EXPRESSION OF HEPATITIS B VIRUS CHIMERIC PROTEINS IN PROKARYOTIC AND EUKARYOTIC SYSTEMS

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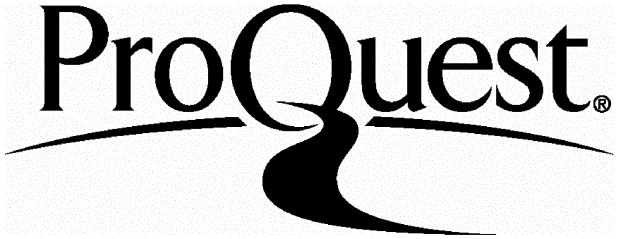

DEDICATION

To my beloved parents,

Khalida and Riaz Nazir Tarar

for giving me the inspiration to be what I am today

AND

To Moazzam and Maham

for being my strength

ABSTRACT

The particulate form of the core antigen of hepatitis B virus (HBcAg), is highly immunogenic. It has been used as a carrier molecule, for expression and presentation of heterologous viral epitopes on the surface of hybrid core particles, in immunogenicity studies. The aim of this project was to produce a hybrid antigen comprising HBcAg and an immunogenic epitope of human cytomegalovirus (HCMV). A direct comparison was made of amino and carboxyl terminal fusions, by investigating the influence of position of the foreign epitope on antigenicity, immunogenicity and hybrid core particle formation. A part of the HCMV genome, encoding a neutralizing glycoprotein epitope gp58, was inserted at the amino terminus or fused to the truncated carboxyl terminus of HBcAg in separate constructs and expressed in a prokaryotic system (E.coli). At the same time, in order to express the same fusion proteins in a eukaryotic system, the baculovirus expression vector system (BEVS) was selected and as an initial control two recombinant baculoviruses, containing genes encoding HBcAg and hepatitis B surface antigen were isolated by dot blot hybridization. It was realized that work in BEVS would require more time than previously expected therefore further work was only carried out on the prokaryotic system.

The HBcAg carboxyl terminal fusion (HBc_{3-144}-HCMV) was expressed in high yields in E.coli and assembled into core like particles resembling native HBcAg. A similar fusion in the amino terminus of HBcAg (HCMV-HBc_{1-183}) could not be purified or characterized immunologically, although it formed core like particles. HBc_{3-144}-HCMV displayed HBc antigenicity but HCMV antigenicity could not be detected. Following immunization of rabbits with HBc_{3-144}-HCMV, a high level of anti-HBc specific antibody was produced along with HCMV/gp58 specific antibody. The data presented here provide evidence that the HCMV/gp58 region can be used as a candidate immunogen for an HCMV subunit vaccine and that HBcAg can effectively present this foreign epitope joined to its carboxyl terminus, to the immune system.

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ABBREVIATIONS

aa	Amino acid
amp	Ampicillin
bp	Base pairs
BSA	Bovine serum albumin
cpm	Counts per minute
DNA	Deoxyribonucleic acid
ddNTP	Dideoxynucleoside triphosphate
dNTP	Deoxynucleoside triphosphate
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
DW	Distilled water
EBV	Epstein Barr virus
E.coli	Escherichia coli
EDTA	Ethylene diamine tetracetic acid
ER	Endoplasmic reticulum
EtBr	Ethidium bromide
Hepes	Hydroxyethylpiperazine ethanesulfonic acid
HSV	Herpes simplex virus
HRP	Horseradish peroxidase
IPTG	Isopropyl thiogalacto pyrinoside
kb	Kilobase pairs (1000 bp)
kD	Kilodaltons
LB	Luria Bertani medium
MCE	2-Mercaptoethanol
mRNA	Messenger RNA
$\underline{M}_r$	Molecular mass
moi	Multiplicity of infection
MOPS	Morpholino-propanesulfonic acid
nt	nucleotide
NT	Nick translation
OD	Optical density
p	Plasmid (or pico-)
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
RIA	Radioimmunoassay

RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
Taq	Thermus aquaticus
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
Tris	2-amino-2-hydroxymethylpropane-1, 3-diol
u	unit(s) (of enzyme)
UV	Ultravoilet
v/v	Volume per volume
VZV	Varicella zoster virus
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

Amino Acid Abbreviations

A	Alanine
C	Cysteine
D	Aspartic acid
E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

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"Even the most assidous workman will from time to time stand back to get a more general view of his work and so to comtemplate its wider relations. Indeed, such intermissions are necessary if he is to escape the tyranny of detail."

WILFRED TROTTER, 1909.

CHAPTER I

GENERAL INTRODUCTION

I.A. HEPATITIS B VIRUS (HBV)

I.A.1. Historical Introduction

In 1963 a clinical study was designed to detect the presence of unidentified antigens in blood samples collected from diverse populations, by reacting these with sera obtained from multiply transfused haemophilia patients. During the course of these investigations, it was discovered that a serum sample from an Australian aboriginal contained an antigen {named as Australia antigen (AA)} that reacted with an antibody present in serum of an American haemophilia patient [Blumberg et al., 1965]. Further studies showed that the AA antigen was prevalent among Asian and African populations and in leukaemia, lepromatous leprosy and Down's syndrome patients but was rare in western European and North American populations [Blumberg et al, 1967]. Within a short time, the association of AA antigen {now known as hepatitis B surface antigen (HBsAg)} with hepatitis was established by Prince [1968] when HBsAg was detected in the blood during the incubation period of hepatitis. This was soon followed by visualization of the 42 nm virion in the serum of AA antigen positive hepatitis patients, using electron microscopy [Dane et al., 1970]. Later, Almeida and co-workers [1971] used detergent to separate the surface and core antigens, and Magnius and Espmark [1972] described 'e' antigen, thus completing the discovery of major antigenic determinants of HBV.

