1991). These domains along with flanking amino acids were PCR-amplified from cDNA using specific primers. The primers included restriction endonuclease sites to allow subsequent ligation in frame with the HBsΔN insert. The composition of flanking amino acids differed significantly for these two domains (see Fig. 20). Three residues from the N-terminal side of each hydrophobic domain and five from the C-terminal side were included. Each has one charged amino acid on the N-terminal side, similar to HBsAg.

After isolation of recombinant baculoviruses, SF cells in monolayer were infected. Media from all HBsAg mutant-infected cultures were taken after varying times p.i. and tested for secretion by RIA (Fig. 21). Extracellular HBsA2 mutant was intermediate to that of HBsAg and the HBsK2 mutant. Of the two fusions, HBsΔNPHV exhibited clearly positive levels of

extracellular protein, while HBsΔNRG gave only background levels. Together, these results support our earlier conclusion that a negative charge preceding a TM domain favors 22 nm particle assembly. It is still possible that other sequence characteristics account for the difference between HBsΔNRG and HBsΔNPHV.

Figure 21. Determination of extracellular HBsAg and HBsAg mutants over time by RIA. Cell were infected in parallel with recombinant baculovirus. Media was harvested at day 1, day 2, or day 3 p.i. (see bar legend on figure) and analyzed using the AusRIA II RIA kit. Short forms were used to represent recombinant viruses AcHBsK2 (K2), AcHBsΔN (ΔN), AcHBsΔNMel (ΔNM), AcHBsA2 (A2), AcHBsΔNRG (ΔNRG), and AcHBsΔNPHV (ΔNPHV). Negative control samples (Neg) were from uninfected cells, and were not tested on day 3 p.i.

Radioimmunoassay: HBsAg mutants

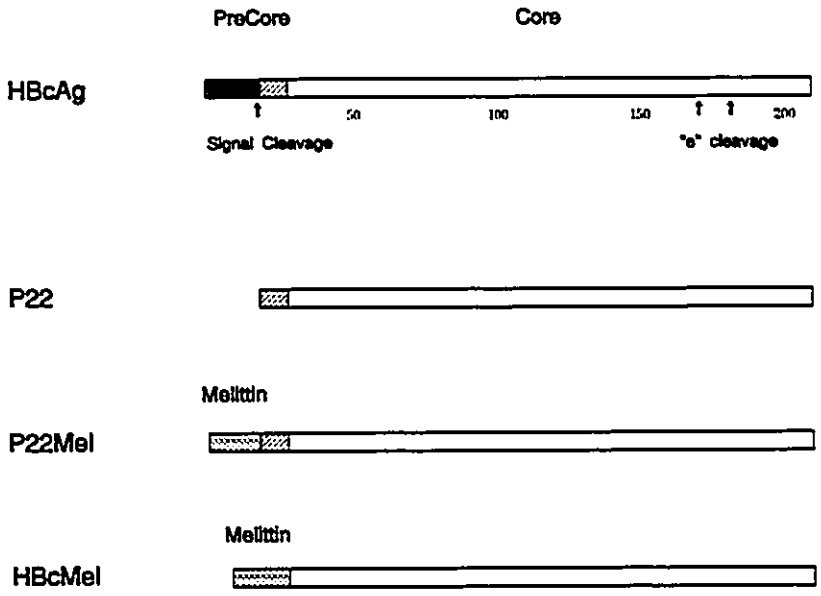


II. HBV Core Antigen Studies

HBcAg has considerable potential as a carrier of foreign epitopes. The high level of expression for HBcAg in the baculovirus system is promising for biotechnological applications. However, certain aspects of HBcAg biosynthesis in insect cells are puzzling. It was previously reported that the precore gene is not capable of elaborating HBeAg to the media from SF9 insect cells (Lanford and Notvall, 1990). In an apparent contrast, expression of the core gene alone did result in HBcAg in the media, as assayed by Western Blot analysis. This situation is the opposite of that seen in mammalian cells (Ou *et al.*, 1986; Roosinck *et al.*, 1986; McLachlan *et al.*, 1987; Jean-Jean *et al.*, 1989a). This apparent deficiency in the precore region and the basis for the appearance of the normally cytoplasmic HBcAg in the medium was investigated.

Signal replacement mutants were constructed by insertion of the appropriate PCR-amplified fragments in the transfer vector pVT-Bac (Fig. 22). For this, both the core gene alone (resulting in HBcMel), and the core gene plus the 10 aa proximal precore region which remains after signal cleavage (designated P22; resulting in P22Mel) were used. The proximal precore region has been implicated in the sorting of protein to the nucleus after it is freed to the cytoplasm by signal peptidase cleavage at the E.R. membrane (Garcia *et*

Figure 22. Schematic representation of HBcAg mutants. The complete HBV core open reading frame is depicted on the top line. The core gene is represented by the open box. The precore region is indicated as a combination of a cleavable signal which directs translocation (distal precore; black region), and a region which remains present in HBeAg after translocation (proximal precore; hatched region). The sites of precore signal cleavage and C-terminal cleavage which occurs during HBeAg biosynthesis are indicated at the appropriate position. The product of deletion of the distal precore region is designated P22. This deletion accompanied by fusion to the mellitin signal peptide is designated P22Mel. Fusion of the mellitin signal peptide to the core gene is designated HBcMel. On the lines below, the sequences of the precore signal peptide and the mellitin signal peptide are compared.



PreCore: M Q L F H L C L I I S C P T V Q A

+

Melittin: M K F L V N V A L V F M V V Y I S Y I Y A

al., 1988; Ou et al., 1989). Comparison of HBcMel with P22Mel will allow investigation of this. Recombinant baculoviruses expressing the HBcAg, precore (abbreviated preC), and P22 proteins were also constructed for comparison with the mellitin signal containing proteins.

SF9 cells were infected in monolayer with recombinant baculoviruses, then labelled 2 days p.i. with [^{35}S]-Met. Cells were solubilized with 1% NP40-containing buffer and microcentrifuged to separate the insoluble and soluble fractions. HBcAg-reactive polypeptides were recovered from both fractions by immunoprecipitation and analyzed by SDS-PAGE (Fig. 23a and b). The pellet samples contain nuclei in addition to insoluble cytoplasmic structures. This fractionation would give a primary indication whether any of the polypeptides show a substantially different distribution.

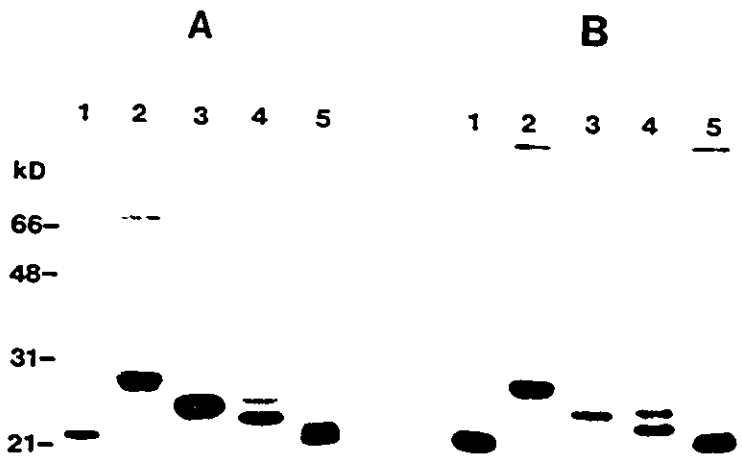
Of the five polypeptides, only HBcAg shows a clearly marked preference for the insoluble fraction (compare Fig. 23a with 23b, lane 1). Only P22 appears to be preferentially located in the soluble fraction (lane 3). No clear distinction between the distribution of P22Mel (lane 4) and HBcMel (lane 5) was seen. Further analysis of subcellular localization was done by electron microscopy (below). The overall levels of expression do not appear to differ greatly among the different polypeptides, although it is possible that they differ in the reactivity with the rabbit anti-HBcAg sera.

The preC gene (lane 2) directs the synthesis of one major

species. Its mobility is most consistent with lack of signal cleavage product, although it appears to migrate more slowly than the predicted 25 kDa molecular weight. This demonstrates that the natural precore signal is not functional in SF cells, as reported (Lanford and Notvall, 1990). A minor band can be seen at the position of the P22 polypeptide (lane 3) upon longer exposure, which may indicate a small amount of cleavage (data not shown). Again, P22 appears to be migrating more slowly than predicted. The uncleaved product of P22Mel should be similar in size to that of precore, but only a minor band is seen at that position (compare lane 2 with lane 4). Instead, a band is seen with a migration similar to that of P22 (compare lane 3 with lane 4). This indicates that cleavage consistent with removal of the mellitin signal peptide is occurring. A predominant species with an even lower molecular weight suggests that a second cleavage event is also occurring. This species appears to be more prominent in the soluble fraction. No species were seen at the expected mobility of e antigen for adw2 (~20 kDa) (Salfeld *et al.*, 1989).

Cleavage was also evident for the product of the HBcMel gene. Only a minor band remains at the expected position of the uncleaved product (lane 5). At the position of migration for HBcAg, three very closely spaced species can be seen in the soluble sample (Fig. 23a; compare lane 1 with lane 5). HBcAg appears to comigrate with the middle of the three bands,

Figure 23. Analysis of HBcAg mutant proteins by immunoprecipitation. Cells were infected with recombinant baculovirus AcYM1-KTc (HBcAg; lane 1), AcPreC (lane 2), AcP22 (lane 3), AcP22Mel (lane 4), or AcHBcMel (lane 5). Cells were labelled at 2 days p.i. with [^{35}S]-methionine (0.5 mCi/ml) for 30 min, then solubilized in 1% NP40 buffer as described. Protein from soluble (A) and insoluble (B) samples was recovered by immunoprecipitation with rabbit anti-HBcAg sera and separated by SDS-PAGE before fluorography.


A
B
1 2 3 4 5
1 2 3 4 5
kD
66–
48–
31–
21–

suggesting that this is the signal peptide-cleaved core product. The smallest species may again be a closely related proteolytic cleavage product. This species is not present in the insoluble sample. The identity of the largest band in the triplet is unknown.