With the availability of serological tests, the epidemiology of the virus infection was established. Although the usual source of infectious virus is blood, transmission can also occur through saliva and semen [Alter et al., 1977]. In addition, perinatal transmission of viral hepatitis B, from an infected mother to her offspring, may be the most important

mechanism of maintenance of an HBV carrier population in certain parts of the world [Merrill et al., 1972; Schweitzer et al., 1973]. A study of 22,707 Chinese men in Taiwan has confirmed that primary hepatocellular carcinoma is higher in carriers of HBsAg than among non-carriers [Beasley et al., 1981; Beasley and Hwang, 1990].

The cloning and expression of HBV DNA in E.coli has added towards understanding of the genomic organization of the virus [Burrell et al., 1979; Charnay et al., 1979; Pasek et al., 1979] and the complete nucleotide sequences of number of cloned HBV isolates have been published [Galibert et al., 1979; Fujiyama et al., 1983; Ono et al., 1983; Vaudin et al., 1988].

I.A.2. Virus Classification

The HBV is the prototype of family Hepadnaviridae, which is a group of closely related, enveloped DNA viruses. The family consists of viruses which share similar ultrastructure, genetic organization, mode of replication and a high degree of host specificity and infect a variety of mammals and birds. Amongst the mammalian hepadnaviruses, woodchuck hepatitis virus (WHV) [Summers et al., 1978; Galibert et al., 1982] and ground squirrel hepatitis virus (GSHV) [Marion et al., 1980] have been well characterized. The avian group includes duck hepatitis B virus (DHBV) which has helped in understanding the replication strategy of hepadnaviruses [Mason et al., 1980; Tuttleman et al., 1986a]. The recently identified heron hepatitis B virus (HHBV) is another addition to the growing list of hepadnaviruses [Sprengel et al., 1988].

I.A.3. Virus Morphology

The 42 nm virion consists of an outer envelope comprised of HBsAg [Dane et al., 1970] and an inner 28 nm electron dense core or nucleocapsid which consists of multiple units of

hepatitis B core antigen (HBcAg) forming an icosahedron [Dane et al., 1970; Cohen and Richmond, 1982]. Within the core is the viral genome, a partially duplex 3.2 kb relaxed circular DNA molecule, and a polymerase involved in its replication [Robinson et al., 1974; Robinson and Greenman, 1974; Summers et al., 1975].

In addition to the 42 nm virion, other morphological forms of HBsAg are also present in the serum of infected patients. These are spheres and filaments of 20 nm diameter that are solely composed of HBsAg and host-derived lipids and are synthesized in vast excess over the 42 nm virions, but have no virion core components [Bayer et al., 1968; Dane et al., 1970].

I.A.4. Genomic Organization

The virus genome is approximately 3.2 kb of partially double stranded DNA with a complete minus strand (complementary to the viral messenger RNAs and the pregenomic RNA) and an incomplete plus strand with variable 3' ends [Robinson et al., 1974; Summers et al., 1975]. The strands are held together in a circular structure by an overlap at the 5' ends. The 5' end of the minus strand has a polypeptide covalently linked to it [Gerlich and Robinson, 1980] and there is evidence from work done on DHBV that this primes the synthesis of the minus DNA strand [Bartenschlager and Schaller, 1988]. An oligoribonucleotide is covalently attached to the 5' end of the plus strand and seems to function as a primer for this DNA strand [Lien et al., 1986]. The region near the 5' end of both strands has the DR1 and DR2 sequence (two 10-12 bp direct repeats, see fig 1) which is the site of initiation of replication of minus (DR1) and plus (DR2) strands of viral DNA [reviewed by Ganem and Varmus, 1987; Miller et al., 1989]. A polyadenylation signal (TATAAA), which is essential for the termination of transcripts and the addition of the poly-A tails to mRNA molecules, lies within the beginning of the Core gene

[Cattaneo et al., 1983] and appears to be used by all major HBV transcripts.

The complete nucleotide sequence of several HBV isolates have been published and although there is some variation in the nucleotide sequence, the genomic organization is conserved [Galibert et al., 1979; Pasek et al., 1979; Fujiyama et al., 1983; Ono et al., 1983]. The comparison of DNA sequence of mammalian hepadnaviruses reveals the presence of four conserved open reading frames (ORF's) in the minus strand which overlap considerably (fig 1) [Pasek et al., 1979] and are described below. Recently ORF5 and ORF6 have been identified by computer analysis [Kaneko and Miller, 1988; Miller et al., 1989]. ORF5 is present on the minus strand but does not have an ATG in DNA [Kaneko and Miller, 1988]. ORF6 is found on the plus strand and can not be translated from previously identified RNA transcripts, although it could be translated from an anti-sense mRNA that would be sense for ORF6 [Miller et al., 1989].

Translation of the Core (C) ORF, which has two in-frame initiation codons, gives rise to two major products. If translation occurs at the precore (preC) AUG, a precursor polypeptide of 212 amino acids (p25) is produced and is processed to give rise to hepatitis B e antigen (HBeAg) [Ou et al., 1986]. When translation starts at the Core AUG, a product of 183 amino acids (p22) is synthesized giving rise to the nucleocapsid protein or HBcAg [Ou et al., 1986]. The Surface (S) ORF has three, in-frame AUG codons and produces three envelope polypeptides of 389-400 (p39/gp42), 281 (gp33/gp36) and 226 (p24/gp27) amino acids in glycosylated and unglycosylated forms. These are the Large, Middle and the Major polypeptides, also called preS1, preS2 and S proteins which share the same carboxyl terminus but have variable amino termini [Heermann et al., 1984]. The Polymerase (P) ORF, which is the largest of all, can encode a polypeptide with viral reverse transcriptase or polymerase activity [Mack et al., 1988]. The X ORF (X) encodes a polypeptide of 154 amino acids which has a transcriptional transactivating function for HBV [Twu and Schloemer, 1987; Spandau and Lee, 1988] and other