Next, the medium was examined for the presence of the different core-related proteins. Cells were continuously labelled for 4 or 8 hrs with [^{35}S]-methionine at 2 days p.i. Protein recovered from the medium by immunoprecipitation and analyzed by SDS-PAGE (Fig. 24). A higher percentage gel was used in this case to allow for resolution of any possible HBeAg-sized species which may be present in the media. Specific protein species were detected for all recombinant baculoviruses. The amounts for P22mel and HBcMel appear to increase significantly between 4 and 8 hrs of steady-state labelling. The preC product is present at the lowest level. Although not clearly resolved at this gel concentration, the smallest P22Mel species appears to be overrepresented in the medium; i.e. little of the P22-sized signal cleavage product is present (compare lane 3 with lane 4). Therefore, this lower molecular weight product may be favoured for secretion. For HBcMel (lane 5), all three species appear to be present with a distribution similar to that seen intracellularly (compare Fig. 24b, lane 5 with Fig. 23a, lane 5).

Only minor species are seen at molecular weights smaller than those seen intracellularly. In particular, two bands are

visible at less than 21 kDa for HBcMel, more notably after 4 hrs of labelling (Fig. 24a, lane 5). It is not clear whether this represents specific proteolytic processing.

I attempted to confirm the presence of core related species in the medium using an HBeAg-specific RIA kit. As mentioned, HBcAg and HBeAg can be antigenically distinguished by a number of private epitopes, but are closely related. This kit uses sera from chronically infected patients, and cannot distinguish between the two antigens. Triplicate samples taken from the medium of infected cells at 3 days p.i. were positive for the presence of all proteins (data not shown). However the level of preC was significantly lower than for the other samples. Recoverable levels of this protein by immunoprecipitation were also consistently the lowest (Fig. 24). Both intracellular and extracellular fractions were then analyzed over a time course (Table II). Surprisingly, all polypeptides were detected levels in both fractions at 8 hrs p.i. The polyhedrin protein is synthesized between 12-15 hrs p.i. These results could not be attributed to background, as a wild type virus-infected control sample (3 days p.i.) gave counts equal to that of the kit negative control (data not shown). HBcAg, P22 and HBcMel all had similar profiles in this assay: counts in both fractions were maximal between 8 and 26 hrs, following which they declined over time. This is likely due to interference by the viral infection and/or cell death.

Figure 24. Analysis of extracellular HBcAg mutants by immunoprecipitation. Cells were infected with recombinant baculovirus AcYM1-KTc (lane 1), AcPreC (lane2), AcP22 (lane 3), AcP22Mel (lane 4), or AcHBcMel (lane 5). Cells were labelled at 2 day p.i. with [^{35}S]-methionine (0.5 mCi/ml) for 4 (A) or 8 (B) hrs continuously. Protein was recovered from medium by immunoprecipitation. In this case, recovered protein was separated by SDS-PAGE in 15% gels (instead of the standard 12%) before fluorography.

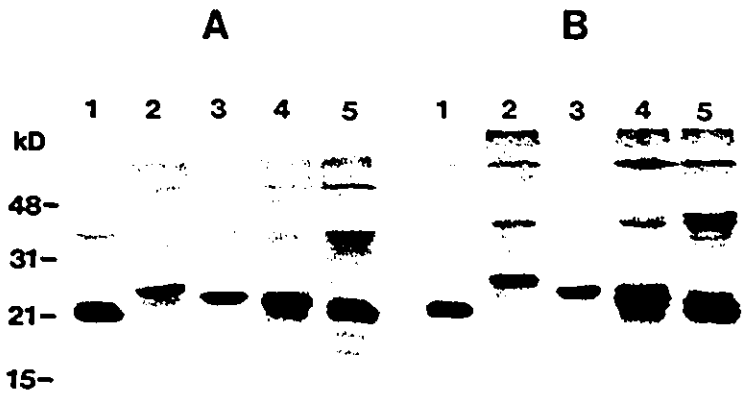

A
B
1 2 3 4 5
1 2 3 4 5
kD
48-
31-
21-
15-

Table II

Hepatitis B c/e antigen RIA

		8h[a]	19h	26h	44h	78h
HBcAg	ex[b]	-	109,995	109,543	92,204	68,474
	in	-	75,487	49,708	42,611	39,542
	ratio	-	1.46	2.20	2.16	1.73
PreC	ex	8,794	9,617	9,754	-	10,667
	in	2,781	3,359	35,123	-	43,429
	ratio	3.16	0.72	0.28	-	0.25
P22	ex	102,546	87,731	88,256	107,156	-
	in	87,619	88,373	47,287	37,838	-
	ratio	1.17	0.99	1.87	2.83	-
P22Mel	ex	17,641	16,472	17,417	33,517	81,542
	in	6,270	90,464	78,138	70,032	61,532
	ratio	2.81	0.18	0.22	0.48	1.32
CoreMel	ex	98,875	107,212	107,996	106,641	88,458
	in	69,134	96,360	58,254	44,467	45,751
	ratio	1.43	1.11	1.85	2.39	1.93

[a] Times post-infection

[b] "ex" refers to activity measured in the medium, "in" refers to activity measured in cell lysates.

For P22Mel, intracellular accumulation was similar to those above. However, a continued accumulation of counts after 26 hrs p.i. is seen in the medium, accompanied by an increase in the extracellular/intracellular ratio. The level becomes equivalent to that of those above after 78 hrs. PreC displayed a profile distinct from that of the others, with low levels in both fractions. There was a moderate rise in the intracellular levels up to 78 hrs p.i. The low levels of preC in the medium are likely related to the observed defect in signal peptide processing (see discussion). The levels seen could therefore be due to a minor degree of processing, or to a background contribution from lysed cells.

These results are consistent with those seen by immunoprecipitation analysis, but do not explain the patterns of extracellular protein accumulation. Only P22mel shows a profile consistent with the accumulation of a secretory protein. This may just be a result of a less toxic infection by the AcP22Mel recombinant virus, or of greater stability for P22Mel polypeptide species. However the early appearance of HBcAg, P22 and HBcMel which then remain constant over time is puzzling, especially as HBcAg and P22 do not have signal peptides. Also, the clear distinction in levels between these proteins and preC is most consistent with elaboration by some active mechanism as opposed to background lysis.

These results generally support the previous observations mentioned earlier (Lanford and Notvall, 1990). The discrepancy

between the low levels of precore in the medium seen here and the absence of precore-related species (by ELISA) previously reported is likely due to the difference in sensitivity of the assays used. The precore signal peptide is clearly not functional in insect cells, while HBcAg and a related protein (P22) are secreted by an unknown mechanism.

As mentioned, HBcAg is a phosphoprotein *in vivo* (Roosinck and Siddiqui, 1987). Phosphorylation of HBcAg in insect cells has also been confirmed (Lanford and Notvall, 1990). HBeAg has not been seen to be phosphorylated (Roosinck and Siddiqui, 1987). The region believed to be phosphorylated (Yang *et al.*, 1992) corresponds closely with the domain removed during HBeAg processing (Takahishi *et al.*, 1983), although a proposed site of processing closer to the C-terminus for HBV *adw2* makes this somewhat uncertain (Salfeld *et al.*, 1989). The phosphorylation status of the various HBcAg-related species was analyzed.

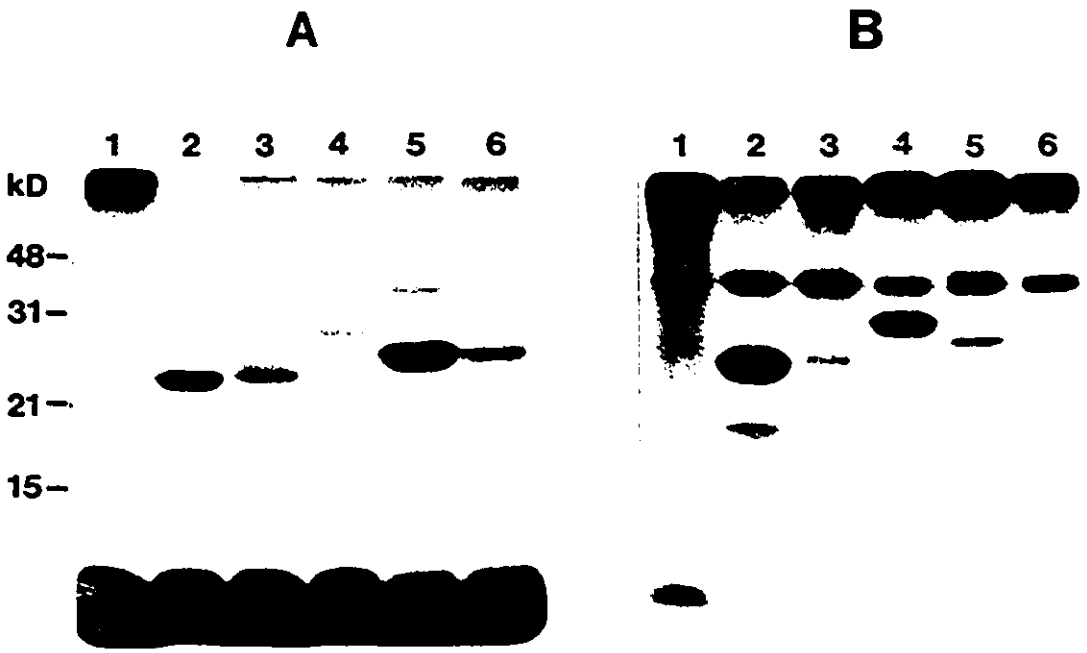
Infected cells were labelled with inorganic ^{32}P at two days p.i., and total cell protein was analyzed by SDS-PAGE. As in previous experiments, 1% NP40 soluble and insoluble fractions were prepared (Fig. 25a and b). All recombinant baculoviruses directed the synthesis of prominently labelled proteins with the expected mobilities. HBcAg was more heavily labelled in the insoluble fraction (Fig. 25a and b, lane 2), while P22 was more heavily labelled in the soluble fraction (lane 5), consistent with [^{35}S]-methionine-labelled profiles (Fig. 23). This suggests that these species are phosphorylated to an

equal degree. PreC was not phosphorylated in the soluble fraction (Fig. 25a, lane 4) to the degree expected from its methionine labelling. The significance of this is unknown. HBcAg, PreC and P22 migrate as homogenous bands (Figs. 23 and 25) suggesting there are no variant phosphorylated forms.