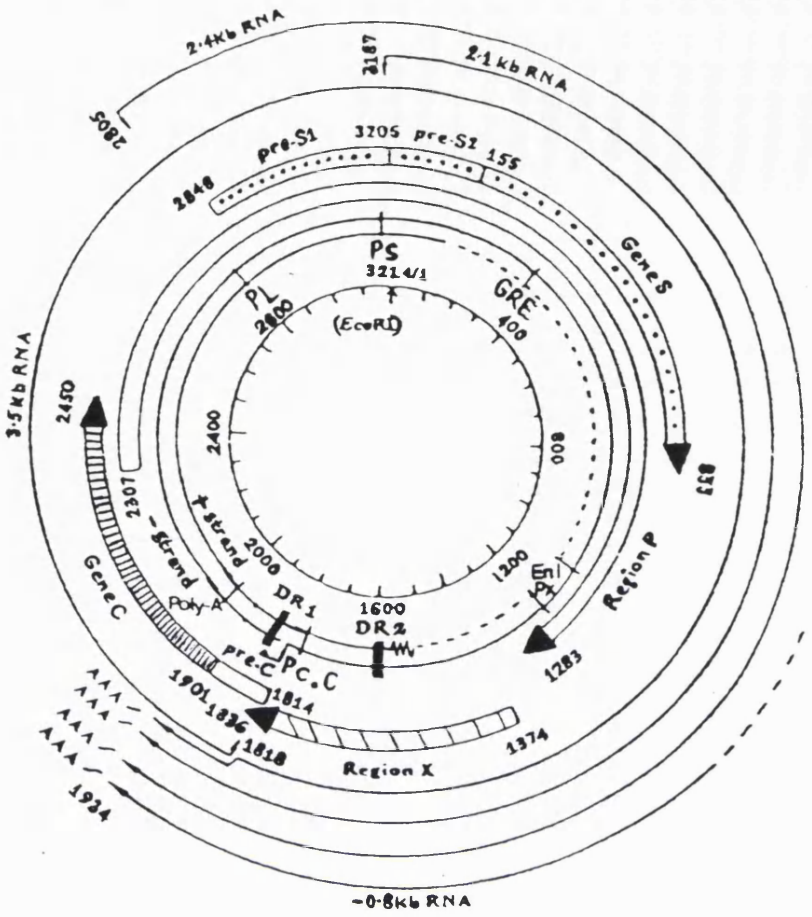


FIG 1: Genetic organization of the HBV genome.

Numbers outside the inner circle represent nucleotide numbers starting from the hypothetical EcoRI restriction enzyme site in HBV subtype adr [Fujiyama et al., 1983]. The partially double stranded genome is shown as two circles with one as complete minus (-) strand DNA and the second half dashed circle as incomplete plus (+) strand DNA. DR1 and DR2 at positions 1824-1834 and 1590-1600, respectively, are the direct repeats. The minus strand DNA synthesis begins at DR1 using a protein primer, represented as a solid triangle (▲) and the plus strand DNA synthesis starts at DR2 which has an oligoribonucleotide primer attached to its 5' end, represented as a wavy line. The broad arrows indicate the four ORFs with direction of transcription indicated on the minus strand. The position of initiation and termination of codons along with number of amino acids is also shown. The outer circles correspond to 3.5, 2.4, 2.1 and 0.8 kb transcripts, all terminating at poly-A transcription termination signal (Poly-A). Other genetic elements are: C promoter (Pc), preS promoter (PL), S promoter (PS), X promoter (Px), enhancer I (EnI), enhancer II (EnII) and glucocorticoid responsive element (GRE) [modification of figure from Matsubara and Tokino, {1990}].

viral promoters [Seto et al., 1988]. The translation of the proteins and their functional and immunological properties are discussed in section I.A.5. The other functional elements of HBV genome, are discussed below.

I.A.4.a. Promoter Elements

A promoter is a nucleotide sequence that serves as a binding site for a host cell derived RNA polymerase, thus synthesizing RNA from a DNA template. Four promoters, namely, C, preS, S and X have been described so far (fig 1). The C promoter is located within the coding region of the X gene, just upstream of the C gene, and is involved in regulating the transcription of 3.5 kb mRNAs. These are translated to give rise to HBcAg, HBeAg, polymerase protein or function as the pregenome for reverse transcription and production of DNA minus strands [Enders et al., 1985; Ganem and Varmus, 1987; Will et al., 1987]. Under experimental conditions, when the C promoter is examined under the control of the HBV enhancer (section I.A.4.b), activity can only be observed in differentiated human liver cells, whereas under the control of a heterologous enhancer the promoter exhibits low activity in non-liver cells (for example, CV-1 and HeLa cell lines) [Roossinck et al., 1986; Honigwachs et al., 1989]. The preS promoter is located upstream of the preS1 start codon, and regulates the transcription of an RNA transcript of 2.4 kb, which is translated to produce preS1 surface proteins. This promoter is transcriptionally less active than the other promoters [Siddiqui et al., 1986; Will et al., 1987]. The S promoter is located upstream of the preS2 region of the S gene and controls the transcription of an abundantly produced family of 2.1 kb preS2/S RNA transcripts which are translated into preS2 and S proteins [Rall et al., 1983; Enders et al., 1985; Siddiqui et al., 1986]. The X Promoter is located upstream of the X gene, within the P ORF. The transcripts from this promoter form less than 1% of the total virus-specific transcripts in the infected liver [Kaneko and

Miller, 1988].

I.A.4.b. Enhancer Elements

An enhancer can stimulate the transcriptional activity of a (viral) promoter(s). A transcriptional enhancer element (enhancer I, fig 1) discovered between the S and X genes (nt 1100-1250), has been shown to upregulate the transcriptional activity of the surface, preS and C promoters using the chloramphenicol acetyltransferase (CAT) gene expression system in vitro [Shaul et al., 1985; Roossinck et al., 1986; Honigwachs et al., 1989]. It has been shown that the HBV promoters under the control of this enhancer are more active in liver cells than other cell types [Honigwachs et al., 1989; Antonucci and Rutter, 1989]. A second viral enhancer (enhancer II, fig 1) has been located in the downstream half of the X gene (nt 1627-1732). This is distinct from the viral enhancer I and has been shown to activate the S promoter selectively [Zhou and Yen, 1990]. It has also been postulated that loss of this enhancer during integration of HBV into the host chromosome in chronic active hepatitis B, can lead to alteration in the relative amounts of the three forms of surface antigen in infected hepatocytes, contributing to cellular damage [Zhou and Yen, 1990].