A phosphorylated band was noticeably absent for the smallest P22Mel species. It also appears that only the upper two of the three HBcMel species are labelled (the largest species is visible upon longer exposure; data not shown). If the smallest species represent further processing events subsequent to signal peptide cleavage, they may be segregated from the kinase activity or have the sites for phosphorylation removed. It is also possible that the larger species seen are phosphorylated variants of the smallest species. However, this would imply that signal peptide-cleaved products are variably recognized by, or have variable access to, the responsible kinase(s), while the closely related HBcAg and P22 proteins are not.

The mechanism by which HBcAg and P22 are secreted to the medium without a signal peptide is unknown. *Gag* precursor protein for both HIV-1 (Gheysen *et al.*, 1989) and HIV-2 (Luo *et al.*, 1990) expressed in insect cells have been shown to bud through the cytoplasmic membrane as particulate structures with high efficiency. The appearance of insect cells infected with HBcAg, P22Mel and HBcMel recombinant baculoviruses was therefore analyzed by electron microscopy (E.M.). Infected

Figure 25. Total infected cell [^{32}Pi]-labelled protein for HBcAg mutants. Cells were infected with wild type AcNPV (lane 1), recombinant baculovirus AcYM1-KTc (lane 2), AcHBcMe1 (lane 3), AcPreC (lane 4), AcP22 (lane 5), or AcP22Me1 (lane 6), and labelled at day 2 p.i. with [^{32}Pi] for 10 min. Cells were solubilized in 1% NP40 buffer, separated into soluble (A) and insoluble (B) samples, and separated by SDS-PAGE before fluorography.


A
B
1 2 3 4 5 6
1 2 3 4 5 6
kD
48–
31–
21–
15–

cells were fixed at two days p.i. and prepared for E.M. by thin-sectioning. Expression of HBcAg resulted in the appearance of a dense array of spherical particles (Figs. 26 and 27). These particles were measured at ~20 nm in diameter. It is not known if this difference in size from the normally reported 27 nm is significant. These particles appeared to have a distinct perinuclear localization (Fig. 26). There were also abundant particles within the nucleus (Fig. 27). Infected cells were examined extensively for cytoplasmic membrane budding of similar particles, but none was noted. Lysed cells with HBcAg particles released to the exterior were easily visible. However, these were in the minority, and were probably primarily due to the fixation procedure.

P22Mel and HBcMel were not present as spherical particle structures in the cytoplasm. Surprisingly, spherical forms for both signal containing constructs were seen within distended E.R. cisternae (Figs. 28 and 30), although these forms were more difficult to locate for HBcMel. Nuclear particles were clearly visible for P22Mel at a lower abundance than for HBcAg (Fig. 29), and possibly also for HBcMel (data not shown). These unusual results suggest that association of core-related proteins into nucleocapsid-like structures can occur within the E.R. after cleavage of a signal peptide.

The mechanism which accounts for HBcAg release into the medium was not observed (see Discussion). The presence of HBcAg as abundant spherical forms intracellularly suggests

Figure 26. Electron microscopy of AcYM1-KTc-infected cells. Cells were infected with AcYM1-KTc and fixed in glutaraldehyde at day 2 p.i. Cells were imbedded and prepared by thin-sectioning (see Materials and Methods) for visualization by electron microscopy. The cytoplasm (C) and nucleus (N) are indicated. The arrow indicates spherical particles present in the cytoplasm. Magnification 20,000X.

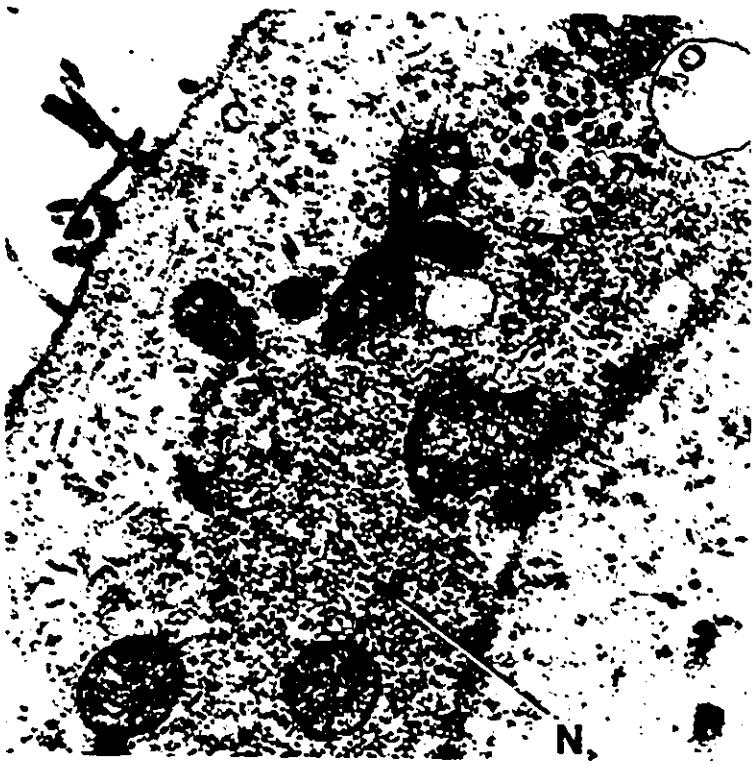
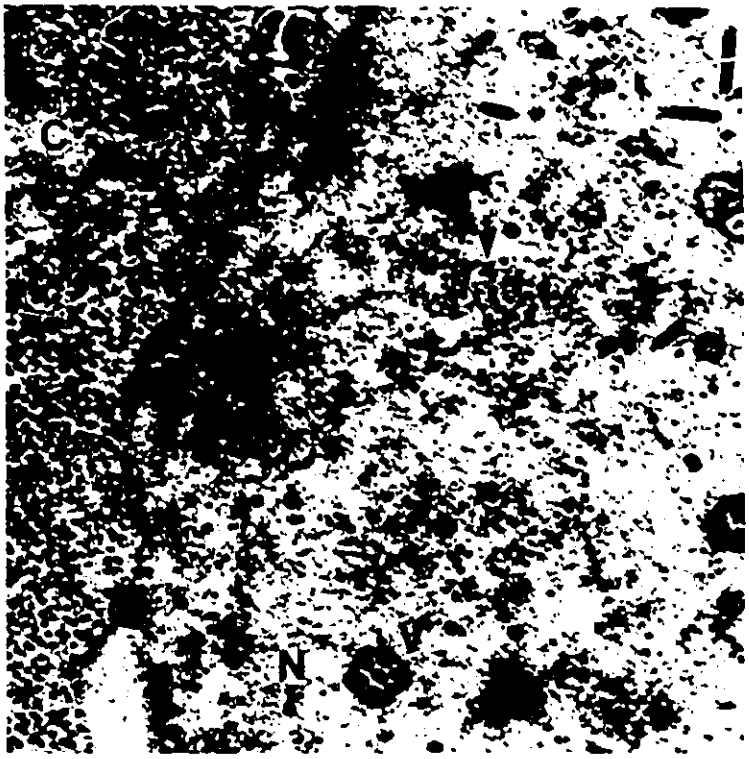

N

Figure 27. Electron microscopy of nuclear HBcAg particles. Cells were infected and prepared as in Fig. 26. The cytoplasm (C) and nucleus (N) are indicated. A cross section of recombinant baculovirus in the nucleus in the form of enveloped multiple nucleocapsids is indicated (V). The arrow indicates the presence of nuclear spherical particles. Magnification 30,000X.


C
N

that released material may also be assembled. Its presence in the medium at early times p.i. can not be accounted for by cell lysis due to baculovirus infection. It may be assumed that P22 is released in a similar fashion, although its intracellular appearance was not investigated. The cleavage events for P22Mel and HBcMel, the apparently distinct RIA profile for P22Mel, and the appearance of these species in the E.R. suggests that they may be actively released by secretion. Whether extracellular protein for these species also has a contribution from a mechanism similar to one used by HBcAg is not known. One important outstanding experiment would be the characterization of extracellular material by velocity gradient centrifugation. The nature of HBcAg-related species in the medium (i.e. particulate vs. monomer forms) would provide important information on the relative contributions of preassembled particle release vs. active secretion.

Figure 28. Electron microscopy of AcP22Mel in the E.R. Cells were infected with recombinant baculovirus AcP22Mel and prepared as in Fig. 23. The nucleus (N) and an element of rough E.R. (RER) present in the cytoplasm are indicated. The arrows indicate the presence of spherical particles present in the lumen of the E.R. Magnification 50,000X.

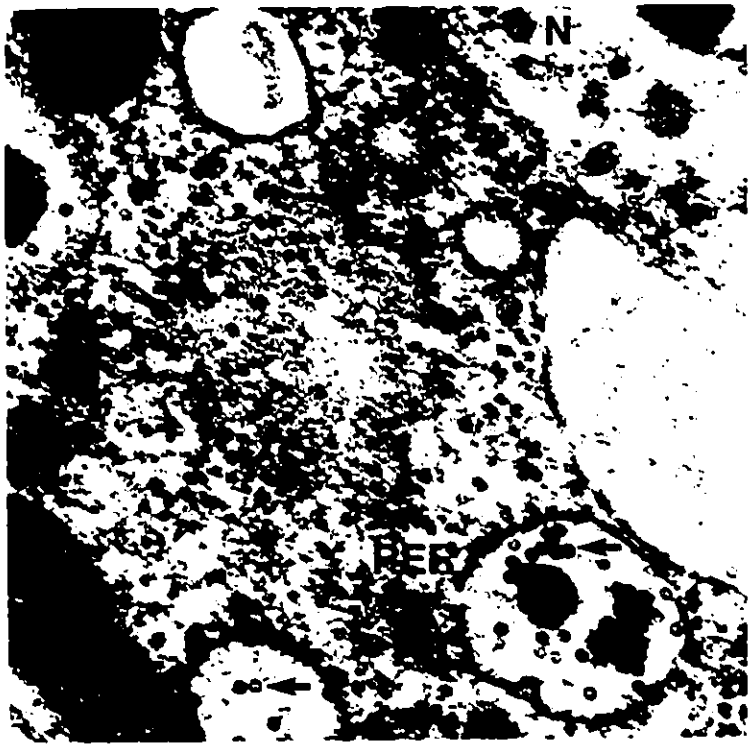

N
RER

Figure 29. Electron microscopy of nuclear AcP22Mel. Cells were infected with AcP22Mel and prepared as in Fig. 23. The nucleus is indicated (N). Arrows indicate the presence of spherical particles in the nucleus. Magnification 50,000X.


N

Figure 30. Electron microscopy of AcHBcMel in the E.R. Cells were infected with AcHBcMel and prepared as in Fig. 23. An element of the rough E.R. present in the cytoplasm is indicated (RER). The arrows indicate the presence of spherical particles present in the lumen of the E.R. Magnification 60,000X.


BEB

III. HBV Core Antigen as a Carrier

The potential of HBcAg particles as carriers of foreign protein epitopes was investigated. A structural model of HBcAg suggest that both termini are not involved in a central folding domain (Argos and Fuller, 1988). The disposition of the termini with respect to the 27 nm particle is not clear, but it has been suggested that both are exterior, making them accessible for stimulation of immune components. In addition, the C-terminus is dispensable to particle formation (Gallina *et al.*, 1989). It may therefore be possible to make substantial additions to either terminus without affecting particle formation.

We attempted to use HBcAg particles to carry epitopes derived from the human immunodeficiency virus type 1 (HIV-1). Our approach was to join a series of epitopes from HIV-1 to HBcAg in the hope of stimulating a more complete immune repertoire active against HIV-1. We selected three antigenic regions. Two were derived from constant regions of the *env* gp120 protein (Starcich *et al.*, 1986). These constant regions might represent a good target for neutralization; the more immunogenic hypervariable regions could be bypassed using this strategy. The third region was taken from a region of the *gag* p17 protein predicted to be antigenic on the basis of its relative hydrophilicity. Although *gag* proteins do not appear to elicit a protective response (Emini *et al.*, 1990), they may

aid in a vigorous immune response by stimulating components of the cellular immune response (Sarin *et al.*, 1986; Pepsidero *et al.*, 1989).

Synthetic oligonucleotides encoding these three epitopes were designed according to the codon preference of the AcNPV polyhedrin protein. They were linked together in-frame and fused at either the N or the C-terminus of HBcAg (Fig. 31). The construction and isolation of the corresponding recombinant baculoviruses and preliminary analyses were carried out by Anthony Tam, a post-doctoral fellow in this laboratory. Cross-reactivity with native HIV-1 proteins as assayed by immunoprecipitation and Western Blot was performed by the author.

Viruses AcHBcHIV and AcHIVHBc (Fig. 31) directed the synthesis of recombinant 27 nm particle-like structures when expressed in insect cells (Fig. 32). We found that these particles could be partially purified based on a similar buoyancy to that of authentic HBcAg particles. E.M. examination of the recovered particles revealed spherical structures of the expected size (Fig. 32). Significant differences in the appearance of chimeric particles from HBcAg particles suggested the disposition of the HIV sequences. HBcAg particles (Fig. 32a) are clearly distinct from HBcHIV particles (Fig. 32b), which have an opaque interior. This suggests that the HBcHIV particle interior may be densely packed with the HIV polypeptide.

Figure 31. Schematic representation of HBcAg/HIV-1 fusions. The HBcAg gene is represented as having a segment encoding portions of HIV-1 structural genes fused in frame at either the N- (HIVCore) or C-terminus (CoreHIV). The restriction endonuclease site of fusion is indicated at the appropriate position. The HIV-1 segment is represented in the expanded portion below. It was constructed from three separate synthetic double-stranded oligonucleotides (represented by three separate lines) which could be ligated together by compatible restriction endonuclease sites (the *Kpn*I and *Eco*RI sites indicated). Oligonucleotides were synthesized on the basis of preferred codon usage for the AcNPV polyhedrin gene. Sites at N- and C-termini (*Acc*I) of the ligated product allowed fusion to the core gene. One coding segment was from the HIV-1 *gag* p17 gene, and the remaining two were from the *env* gp120 gene. The encoded amino acids are indicated with the corresponding amino acid numbers for the mature protein indicated. A methionine codon was provided at the extreme N-terminus to allow initiation for HIVCore.

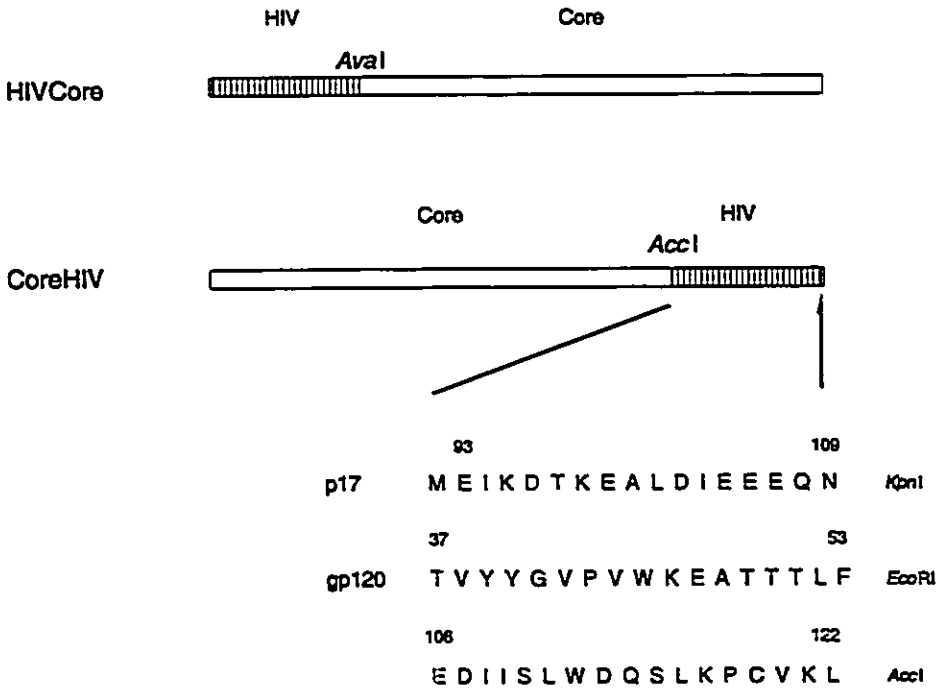
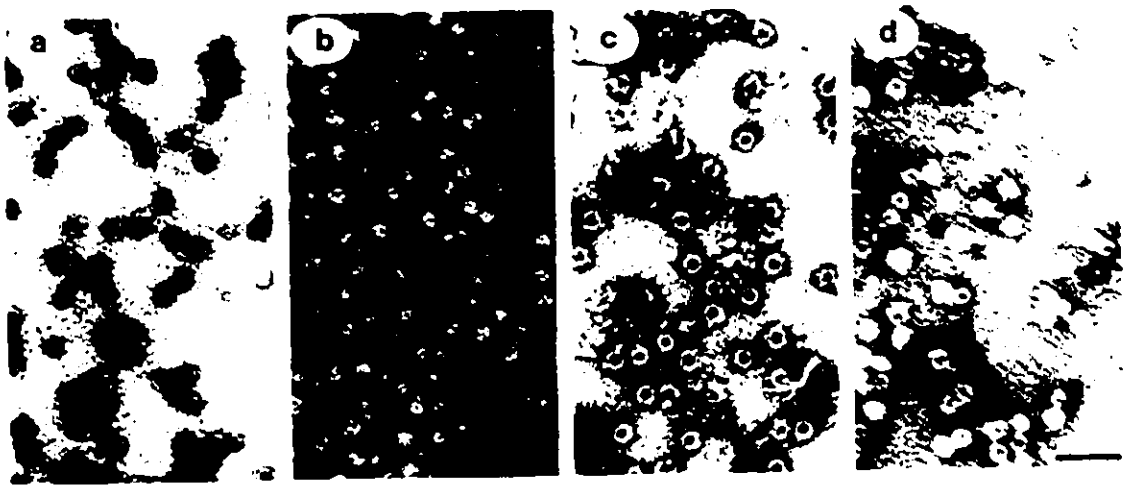

HIV
Core
AvaI
HIVCore
Core
HIV
AccI
CoreHIV
93
109
p17
M E I K D T K E A L D I E E E Q N
KpnI
37
53
gp120
T V Y Y G V P V W K E A T T T L F
EcoRI
106
122
E D I I S L W D Q S L K P C V K L
AccI