I.A.4.c. Glucocorticoid Responsive Element (GRE)

Steroid receptors complexed with their cognate hormone (glucocorticoids) can bind to specific DNA sequences, thus increasing the efficiency of transcription initiation from nearby promoters. The steroid receptors are soluble intracellular proteins that bind to the hormone in a process called activation or transformation and this activated complex then associates with specific sites in the DNA called receptor binding region (collectively termed as steroid or glucocorticoid response element or GRE) [reviewed by Yamamoto,

1985]. It has been shown that glucocorticoids can stimulate the expression of the CAT gene, driven by the SV40 early promoter, in cells transfected with a plasmid containing specific fragments of the HBV genome (nt 354-371) linked to the CAT gene [Tur-Kaspa et al., 1986]. Analysis of this specific region has revealed extensive homology to several known GREs and contains two tandem copies of the GRE core sequence: 5'-TGTCCT-3' [Tur-Kaspa et al., 1986 & 1988]. Plasmids containing both the GRE of the HBV genome and the HBV enhancer based on SV40 early promoter CAT expression system, show increased CAT expression in response to glucocorticoids, but no CAT activity is observed if the enhancer region is deleted [Tur-Kaspa et al., 1988]. Therefore, it seems that the HBV glucocorticoid receptor binding sequence can serve as a regulatory signal or functional GRE for augmenting glucocorticoid dependent activity of the HBV enhancer I, located about 730 nucleotide downstream in the HBV genome [Tur-Kaspa et al., 1988].

I.A.4.d. U5-like Sequences

The U5-like sequence is located between the carboxyl terminal end of the X ORF and the C ORF (nt 1855-1916) [Miller and Robinson, 1986]. The sequence has been named because of its homology to the unique region of long terminal repeats (LTR) of the retroviruses. Although the biological function of this U5-like region remains unknown, it has been suggested on the basis of these homologies that HBV and retroviruses have a common evolutionary origin [Miller and Robinson, 1986].

I.A.5. Viral Proteins and Their Immunogenicity

I.A.5.a. Core Proteins (HBcAg/HBeAg)

The core proteins are translated from a family of 3.5 kb mRNA transcripts (also called preC/C mRNAs) which are

generated from preC or C initiation codons (fig 1) [Enders et al., 1985; Will et al., 1987]. This mRNA family has been described in detail in section I.A.6.

I.A.5.a.(1). HBcAg

HBcAg is found inside HBV infected hepatocytes but is not found free in the serum [Hoofnagle et al., 1981]. However, free antigen has been detected in sera of some prospectively followed patients during acute hepatitis [Hollinger et al., 1975].

HBcAg is the major nuclecapsid protein that is translated from the second in-frame initiation codon of the C ORF [Pasek et al., 1979]. The 28 nm viral core particles are formed by spontaneous self assembly of multiple copies of the core polypeptide, in the absence of other viral proteins and nucleic acid [Dane et al., 1970; Cohen and Richmond, 1982]. HBcAg has been expressed in variety of expression systems such as mammalian [Ou et al., 1986], bacterial [Mackay et al., 1981; Cohen and Richmond, 1982; Stahl et al., 1982; Murray et al., 1987], yeast [Miyanohara et al., 1986] and insect cells [Takehara et al., 1988] producing nucleocapsids which are indistinguishable morphologically from those made in the infected hepatocytes. This assembly process is not affected by addition of short heterologous sequences at the amino (up to 20 aa) or carboxyl (up to 65 aa) termini of the chain [Clarke et al., 1987; Stahl and Murray, 1989].

A protein kinase activity has been associated with HBV core particles purified from liver [Albin and Robinson, 1980]. This reaction results in phosphorylation of core particle protein in vitro and in vivo [Albin and Robinson, 1980; Roossinck and Siddiqui, 1987] and cannot be abolished by treatment with exogenous alkaline phosphatase, suggesting that it is an endogenous reaction [Gerlich et al., 1982]. The amino acid sequence of core antigen does not show homology to any of the known protein kinase domains of serine/threonine kinases [Ganem, 1991]. Therefore, the precise identity and

origin of the protein kinase activity residing in the core particle remains uncertain but it could be of host origin as DHBV core antigen produced in E.coli is unphosphorylated [Schlicht et al., 1989]. In addition, mutation within the P gene does not influence phosphorylation supporting the view that kinase activity is not encoded by the viral P or C gene [Schlicht et al., 1989]. In another study, DHBV core antigen phosphorylation, distinct from endogenous protein kinase activity, has been described [Pugh et al., 1989]. Core particle protein, purified from the cytoplasm of DHBV-infected duck hepatocytes exhibits heterogeneity in SDS-PAGE, with at least three distinct bands detected by western blotting. This heterogeneity is abolished by treatment with alkaline phosphatase suggesting that the phosphate moieties reside on the exterior of the particles. The core protein isolated from mature DHBV does not exhibit the heterogeneity displayed by intracellular core particle protein, implying that dephosphorylation must occur before or after secretion of mature virus [Pugh et al., 1989]. The significance of dephosphorylation is not known but it has been speculated that it could act as a signal for envelopment or export of mature core particles [Pugh et al., 1989].

It has been established that the region of protein-protein interaction between the core proteins, extends from the amino terminus to around amino acid 144 [Stahl and Murray, 1989; Borisova et al., 1989; Birnbaum and Nassal, 1990] and the truncation of the protein beyond amino acid 140 is not tolerated [Birnbaum and Nassal, 1990]. The 34 carboxyl terminal amino acids of the core polypeptide contain four arginine rich clusters which render the protein highly basic and this region is thought to be the nucleic acid binding domain [Petit and Pilot, 1985]. This observation has been extended in that out of four clusters of arginine residues, two [clusters 1 (residues 172-183) and 4 (residues 145-156)] represent independent nuclear localization signals [Eckhardt et al., 1991]. The presence of two nuclear localization signals and the reason for the protein transport to the nucleus is not understood. The remaining two arginine

clusters (clusters 2 and 3) do not appear to influence the cellular localization of the core polypeptide [Eckhardt et al., 1991]. The presence of basic terminus enhances encapsidation of the nucleic acid and is thought to make an important contribution to capsid stability through protein-nucleic acid interactions [Birnbaum and Nassal, 1990]. In DHBV, deletions in the carboxyl terminus do not affect particle formation or genomic RNA packaging, but produce noninfectious particles [Schlicht et al., 1989].