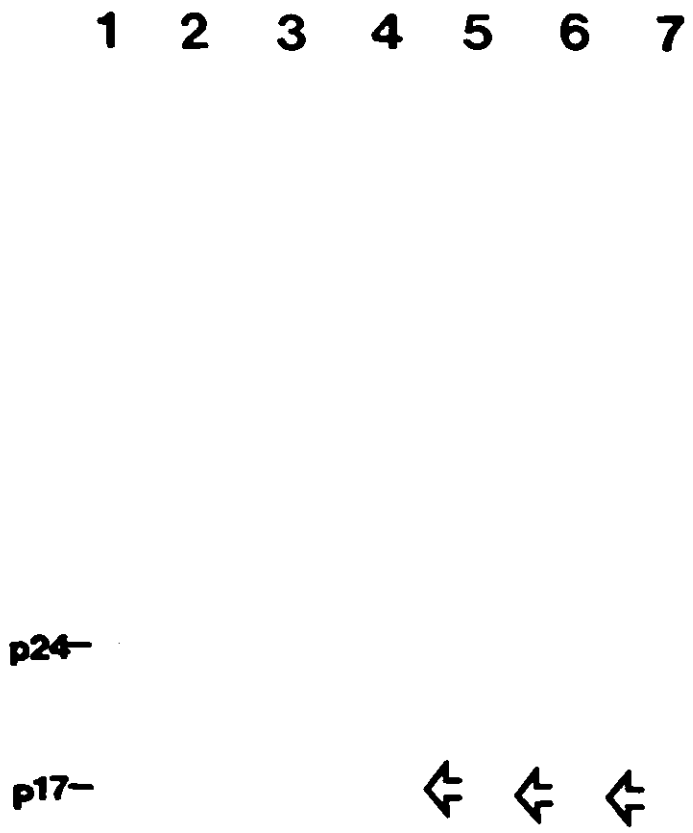
Figure 32. Electron microscopy of HBcAg/HIV-1 chimeric particles. Cells were infected with recombinant baculovirus AcYM1-KTc (a), AcHBcHIV (b), or AcHIVHBc (c and d). Cells were harvested in medium and lysed directly (a, b and c) or washed first in PBS (d) prior to lysis. Spherical particles were isolated from cells lysates based on their positions in renograffin-76 buoyant density gradients. Particles were fixed and negatively stained for microscopic examination.


a
b
c
d

In contrast, HIVHBc (Fig. 32d) particles are similar to HBcAg particles, but have an irregular surface appearance. In addition, HIVHBc particles were found to be more sensitive to proteolytic degradation. A predominant smaller species with HBcAg reactivity was seen by Western Blot analysis (data not shown). The stability of these chimeric particles was found empirically to be enhanced by heating at 50-60°C prior to washing infected cells with PBS, while HBcHIV particles were highly stable and heating did not affect their morphology. HIVHBc particles prepared in this manner (Fig. 32c) had an increase in the irregular projections on their surface. Together, these experiments suggest that the HIV-1 polypeptide is at the particle exterior for HIVHBc, and is sensitive to proteolytic degradation in this position.

It was found that both chimeric particles were fully reactive with polyclonal HBcAg antiserum by Western Blot analysis (not shown). Rabbit antisera against these particles was prepared in order to study whether they were immunologically similar to authentic HIV-1 proteins. A Western Blot with immobilized HIV-1 virion proteins (DuPont strips) was tested with various sera prepared against chimeric HBcAg particles (Fig. 33). We found that anti-HIVHBc sera could detect blotted p17 on the strips (lanes 4 and 5). In contrast, anti-HBcHIV sera did not recognize p17 in the same assay. This suggests that the p17 epitope on the HBcHIV polypeptide is either sequestered in the particle interior, or present in a

Figure 33. Anti-chimeric particle sera reactivity to HIV-1 virion proteins by Western Blot. Biotech/Dupont Western Blot strips were incubated with rabbit anti-HIV-2 *gag* precursor protein sera (lane 1), anti-HBcAg sera (lane 2), preimmune serum (lane 3), second inoculation anti-HIVHBc sera (lane 4), third inoculation anti-HIVHBc sera (lane 5), second inoculation anti-HBcHIV sera (lane 6), or third inoculation anti-HBcHIV sera (lane 7). Strips were incubated with sera diluted 1/200 before incubation with mouse anti-rabbit IgG diluted 1/3,000. The open arrows indicate a position corresponding to the mobility of p17 as detected in lane 1.


1 2 3 4 5 6 7
p24
p17

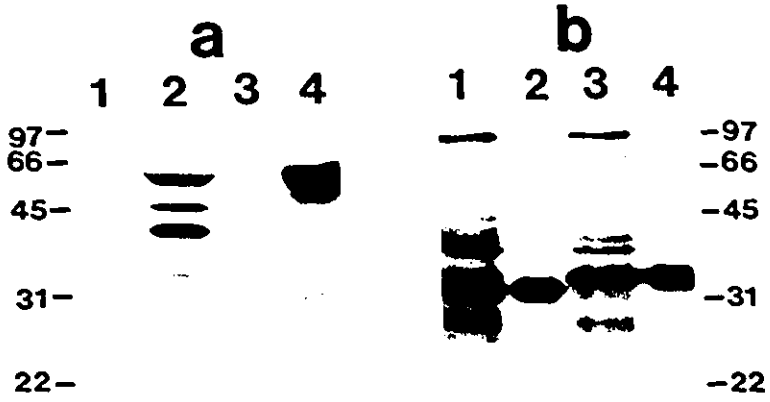
conformation which differs from that of the corresponding region in HIV-1 p17. The former would be consistent with the E.M. analysis (above). Neither antisera could recognize gp120 in this assay. Also, neither chimeric particle antisera could recognize p56 expressed from a recombinant baculovirus by Western Blot (not shown).

We attempted to further characterize the chimeric proteins by immunoprecipitation analysis. For this, a recombinant baculovirus containing the HIV-1 gp120 gene with the signal peptide deleted was used. This results in the expression of a non-glycosylated, cytoplasmic form of gp120 (hereafter referred to as p56 according to its apparent molecular weight on SDS-PAGE). Our laboratory has found that glycosylation is a necessary prerequisite for the folding of gp120 into a form recognized by CD4 (Li *et al.*, 1993). However, antiserum against p56 still strongly recognizes authentically glycosylated gp120 (Li *et al.*, 1993). AIDS patients' sera also recognize p56 (Y. Li, unpublished observations). Thus p56 represents an appropriate target for cross-reactivity testing.

Antiserum raised against p56 was used to immunoprecipitate chimeric HBcAg/HIV polypeptides from labelled insect cells lysates two days p.i (Fig. 34b). This antiserum was able to recover both polypeptides (lanes 2 and 4) from cell lysates. These results demonstrate that our chimeric particles express epitopes also found on p56. In addition, antiserum against our chimeric particles was able to immunoprecipitate p56 from

recombinant baculovirus infected cells (Fig. 34a). Anti-HIVHBc sera clearly recognized p56 although background reactivity was high (lane 2). Anti-HBcHIV showed only weak reactivity (lane 3). This again demonstrates shared gp120 epitopes present in chimeric particles and p56. The greater reactivity of anti-HIVHBc sera may again be related to an exterior disposition of the HIV-1 polypeptide. We were unable to immunoprecipitate p17 expressed from a recombinant baculovirus using antisera raised against either of the chimeric particles (data not shown).

Figure 34. Chimeric particle cross-reactivity to HIV-1 p56 by immunoprecipitation. (A) Cells were infected with recombinant baculovirus Acgp120-ΔS. At day 2 p.i. cells were [^{35}S]-methionine-labelled (0.5 mCi/ml) for 30 min. Lysates were immunoprecipitated with rabbit preimmune serum (lane 1), anti-HBcHIV serum (lane 2), anti-HIVHBc serum (lane 3), or anti-gp120Δs serum (lane 4). (B) Cells were infected with recombinant virus AcHBcHIV (lanes 1 and 2) or AcHIVHBc (lanes 3 and 4). At day 2 p.i., cells were [^{35}S]-methionine-labelled (250 μCi/ml) for 30 min. Whole samples were retained (lanes 1 and 3), or immunoprecipitated with rabbit anti-gp120-ΔS serum (lanes 2 and 4). All samples were separated by SDS-PAGE before fluorography.


a
1 2 3 4
97–
66–
45–
31–
22–
b
1 2 3 4
–97
–66
–45
–31
–22

Discussion

I. HBV Surface Antigen as a Carrier

Our strategy for the use of HBsAg as a carrier was in part based on the previous observation that HBsAg was secreted from recombinant baculovirus-infected insect cells (Kang *et al.*, 1987). It was therefore necessary to resolve this study with another in which a contradictory conclusion was reached (Lanford *et al.*, 1989). I was also unable to detect HBsAg in the medium by Western Blot (data not shown). The RIA assay used here may be more sensitive, or denaturation of HBsAg by immunoblotting may lower its recognition by polyclonal antisera. However, HBsAg could easily be detected intracellularly by Western Blot (e.g. Fig. 8). In fact, from the ratio of extracellular to intracellular (E/I) HBsAg detected by RIA, it seems surprising that extracellular HBsAg is below the level of detection for Western Blotting; i.e. the E/I ratio would seem to predict a visible band on Western Blot based on the intracellular band intensity. This can be explained if the RIA assay is underestimating the amount of intracellular HBsAg. The levels of intracellular HBsAg may exceed the linear response phase of the RIA assay. There could also be some masking of reactive HBsAg after disruption of the cells by aggregation of intracellular material.

It was found that the levels of HBsAg in the medium from cells cultured in monolayer were comparable with those from cells in spinner culture (Kang *et al.*, 1987). It was also found, however, that the secretion of HBsAg from insect cells was very inefficient. Our results can be compared to those from one mammalian system (mouse L cells), in which greater than 95% of the synthesized HBsAg was secreted within a 10 hr period (Simon *et al.*, 1988a). Our results were more comparable to those for TPA expressed in insect cells (Jarvis and Summers, 1989). These authors reported an extracellular to intracellular ratio of ~0.3 at 20 hrs p.i., similar to that observed here. They suggested that the recombinant baculovirus infection was the cause of the inefficient secretion. It has also been suggested that the temperature of culture for insect cells (27°C) is inhibitory to the secretion of HBsAg (Lanford *et al.*, 1989). In support of this latter possibility, our results were strikingly similar to those seen for expression of HBsAg from *X. laevis* oocytes, which are maintained at 19°C (Simon *et al.*, 1988b). The hypothesized lipid-phase reorganization at the E.R. membrane was proposed as the limiting event (Patzer *et al.*, 1984; Simon *et al.*, 1988b). Thermodynamic-dependent processes are known to be critical to the normal folding and translocation of secretory proteins (e.g. Machamer and Rose, 1988). In any case, highly efficient secretion of HBsAg is not typically seen (Davis *et al.*, 1985; Ou and Rutter, 1987; Patzer *et al.*, 1986). This may be

restricted to differentiated hepatocytes, which specialize in the secretion of serum lipoprotein particles.

No difference was seen between 22 nm particles isolated from insect cells and those from other systems (Kang *et al.*, 1987). Therefore, although the release of HBsAg from our system appears to be inefficient, it nonetheless appears authentic. HBsAg expressed in yeast cells is not secreted at all, but 22 nm particles can be recovered from lysates which appear authentic by all criteria (Valenzuela *et al.*, 1982). This suggests that assembly and secretion of 22 nm particles are separable processes. In other words, inefficient secretion does not imply aberrant membrane insertion and/or subunit association. Coupled with a sensitive assay system, these results suggested that the baculovirus/insect cell system used for expression was adequate for our purposes.

In my initial studies, I attempted unsuccessfully to create HBsAg fusion polypeptides using portions of the rabies G protein which could assemble and secrete chimeric 22 nm particles. Our criterion for failure was a lack of secretion for the recombinant product, although this is not necessarily a prerequisite for a economically viable vaccine. 22 nm particles derived from yeast are currently used as a recombinant hepatitis vaccine. However, secretion would be a significant advantage in the purification and analysis of a potential chimeric vaccine.

Four different peptides derived from the rabies G

glycoprotein were fused at various points to HBsAg. It was hoped that by this approach nonessential regions of HBsAg could be replaced by structurally compatible peptide segments. It has now been conclusively demonstrated that only the C-terminal hydrophobic region of HBsAg is not required for particle assembly. In one study, it was found to be involved in the subsequent stage of secretion (Bruss and Ganem, 1991b), while in another it was completely dispensable (Prange *et al.*, 1992). In any case, even the presence of the complete HBsAg sequence did not allow for secretion of our fusion polypeptides. HBsAg was equally sensitive to foreign peptide fusion at both termini. Four different peptides inhibited secretion of fusion proteins in an indistinguishable fashion, which suggests that something other than their primary sequence was responsible. Of course, not all combinations of HBsAg fusion points and rabies G fragments could be attempted. Only C-terminal fusions with the second group of rabies G peptides were attempted. It may be that one or more of these peptides would have been successful when placed at the N-terminus, however the results obtained did not warrant further attempts.

Generally, small peptides fused at, or near (i.e. within the pre-S2 domain) the N-terminus have allowed the secretion of chimeric particles (Delpeyroux *et al.*, 1986; Michel *et al.*, 1988). Small peptides could also be inserted within the second hydrophilic domain (Delpeyroux *et al.*, 1986). However, another

chimeric particle involving 300 amino acids of the HSV glycoprotein D fused within preS2 (expressed in yeast) was reported (Valenzuela *et al.*, 1985a). In addition, secretion of a chimeric particle involving 100 amino acids of α-globin fused within preS2 (from COS7 cells) was possible if an additional signal peptide was provided at the globin peptide N-terminus (Bruss and Ganem, 1991b). It was inferred from these results that the signal peptide-directed insertion of the N-terminus of the globin peptide into the E.R. lumen, and thus allowed the normal topological disposition of the remaining HBsAg sequence to proceed. Note that this type of construction is very similar to the N-terminal fusions HBsRG1 and HBsRG2 studied here, as the rabies G peptide carried its own native signal peptide. The disposition of the preS peptides also have a bearing on this discussion. PreS2 (55 aa) can be incorporated into 22 nm particles, and does not appear to inhibit particle assembly (Michel *et al.*, 1984; Persing *et al.*, 1985; Cheng and Moss, 1987). L protein is inhibitory to 22 nm particle formation, but it can be secreted from COS7 cells when a specific region of preS1 responsible for its retention is deleted (Kuroki *et al.*, 1989). Finally, it was found that large preS1-derived peptides could be fused to a truncated C-terminus and chimeric protein efficiently secreted (from HepG2 cells) (Streeck *et al.*, 1991), although the physical nature of the extracellular product was not examined. These constructions are similar to many of the C-terminal

fusions attempted here.

Together, these results create an obscure picture of what additions HBsAg can successfully tolerate. Limited acceptance of larger peptides at the N-terminus does appear possible, however these are likely part of a small subset that meet specific structural criteria by chance. In this regard, the preS2 peptide has likely evolved such structural criteria as part of its functional role. The initially promising approach of using N-terminal fusions with signal peptides was found to be not generally applicable (Bruss and Ganem, 1991b; our results). At this time, then, the most promising approach for longer peptides appears to be fusion at the C-terminus, as referred to above (Streeck *et al.*, 1991). The complete dispensability of the C-terminus in their system may be a key to their success. The use of a differentiated hepatoma cell line may explain this and the success of their C-terminal fusions as compared to those reported here.

At which stage of 22 nm particle biogenesis are foreign peptides likely to interfere? As referred to above, this process can reasonably be divided into particle assembly (including membrane insertion, subunit association, and possibly reorganization within the membrane), and particle secretion (release of the formed lipoprotein particle to the E.R.lumen). Our data and that of others (Bruss and Ganem, 1991b) show that the extent of glycosylation for nonsecretory fusion polypeptides is often similar to that of wild type,

suggesting that membrane insertion is normal. Our intracellular fusion proteins were recognized by antibody to HBsAg. This suggests that some structuring similar to that of native HBsAg is occurring. In addition, a nonsecretory fusion protein from a cell lysate had the same hydrodynamic properties as purified 22 nm particles (Delpeyroux *et al.*, 1987). Together, these results suggest that inhibition by a foreign peptide is often at the final stages of particle formation, i.e. subunit reorganization or release to the E.R. lumen.

Lack of secretion for fusion proteins was correlated with lowered solubility. Although this suggests that these polypeptides are in a more aggregated state or one which reflects a more irreversible association with membrane, the physical basis for this insolubility is not known. For HBsAg (and also HBsK2 and HNsΔN), glycoprotein was present in both fractions, so the distinction is not simply cytosolic vs. membrane-bound. There may be a physical distinction between the soluble/insoluble states which is reflective of some process involved in 22 nm particle formation, or there may simply be a difference in the degree of association with other microsomal components. Longer peptides presumably perturb normal HBsAg structure more readily than shorter peptides, but how this results in the inhibition of particle release is not known. The instability observed for some peptide fusions may be significant in this regard. Degradation is a common fate

for misfolded or improperly aggregated proteins (Klausner and Sitia, 1990). However, it is not known whether recognition and targeting for turnover of aberrant fusion protein would occur in the more unusual context of lipoprotein particle formation.

II. Mutagenesis of HBV Surface Antigen

In investigating the role of the first TM domain in particle assembly, minor levels of HBsAg secretion could still be detected in its absence. In contrast, the second TM domain appears to be critical (Araki *et al.*, 1990; Bruss and Ganem, 1991b). What is the exact function of the first domain? Being the first domain to emerge from the ribosome, it seems reasonable to assume that it initiates translocation, as suggested (Eble *et al.*, 1987). It may also specify the N-terminal lumenal orientation of the emerging polypeptide. Our data support the first possibility. Deletion mutant HBsΔN was delayed in its entry to membranes relative to the other HBsAg species. In contrast, HBsΔNMel had kinetics of membrane accumulation similar to HBsAg, demonstrating that a heterologous TM domain can substitute for this function.

A role in topological specification for domain I could not be definitively supported. Mutant HBsK2 was inhibited for secretion. This result, and the fact that a neutral residue (HBsA2) was less inhibitory than a positive one, initially

suggests that the charge balance about domain I is important. However, no difference in the pattern of proteinase K protected fragments between native HBsAg and these mutants was seen, even in the case of HBsΔNMel, which has the charge balance perturbed even further by the loss of residue Arg24. While the proteinase K protection pattern was not assessed over time, the time used for labelling was short. This suggests little or no delay in the adoption of wild-type topology, even for HBsΔN, which was delayed in membrane entry. Therefore, wild-type topology can be specified in the absence of domain I, presumably by domain II. This would be compatible with the series of positively charged residues which precede it (Fig. 12). However, coupled with our data, this would require that the topology of domain I is not fixed until the emergence of domain II, and that the charges about domain I can do little to override the dominance of domain II. It is hard to envision such ambivalence about topology during initial membrane insertion, especially as domain I has been demonstrated to interact with signal recognition particle and to act cotranslationally (Eble *et al.*, 1987). It has been proposed that TM domains are initially inserted into the E.R. membrane as hairpin loops, following which either the N- or C-terminus is translocated across the membrane (Engleman and Steitz, 1981; Shaw *et al.*, 1988). Perhaps domain I is retained in a hairpin conformation until the emergence of the charged residues adjacent to domain II.

Our data do clearly demonstrate, however, that a negative charge in position 2 is still important to particle formation. In particular, the two fusions HBsΔNPHV and HBsΔNRG demonstrate the importance of the N-terminal negative charge in the context of a heterologous TM domain. One possibility is that this residue is involved in subunit-subunit contacts which are essential to the final stages of particle formation. Alternatively, the charge of this residue may be important to maintaining a conformation compatible with particle secretion.

Our data differ from that previously obtained for HBsAg in mammalian cells. Using isolated microsomes from mouse L cells, HBsAg became carbonate soluble after a period of hours, suggesting a slow release of complete particles to the E.R. lumen (Eble *et al.*, 1986). Using whole cell lysates in this study, it was surprising to see that HBsAg was detected in the soluble fraction after a short pulse time. No convincing demonstration of glycosylated HBsAg was seen here. Although translocation has been shown to be cotranslational *in vitro* (Eble *et al.*, 1986), this suggests that much of the newly synthesized HBsAg and related species is cytosolic in our system. This may be a consequence of the proposed inefficiency of secretion in insect cells. This, and possibly also the high level of expression, could saturate the cytoplasmic membrane compartments and promote accumulation in other compartments. It would be informative to examine mammalian cells for the presence of HBsAg in the cytosol.