A predictive model of the secondary structure and folding of the polypeptide has been published by Argos and Fuller [1988]. It has been shown that extensive sequence homology exists between hepadnavirus and picornavirus capsid proteins including the VP3 capsid protein of the mengovirus. The homology also suggests that the HBV core protein will adopt the same beta sheet architecture as this picornavirus protein [Argos and Fuller, 1988].

The core polypeptide is not processed proteolytically and in vitro, localizes either in the nucleus [McLachlan et al., 1987; Roossinck and Siddiqui, 1987], or the cytoplasm [Roossinck and Siddiqui, 1987] depending on the expression system used. In vitro, HBcAg can also be converted into a polypeptide with HBeAg activity by proteolytic degradation [Mackay et al., 1981].

I.A.5.a.(2). HBeAg

HBeAg is a nonparticulate antigen which can be detected in the serum of infected individuals [Magnius and Espmark, 1972] and is correlated with high infectivity and presence of elevated titre of HBV DNA [Hoofnagle, 1981].

Initiation of translation from the first codon of the C ORF results in an additional 29 amino acids to the amino terminus of the core polypeptide resulting in a 25 kD precore-core precursor protein of 212 amino acids (p25) [Ou et al., 1986]. The first 19 amino acids of this precore protein form a signal sequence which direct this protein to the

endoplasmic reticulum (ER). Once targeted to the ER membrane, p25 is engaged in the translocation machinery and becomes a substrate for a signal peptidase, after the cleavage site has been exposed on the lumenal side of the membrane [Garcia et al., 1988]. Removal of the 19 amino acids, thus forms the precore protein derivative p22 [Garcia et al., 1988; Bruss and Gerlich, 1988]. The p22 is secreted through the ER and golgi apparatus and further removal of carboxyl terminal arginine rich domain, probably by an unidentified cellular enzyme, results in secretion of 16-18 kD HBeAg [Ou et al., 1986; McLachlan et al., 1987; Salfeld et al., 1989]. An unusual aspect of the translocation of p25 is that the process can be halted after the protein has been processed by the signal peptidase and the processed chains can be transported back to the cytoplasm [Garcia et al., 1988].

In addition to the secretion products described above, a group of precore proteins of 24-25 kD have been detected in xenopus oocytes following injection with precore mRNA [Yang et al., 1992]. Despite the presence of uncleaved signal peptide, the largest of these P25 precore proteins is not translocated into the lumen of ER and does not appear to associate with intracellular membranes. Thus, while many precore molecules are correctly targeted to intracellular membranes and translocated, a significant proportion of these molecules can evade translocation and processing [Yang et al., 1992].

Expression of recombinant vaccinia viruses encoding full length or carboxyl terminal truncated DHBV precore proteins in HepG2 cells has shown that carboxyl terminal deficient precore proteins are not secreted [Schlicht, 1991]. This suggests that the carboxyl terminal proteolytic processing of the precore protein represents an obligatory step for DHBV e antigen synthesis [Schlicht, 1991]. HBeAg can be expressed on the surface of cells infected with recombinant vaccinia virus encoding HBeAg and this might serve as a target for elimination of HBV-infected cells by antibodies [Schlicht and Schaller, 1989]. The same phenomenon has also been observed for DHBV e antigen, indicating that this property may be shared by other hepadnaviruses [Schlicht, 1991]. The pathway

by which HBeAg can reach the cell surface still remains to be elucidated.

I.A.5.a.(3). Immune Response to HBcAg

HBcAg is a powerful immunogen in humans as high titres of anti-HBc are detected in infected patients during acute hepatitis B [Hoofnagle et al., 1974]. The titre of anti-HBc IgM declines with recovery but it is replaced with anti-HBc of IgG class which usually persists for life [Hoofnagle, 1981]. Although anti-HBc is the most reliable serologic marker of hepatitis B virus infection, it does not correlate with resistance to reinfection or recovery from infection [Hoofnagle, 1981]. Anti-HBc may be harmful in neonates as chronic infection usually develops in those babies where passive transfer of the antibody has occurred perinatally and this postulate has been supported by work done in chimpanzees [Pignatelli et al., 1987]. Some workers have also proposed that anti-HBc may have a protective effect against HBV infection [Iwarson et al., 1985] as immunization of chimpanzees with HBcAg purified from bacterial cells has resulted in partial [Murray et al., 1984] or complete [Iwarson et al., 1985] protection against experimental HBV infection.

The core antigen is similarly immunogenic in mice and induces the production of IgM and IgG class antibodies in both athymic (nude) and euthymic mice, indicating that it can function dually as a T-cell independent and a T-cell dependent antigen [Milich and McLachlan, 1986]. The immunogenicity of HBcAg has been examined in detail in the murine system and this has led to the recognition of epitopes to which antibodies are directed (B-cell epitopes) and epitopes which are crucial for T cell recognition (T-cell epitopes) [reviewed by Milich, 1988]. Examination of the fine specificity of T-cell recognition of HBcAg (using short synthetic peptides) has revealed that the T cells from several mouse strains recognize multiple but distinct sites in the sequence, as defined by short synthetic peptides [Milich et al., 1987a]. Each strain