The behaviour of the HBsΔNMel fusion is interesting in terms of foreign peptide addition. As referred to above, the mellitin signal peptide promoted membrane entry even though its normal topology (N-terminal cytoplasmic) (Boyd and Beckwith, 1990) is opposite to that of domain I. This fusion had decreased solubility in nonionic detergent, and thus behaved very similarly to the RGf fusions. The distinction in behaviour with HBsK2 clearly demonstrates that the presence of the positive charge on the N-terminus of HBsΔNMel cannot account for this. Thus, while aggregation is correlated with an inhibition of secretion, inhibition can occur in the absence of aggregation. Therefore the additional characteristic of insolubility for HBsΔNMel must be due to the foreign peptide addition. This leaves the interesting question of whether decreased solubility colocalizes with the inhibitory effects of the other domain I mutations (HBsA2 and HBsK2) during the same late stages of particle formation. It may also colocalize with the inefficiency of particle secretion in insect cells.

TM domains often do not require specific primary sequence information for their functions (Zerial *et al.*, 1987; Kaiser and Botstein, 1990; Solomon *et al.*, 1991). This is interpreted to mean that the ability to form a hydrophobic membrane-spanning structure is sufficient. However, while HBsΔNPHV clearly restored a secretory phenotype to HBsΔN, demonstrating that domain I function can be fulfilled by a heterologous

domain, the levels did not approach that of HBsAg. Note also that while HBsΔN did eventually accumulate significant amounts of membrane bound polypeptide, secretion was still minimal. Together, this suggests some involvement of domain I (beyond membrane entry) in particle formation which depends upon its primary structure. Perhaps this is also related to subunit contacts as proposed for a negative residue at position 2. Note that the charged residue Arg24 may actually be internal to domain I (Fig. 12). If so, it would imply that, e.g., HBsR1 and HBsR2 are actually distinct in their behaviour: HBsR2 would have an additional loss of function not present for HBsR1 which is masked in our assays by the effects of the foreign peptide (which is completely inhibitory on its own).

III. Modifications of HBV Core Antigen

Expression of HBcAg in recombinant baculovirus-infected insect cells allows the investigation of the capacity of insect cells to make certain post-translational modifications. The apparent lack of HBeAg production from insect cells suggest that they may not be fully competent to process secretory proteins (Lanford and Notvall, 1990). There may be a combination of structural determinants between the core and precore polypeptides specifying post-translational modifications that insect cells are not capable of recognizing. Alternatively, it may be that insect cells are

not as capable as mammalian cells of recognizing "poor" signal peptides, but are capable of making subsequent modifications. While precore signal peptide cleavage is usually efficient in animal cells, there have been reports of 25 kDa unprocessed precore protein, supporting the latter hypothesis (Junker *et al.*, 1987; Jean-Jean *et al.*, 1989b; Yang *et al.*, 1992). Also, phosphorylation of core polypeptides by insect cells has been demonstrated (Lanford and Notvall, 1990). I therefore tested whether precore signal function could be replaced using an insect protein-derived signal peptide, and whether insect cells could secrete processed HBeAg.

Our data were consistent with the efficient cleavage of the mellitin signal peptide from P22Mel and HBcMel, while the precore signal peptide was not utilised. This situation is paralleled by other observations in our laboratory. The HIV-1 gp120 signal peptide is inefficient in mammalian cells, and is even less efficient in insect cells (Li *et al.*, 1993). HIV-1 gp120 secretion can be greatly improved by replacement with the mellitin signal peptide. Experiments have shown that a suboptimal distribution of charged residues in the signal peptide are responsible for its poor function, presumably confusing its topology (Y. Li, unpublished observations). Examination of the precore signal peptide show that it does not have the usual N-terminal positively charged residue, but this is not a prerequisite for signal peptide function (Boyd and Beckwith, 1990). There may be other structural features

which specify inefficient translocation.

When expressed in yeast cells, full length precore/core (p25) is the sole product seen (Miyanohara *et al.*, 1986). In *Xenopus* oocytes, there is an inefficient pattern of processing, intermediate between insect cells and a typical mammalian cell (Standring *et al.*, 1988; Yang *et al.*, 1992). It has been demonstrated that translocation of p25 subsequent to signal processing can be aborted, and the products released to the cytoplasm (Garcia *et al.*, 1988). From there they may be targeted for nuclear import (Ou *et al.*, 1989). This unusual aspect of inefficiency is not understood, but presumably has an important biological role. The mellitin signal peptide does not share this characteristic of inefficiency when fused to HBcAg/P22. It may also be that the mellitin signal peptide is insect-cell specific, although I am aware of no precedent for this.

In addition to signal peptide cleavage, P22Mel appears to undergo further proteolytic modification. The size of the smallest species is consistent with a cleavage of less than 10 aa from the signal peptide-processed species. However, the preC and P22 polypeptides appear to be migrating more slowly than predicted relative to HBcAg. Correspondingly, the major P22Mel species may actually be smaller than estimated. HBV adw produces a relatively homogenous HBeAg species with an apparent molecular mass of approximately 20 kDa (Salfeld *et al.*, 1989). This difference from other subtypes (which produce

smaller, heterogenous species) is due to changes in the primary sequence at C-terminal processing sites. Despite this, the major P22Mel species still appears to be too large to represent authentic HBeAg. However, it may still be modified by a cleavage event very similar to that for authentic HBeAg.

The situation for HBcMel is less clear. It also exhibits more than one species smaller than the full-length predicted size, but all are very close in apparent molecular weight. If cleavage subsequent to signal processing is occurring for the smallest species, it is not the same event as for P22Mel. There also appears to be a species larger than HBcAg present in this population. These intriguing differences can only be accounted for by the 10 aa proximal-precore region. Families of species closely related in size have been seen when precore/core is expressed in *Xenopus* oocytes (Yang *et al.*, 1992). It was speculated that signal-peptidase cleaved species which are released back to the cytoplasm might be differently structured or modified than the same species which were completely translocated. Therefore, the heterogeneity of HBcMel may be accounted for by less inefficient translocation of processed HBcMel species compared to P22Mel species. Furthermore, recent experiments have localized the secondary proteolytic cleavage events in HBeAg formation to a post-E.R. compartment (Wang *et al.*, 1991). If signal-peptide cleaved P22Mel is favoured for translocation over cleaved HBcMel, this could also explain the apparent distinction in proteolytic

processing. Why this might occur is unknown. Perhaps the proximal precore domain promotes a conformation subsequent to signal peptide cleavage compatible with further translocation which HBcAg alone cannot achieve. In an apparent contradiction, the proximal precore domain has also been implicated in the resorting of signal peptide-cleaved to the nucleus after aborted translocation (Ou *et al.*, 1989).

HBcAg-related proteins which are properly translocated and proteolytically processed should be specifically present in the secreted population. The populations present in the medium were therefore examined. Surprisingly, polypeptides were found in the medium for all expressed constructs. This is unusual in the case of HBcAg, which is expected to be cytoplasmic. However, this has been observed before (Sureau *et al.*, 1986; Jacob *et al.*, 1989; Lanford *et al.*, 1990), and in one case it was demonstrated that the release of HBcAg was not lytic (Jean-Jean *et al.*, 1989a). It was also found in the latter study that release of HBcAg occurred subsequent to an accumulation of intracellular protein. For the preC protein, secretion was not expected as the signal peptide was apparently not processed. In this case, there may be some background contribution from the handling of infected cells. In any case, these results do not allow the conclusion that the mellitin signal peptide specifically promoted secretion. It was noted, however, that while the two major HBcMel species were present in the media, only the smallest P22Mel species

was convincingly present. This may indicate the expected linkage between secondary cleavage and active secretion.

An analysis of phosphorylation was carried out in an attempt to provide further information on translocation. HBeAg is not phosphorylated (Roosinck and Siddiqui, 1987). Phosphorylation of DHBV HBcAg occurs at sites in the basic C-terminal region (Schlict *et al.*, 1989b), and probably in the same region for HBV (Yang *et al.*, 1992). This activity is localized to both the cytoplasm and the nucleus of host cells. Phosphorylated protein species were present for all constructs. However, only the 22 kDa-sized species was phosphorylated for P22Mel. The same appears to be true for the HBcAg-sized species of HBcMel. This suggests that the smaller species of both are either segregated from the kinase activity, or that their sites of phosphorylation have been removed. It also remains a possibility that the phosphorylated species seen are phosphovariants of the smallest species, although I do not favor this interpretation. The phosphorylated species HBcMel and P22Mel species may be partially translocated and/or released species present in the cytoplasm.

In summary, it was found that the mellitin signal peptide in the case of P22Mel does appear to promote secondary proteolytic cleavage, suggesting translocation at least as far as a post-E.R. compartment. Further, the selective appearance of the smallest species in the media and the segregation of

this species from the larger phosphorylated form support active secretion. The basis for the distinction between P22Mel and HBcMel is not known, but must be due to the proximal precore region; active secretion is not ruled out for HBcMel, as it did display selective phosphorylation similar to P22Mel. RIA analysis confirmed that all species could be detected in the medium by an alternate technique. Most surprising was the presence of HBcAg in the medium after very early times p.i., well before lytic death of baculovirus-infected cells occurs. Instead of a continued accumulation over time, levels decreased along with intracellular HBcAg. Although HBcAg is generally quite stable, degradation in a milieu of dying cultured cells is quite feasible. HBcMel showed much the same kinetics as HBcAg did. Only P22Mel had a profile which reasonably suggested active secretion, consistent with the other data obtained. Thus, there may be two modes of secretion occurring: signal-dependent and signal independent. If the low levels of precore present in the medium are accounted for by background release, it suggests that it is unable to be secreted by either mechanism. Perhaps precore becomes irreversibly associated with the secretory system by a recognized, but non-cleavable signal peptide. Alternatively, there may be a conformational prerequisite for the release of cytoplasmic HBcAg which precore cannot fulfil.

Electron microscopy confirmed the essential distinction between HBcAg and the mellitin signal peptide-containing

polypeptides. Cleavage of the signal peptide is consistent with the presence of dilated E.R. elements containing spherical particles. No clear distinction between P22Mel and HBcMel could be seen which might support a release mechanism for HBcMel similar to that of HBcAg. It was more difficult to see E.R. cisternal HBcMel, but this may be just related to the generally lower level of expression seen. A further level of resolution, using immunoelectron microscopy, would be necessary to study this in detail. It is clear that both are capable of forming particulate structures in the E.R. To our knowledge, this has not been reported previously with respect to HBeAg biosynthesis, although low levels of HBeAg-reactive particulate forms have been visualized in the E.R. of infected hepatocytes (Yamada *et al.*, 1990). Note also that these particles are too large for diffusion through nuclear pores. A recent report has suggested that core particle assembly in the nucleus may occur after diffusion or transport of monomers (Zhou and Standring, 1991). Thus, association of monomers appears to occur freely after membrane transit. Our results may be accounted for by the high levels of protein expression in these cells, especially if assembly is a cooperative process.