recognizes a different T-cell site and the recognition is dependent upon the haplotype of the responding strain, for example, H-2^b strain recognizes p129-140 (a synthetic peptide corresponding to residues 129-140 of HBcAg sequence), H-2^s recognizes p120-131, and H-2^q recognizes p100-120 [Milich et al., 1987a; Milich et al., 1988a]. These 12-21 residue peptides have also been shown to stimulate HBcAg-primed T cells and reciprocally to prime peptide-specific T cells that cross reacted with HBcAg [Milich et al., 1988a]. The HBcAg specific T cells are also able to provide T helper cell activity for anti-S, anti-preS2 and anti-preS1 specific antibody production [Milich et al., 1987b]. Furthermore, HBcAg specific T_H (T helper cells) have been shown to induce anti-S antibody production in S region nonresponder mice [Milich et al., 1987b]. Although H-2 linked genes influence the anti-HBc response, no nonresponder strains have been identified so far [Milich, 1988]. Comparative studies of murine antibody production have shown that the anti-HBc response is at least 80 times greater than the anti-HBs response in all the strains tested [Milich and McLachlan, 1986]. The core particle, as compared to HBsAg, not only stimulates a greater magnitude of antibody production [Milich and McLachlan, 1986] but also activates the T cells more efficiently [Milich et al., 1987a]. These findings in the murine model emphasize the importance of HBcAg and advocate its inclusion in future HBV vaccines with the idea that it may modulate the immunological response of the S region non-responder population. In addition, further evidence of the suitability of HBcAg to act as a presentation system for expression of different immunogenic epitopes on its surface as a hybrid core particle, has accumulated [Clarke et al., 1987; Borisove et al., 1989; Stahl and Murray, 1989; Beesley et al., 1990]. Therefore it is possible that a recombinant core particle could provide immunity to several virus strains in one vaccine.

I.A.5.a.(4). Immune Response to HBeAg

During early stages of convalescence from hepatitis B virus infection, anti-HBe replaces HBeAg and indicates the resolution of disease and reduction of virus replication. The anti-HBe level declines within 6 months in half of the patients [Hoofnagle, 1981].

The immune response to HBeAg is regulated independently as it is a T cell dependent antigen. This differential immunogenicity cannot be explained by the particulate structure of HBcAg alone because a particulate form of HBeAg is incapable of eliciting antibody response to the same level as HBcAg [Milich et al., 1988b].

It has been shown that antibodies present in anti-HBe positive sera can bind efficiently to cells expressing a membrane bound form of HBeAg, whereas anti-HBc positive sera cannot bind to such cells [Schlicht and Schaller, 1989; Schlicht et al., 1990]. Thus, it is suggested that anti-HBe antibodies could be involved in virus clearance during acute HBV infection, whereas the liver tissue destruction observed during chronic anti-HBe negative infections may be caused by cytotoxic lymphocytes [Schlicht et al., 1990].

I.A.5.a.(5). HBV Core Variants

In HBV infection, the presence of HBeAg in serum is usually associated with ongoing virus replication and often with liver disease. Clearance of HBeAg followed by a rise in anti-HBe usually indicates remission of liver disease and termination of HBV replication [Hoofnagle, 1981]. However, fluctuating levels of HBV DNA, measurable by dot blot hybridization in the serum of patients positive for anti-HBe and negative for HBeAg, have been reported [Hadziyannis et al., 1983]. This has been recently attributed to the presence of virus isolates with mutant preC regions that prevent the synthesis of HBeAg [Vaudin et al., 1988; Carman et al., 1989; Brunetto et al., 1990; Okamoto et al., 1990]. In one study,

HBeAg negative patients have been found to have two mutations (substitution of guanosine by adenosine) in the last two codons of the precore region resulting in a new translational stop codon [Carman et al., 1989]. Similar results have been reported by other workers [Tong et al., 1990; Tran et al., 1991]. Out of 10 possible sites in the precore region where a mutation can result in an in-phase stop codon, the commonest position for the mutation has been observed to be nucleotide 1896 [Carman et al., 1989; Brown et al., 1992]. In some patients, HBeAg negative precore HBV mutant becomes predominant at the time of anti-HBe seroconversion and this could be due to immune elimination of hepatocytes bearing HBeAg by cytotoxic T cells or antibodies directed against these cells [Okamoto et al., 1990]. The precore HBV mutants have been closely associated with fulminant hepatitis and whether patients select this variant during aggressive clearance of HBeAg or if it is a major fraction of initial inoculum is unknown at the moment [Brown et al., 1992]. Other changes resulting in an inactive precore region such as the loss of precore start codon [Okamoto et al., 1990] and a nucleotide insertion resulting in a frameshift mutation [Tong et al., 1990] have also been described in some patients who are anti-HBe positive. A point mutation in the precore gene creating a stop codon has also been accompanied by an insertion of 36 nucleotides in the core gene [Tran et al., 1991].

I.A.5.b. Surface or Envelope Proteins (preS1, preS2 and S)

Two mRNAs of 2.4 (preS1 transcript) and 2.1 kb (preS2/S transcript) are used for translation of the envelope proteins (see fig 1).

The 5' end of the preS1 transcript, in HBV infected liver, has been mapped to a position in the viral genome about 38 nucleotides upstream of the preS1 start codon [Will et al., 1987]. The transcript appears to serve as mRNA for the production of preS1 protein, but is produced in small amounts.

The transcript has been identified in HBV and DHBV infected tissues but not in the WHV and GSHV infected tissues [Enders et al., 1985; Moroy et al., 1985]. The transcript is regulated by the preS1 promoter which is liver specific and is considered to be the least active promoter of HBV [Rall et al., 1983; Siddiqui et al., 1986; Antonucci and Rutter, 1989].

The 5' end of the abundantly produced family of 2.1 kb preS2/S transcripts has been mapped to the preS1 region and serves as mRNA for preS2 and S proteins (Cattaneo et al., 1983]. It has been noticed that in this group slightly shorter mRNAs (initiating between positions 1274-1395) are used for translation of the S protein, whereas the longer mRNAs (initiating between positions 1256-57) are used for preS2 protein translation [Schaller and Fischer, 1991]. The production of these transcripts is regulated by the Surface promoter [Rall et al., 1983].

I.A.5.b.(1). Surface Proteins

Three polypeptides (preS1, preS2 and S {see section I.A.4}) are translated by the use of three initiation codons contained in one S ORF, where S polypeptide is common to all envelope proteins, and each protein exists in two isomeric forms differing only in the glycosylation of the S domain. The particles containing HBsAg have different relative composition of the three polypeptides [Heermann et al., 1984]. Closer examination of the particle preparations reveal that the 22 nm subviral particles are mainly made up of the major S polypeptides (p24 and gp27), whereas the filamentous type of particles contain some middle, preS2 (gp33 and gp36) polypeptides in addition to the S proteins and the virion contains all three types of polypeptides [Heermann et al., 1984]. HBsAg is a complex antigen which has a common group specific determinant or antigen 'a' which is defined serologically, and two groups of subtype specific determinants, d/y and w/r. Therefore HBsAg can be classified into four subtypes; adr, ayr, adw, and ayw, which are also the

HBV subtypes [Le Bouvier, 1973]. These subtypes act as useful epidemiological markers of HBV.

The secondary structure of HBsAg has been predicted using computer algorithms. The S protein has been studied for the presence of alpha helices using the helix wheel principle which divides the protein into interproline lengths, where each proline residue initiates and breaks the helix [Howard et al., 1988]. In addition, extensive sequence analysis and biochemical data has been used for deriving a topological model of HBsAg [Stirk et al., 1992]. By these methods, four transmembrane helices (residues 8-28, 78-100, 160-184 and 189-210; see fig 2a), one major loop in the extracellular space (residues 101-159) and one loop inside the particle (residues 28-77) have been predicted [Howard et al., 1988; Stirk et al., 1992] (fig 2a). It has also been suggested that the transmembrane helices may pack to form a channel, that might be involved in the mechanisms of cell entry [Stirk et al., 1992] (fig 2b). Another model for the S protein arrangement in HBsAg particles, has been predicted by Guerrero et al., [1988] (fig 3). The pattern of disulphide bonding has also been examined for the S protein and it has been suggested that 2-4 interchain disulphide bonds exist which lead to extensive cross linking of the protein [Guerrero et al., 1988].

The S protein can assemble into 22 nm subviral particles as shown by efficient expression, secretion and assembly of the S protein into particles in the absence of any other viral proteins [Laub et al., 1983]. This particle assembly occurs through a series of steps and involves insertion of nascent S polypeptides into ER bilayer as a transmembrane protein which is resistant to exogenous protease [Eble et al., 1986 and 1987]. This is followed by aggregation of the S polypeptides in the plane of the ER bilayer and secretion into the ER lumen [Eble et al., 1986 and 1987]. During this process, part of the membrane lipids incorporates into the particle, which is then secreted from the cell through constitutive vesicular transport system [Kelly, 1985]. The S polypeptide contains three hydrophobic regions located between residues 4-28, 80-100 and 164-221 respectively and two hydrophillic areas

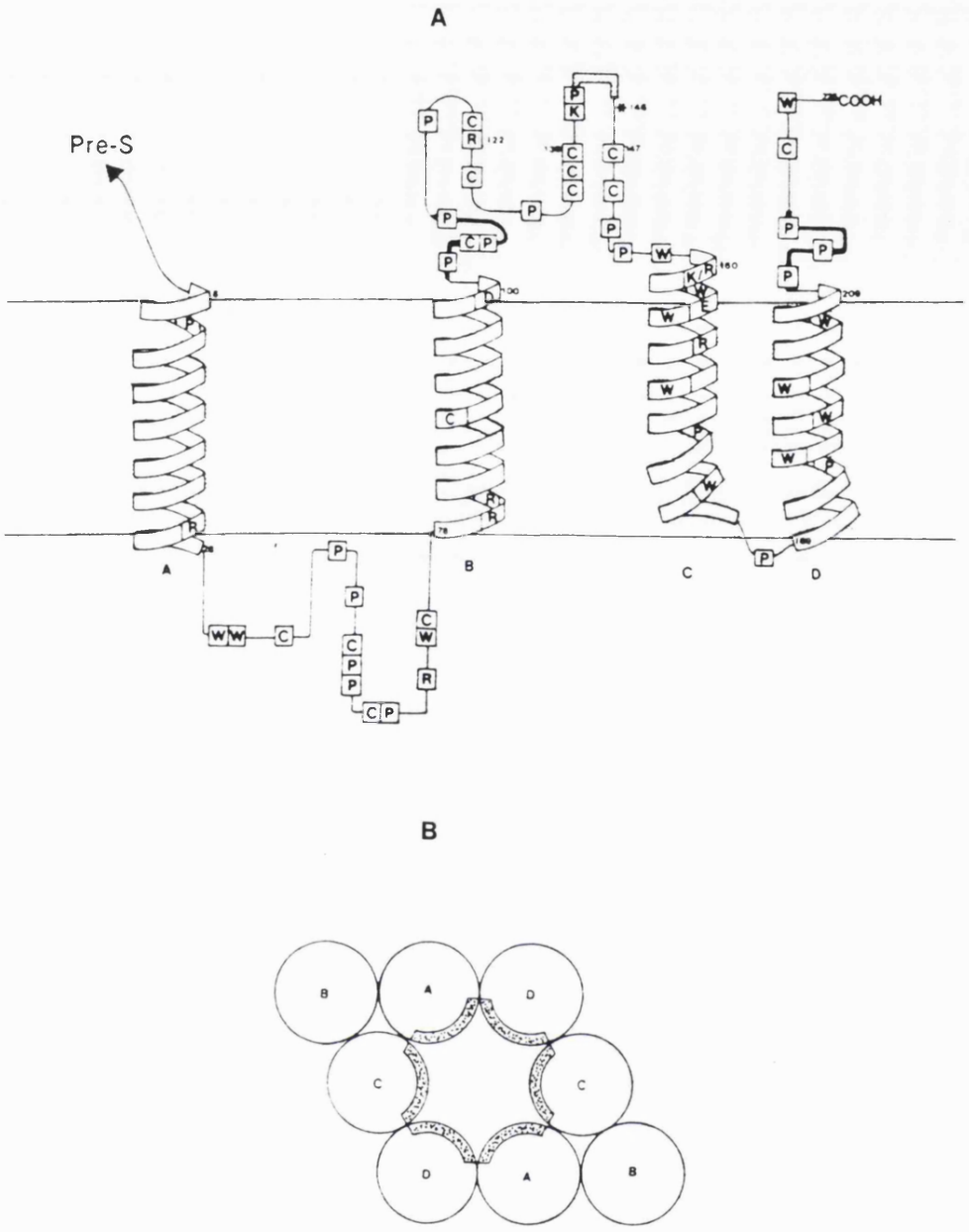


FIG 2: Predicted secondary structure of major S protein (p24/gp27) of HBV.