HBcAg particles at the cytoplasmic membrane were not seen. In fact, they appeared to be largely restricted to the perinuclear regions. The simian immunodeficiency virus, HIV-1 and HIV-2 *gag* precursor proteins have all been shown to bud

through the cytoplasmic membrane as immature nucleocapsids to form extracellular particles (Delchambre *et al.*, 1989; Gheysen *et al.*, 1989; Karakostas *et al.*, 1989; Luo *et al.*, 1990). It has also been shown that the secretion of SV40 virions (naked capsids) occurs via large cytoplasmic vesicular structures (Clayson *et al.*, 1989). Further work is necessary to determine whether the release of HBcAg particles occurs by a mechanism such as these or by a novel one, and whether there is a distinction between release of HBcAg particles and the mellitin signal-containing polypeptides.

IV. HBV Core Antigen as a Carrier

We have been able to synthesize chimeric HBcAg particles, adding foreign peptides from structural proteins of HIV-1. A series of three different HIV-1 peptides which were in frame with each other (totalling ~60 aa) could be fused at either the N- or C-terminus of HBcAg. In both cases, the assembly of the chimeric particles did not appear to differ from that of authentic 27 nm particles. This demonstrates that lengthy peptides can be fused to HBcAg without disruption of the normal quaternary structure, which improves the chances of full cross-reactivity with the protein from which the peptide was derived.

HBcAg appears to be ideal as a common carrier for foreign

epitopes. Its particulate structure allows for presentation of a high copy number of epitopes in a repeating array. In addition, HBcAg is highly immunogenic. It has multiple Th epitopes (Milich *et al.*, 1987a), and can act as both a T-cell dependent and T-cell independent antigen (Milich and McLachlan, 1986). It should therefore be capable of providing help for the fused foreign peptides, and it may limit the necessity for adjuvant.

The purified chimeric particles had interesting morphological distinctions from native 27 nm particles. The irregular surface projections present on HIVHBc particles suggested an exterior disposition for the HIV-1 oligopeptide. We also found that this polypeptide was susceptible to proteolytic breakdown if cells were not harvested directly from medium, or were not heated prior to washing with PBS. This could be visualized by E.M. as a loss of surface projections. In contrast, the HBcHIV particles had an opaque central cavity not seen for HBcAg or HIVHBc particles, suggesting the oligopeptide was densely packed in the spherical particle interior. Together, our results suggest that the HBcAg N-terminus is exposed at the particle exterior, while the C-terminus is in the interior cavity. This interpretation also seems consistent with the antigenicity profiles of the chimeric particles.

This structural disposition of the termini is also in accordance with the proposal that the C-terminus has DNA-

binding activity in its protamine-like tract which sequesters nucleic acids to the particle interior (Petit and Pillot, 1985; Gallina *et al.*, 1989). However, other studies have put this simple model in doubt. Chimeric particles with C-terminal peptide fusions were examined for their morphology (Borisova *et al.*, 1989; Schodel *et al.*, 1992). While some particles had an opaque interior cavity, others clearly did not. In one case, immunogold-labelled antibody to a C-terminally-fused peptide formed a halo about the particle, suggesting availability of the peptide at the particle exterior (Borisova *et al.*, 1989). Another study found that an HBsAg pre-S1 peptide was not available at the particle exterior when fused to the N-terminus, but was available when fused to the N-terminus via a short precore linker (Schodel *et al.*, 1992). Together, this data suggests that the disposition of the N- and C-termini may not be fixed. The particular physical characteristics of a fused peptide epitope may determine its disposition. This may have an important influence on the antigenicity of the foreign peptide. A internal disposition may be superior for a vaccine as it may inhibit degradation or neutralization of the foreign epitopes.

Antisera raised against both the N-terminal and C-terminal fusions were able to immunoprecipitate the non-glycosylated form of HIV-1 gp120 (p56). This suggests that epitopes within the HIV-1 oligopeptides elicited antibodies directed against the non-glycosylated gp120 backbone. Furthermore, antisera to

p56 immunoprecipitated both chimeric fusion particles. However, a similar profile was not seen by Western Blot assay. Anti-chimeric particle sera was not able to recognize p56 expressed from a recombinant baculovirus, and anti-p56 sera was not able to recognize chimeric particles. In addition, chimeric particle antisera could not recognize virion gp120 by Western Blot. Presumably the epitopes recognized by immunoprecipitation were denatured during the blotting procedure. Together these data suggest that the cross-reactive HIV-1 epitopes present on p56 and the chimeric particles were actually conformational, despite the short length (~20 aa) of the peptides.

The cross-reactivity profile for p17 was in sharp contrast. There was no reactivity of chimeric particle antisera to HIV-1 p17 (expressed from a recombinant baculovirus) by immunoprecipitation. However, anti-chimeric particle sera was reactive to virion p17 on Western Blot, suggesting that a linear epitope is exposed only after denaturation of p17 by immunoblotting. The reactivity of anti-HIVHBc, but not anti-HBcHIV sera, seems to provide support for the contention that the HIV-1 oligopeptide is sequestered at the particle interior. However, both chimeric particle antisera were able to immunoprecipitate p56. It may still be that the p17 on HBcHIV is somehow shielded from the immune system. Alternatively, it may adopt a conformation different from that of the oligopeptide fused N-terminally, such that it elicits

antisera not reactive to blotted p17.

In conclusion, we have successfully demonstrated the feasibility of HBcAg fusion particles involving multiple fused foreign peptides. Multiple peptides allow for the selection of a repertoire of epitopes (e.g. Th and Tc cell determinants) which could activate various arms of the immune response and neutralize the pathogen. It also maximizes the chances of targeting regions of proteins which are incapable of mutational escape because of inflexible functional characteristics. These features might be ideal in the case of HIV-1 which can maintain itself in the absence of viremia, and is highly mutable. Of course, peptide-based vaccines still have notable drawbacks, such as the poor cross-reactivity to conformational or inaccessible features on the target. However, the selection of an appropriate peptide repertoire should overcome these limitations. Further experimentation is necessary to prove that chimeric particles such as these are fully reactive to AIDS patients' sera, and capable of eliciting protective immunity.

Conclusions

Recombinant chimeric proteins involving various portions of HBsAg fused to peptides derived from the rabies virus glycoprotein were expressed and analyzed using a recombinant baculovirus system. It was found that the rabies G peptides used were inhibitory to the normal secretion of HBsAg lipoprotein particles. The presence of the chimeric protein in a detergent-insoluble fraction suggests that disruption of normal HBsAg topology by long foreign peptides leads to irreversible aggregation. The glycosylation status and HBsAg-specific RIA reactivity of chimeric protein suggests that E.R. membrane insertion and an HBsAg-like association of polypeptide is occurring normally, and that aggregation must be occurring subsequent to this.

Mutants of HBsAg in which the charge of residue 2 (Glu; normally negative) was changed to neutral (Ala) or positive (Lys) demonstrate that the charge of this residue is important to levels of secretion, and therefore likely to particle assembly. Further support for the importance of this residue came from the ability of a foreign protein transmembrane domain to functionally replace domain I of HBsAg if a negatively charged residue was present in a similar position. However, the role of this residue does not appear to be in

specification of transmembrane topology, as initially proposed.

Removal of domain I demonstrates that it enhances the rate of membrane insertion and greatly increases particle secretion. Membrane insertion, but not secretion, can be restored by the mellitin signal peptide. However, the mellitin signal peptide causes aggregation of the fusion protein similar to that seen for the rabies G fusions. Together, these data suggest that domain I also functions after membrane insertion during the association of HBsAg subunits. Based on the difference in behaviour between HBsΔNMel and HBsK2, I have speculated that this second function may be more dependent upon the primary sequence of domain I.

Expression of the core plus precore polypeptide using a recombinant baculovirus system has confirmed that the precore signal peptide is not functional in SF insect cells. However, this signal can be replaced by the mellitin signal peptide as evidenced by the cleavage of this signal and the high levels of extracellular products. The proximal precore domain did not appear to influence the subcellular localization of polypeptides subsequent to mellitin signal-peptide cleavage (p22Mel vs. HBcMel). All constructions expressed protein species which were phosphorylated.

Proteolytic events subsequent to signal peptide cleavage were evident for both p22Mel and HBcMel. It is not clear if this is the same as authentic HBeAg-related processing which

occurs in mammalian cells. However, we found that these smaller species were present in the medium in relatively higher levels, and that they were not phosphorylated, suggesting a segregation from the larger (presumably cytoplasmic) species.

All species with the exception of precore were found at high levels in the medium. No explanation was found for the secretion of HBcAg by E.M. analysis. However, spherical particles could be seen for both HBcMel and P22Mel in distended E.R. cisternae. It is not clear if this is simply due to high expression levels, or to the efficient processing of the mellitin signal peptide.

A peptide composed of three epitopic domains from the HIV-1 *gag* and *env* genes was fused to either the N- or C-terminus of HBcAg. Chimeric core particles could be easily isolated from infected cell lysates in high yields. The morphology of these particles and the susceptibility of the HIV-1 peptide to proteolysis show that the N-terminally fused peptide was exterior to the chimeric particles, while the C-terminally-fused peptide was interior to the particles. Antisera elicited against the chimeric particles was able to recognize non-glycosylated gp120 (p56) by immunoprecipitation, and *gag* p17 by Western Blot. Antisera elicited against p56 recognized both chimeric particles by immunoprecipitation. These results demonstrate that there are epitopes present within the HIV peptide on chimeric HBcAg particles which are similar to ones

found in native HIV-1 proteins. These particles are thus candidates as HIV-1 vaccines.

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