(A) Alpha helices are shown embedded in lipid and are indicated by letters A, B, C and D. A shaded box represents a possible ß bend. Both proline helices, indicated by dark lines (residues 105-111 and 211-217), are situated at the amino terminal end of the transmembrane helices B and D. A complex carbohydrate side chain is attached to aa residue 146 [C-cysteine, P-proline, W-tryptophan].

(B) A proposed possible mode of packing of transmembrane helices. Lettered circles represent transmembrane helices and the shaded parts show hydrophillic faces (from Howard et al., 1988; Stirk et al., 1992].

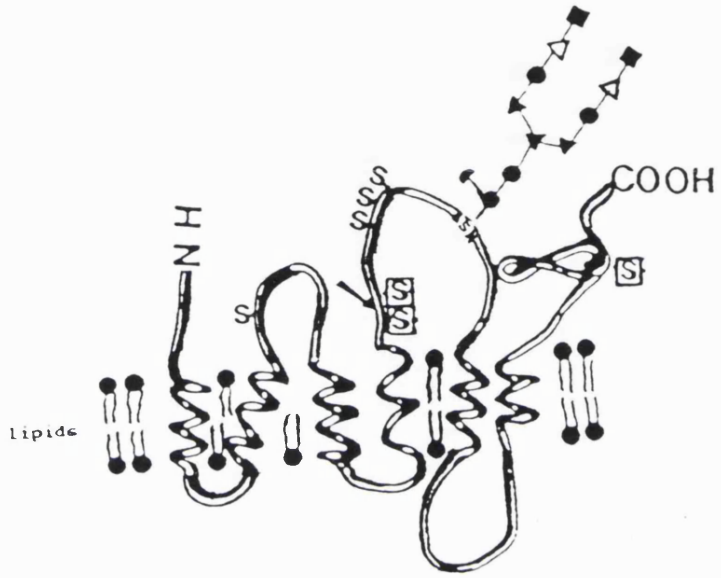


FIG 3: A second proposed model for the secondary structure of S protein.

The position of the potential sulfhydrl groups in exposed hydrophillic domains is indicated (S). The arrows indicate the position that is trypsin sensitive [from Guerrero et al., 1988].

separating these domains [Eble et al., 1987; Bruss and Ganem, 1991a and b]. The amino terminal hydrophobic domain, which has an uncleaved signal sequence called signal I, helps the complete translocation of the upstream and downstream sequences and delivers the amino terminus of the S protein to the ER lumen. In addition, signal I also helps translocation of the preS2 sequences across the ER membrane [Eble et al., 1990]. The second hydrophobic domain, which has signal II, acts as a membrane anchor and further helps translocation of downstream or carboxyl terminal S sequences into the ER lumen. As a result of this arrangement, most of the region between signal I and signal II is thought to reside in the cytoplasm. The disposition of the third hydrophobic carboxyl terminal domain is not yet clear [Eble et al., 1987]. The effects of mutations on these two signal sequences and other regions of the S protein, on particle assembly and export, have recently been examined. The results suggest that the correct transmembrane orientation of the amino terminus of the molecule is important and this region (amino acids 1-80) is directly involved in interchain recognition [Bruss and Ganem, 1991a].

I.A.5.b.(2). preS Proteins

The preS domains are hydrophillic and have been shown to be expressed on the outside of the lipid particle. It has been suggested that the preS and S regions exist as independent domains in the HBsAg particle as the elimination of the preS region by trypsin digestion does not alter the particle structure nor the chemical or immunological properties of S protein in the HBsAg particle [Hu and Peterson, 1988].

The preS1 protein does not have a signal sequence, instead it possesses an amino-terminal signal for the addition of myristic acid, a C_{14} fatty acid, which presumably causes an insertion of the amino end into the ER membrane [Persing et al., 1987]. It has already been shown that a part of these

amino terminal domains extends outside the lipid bilayer [Eble et al., 1986]. Thus, membrane interactions mediated by myristylation could be important in anchoring the amino terminus of the preS1 protein in the membrane of the ER and may affect molecular interactions with other viral components, towards formation of the viral particle [Persing et al., 1987]. The preS2 region lacks the signal sequence but is conveyed across ER bilayer by signals contained in the S protein [Eble et al., 1990].

The preS1 protein is not secreted into the culture medium, despite the presence of secretory information contained in the S protein and furthermore it also inhibits the secretion of the S protein, if the preS1 and S proteins are expressed together [Persing et al., 1986]. Epitope mapping studies with preS1 specific monoclonal antibodies reveal that most of the preS1 domain is present on the lumenal side of the ER [Kuroki et al., 1990]. It is suggested that membrane interactions mediated by the presence of myristic acid could be important in anchoring the amino terminus of the preS1 protein into the ER membrane, thus preventing its spontaneous secretion [Eble et al., 1987]. But, myristylation alone can not account for ER retention as point mutations that selectively ablate this reaction do not result in release of the preS1 protein from ER compartment [Kuroki et al., 1989]. Thus, some features of amino acid sequence may play a role in mechanism of retention. Analysis of deletion mutants of the preS1 region has confirmed this view as amino acids 6-19 have been found to be an essential retention element [Kuroki et al., 1989].

The preS proteins have domains with important antigenic [Milich et al., 1986] and binding functions [Neurath et al., 1986]. The hepatocyte receptor binding site on HBV, is located in the preS1 region between amino acid residues 21-47 and antibody to a peptide corresponding to this region blocks binding of HBsAg particles to HepG2 cells [Neurath et al., 1986]. The preS1 region is also involved in attachment of HBV to peripheral blood mononuclear cells [Pontisso et al., 1991].

I.A.5.b.(3) Immune Response to Surface Proteins

In humans, anti-HBs appears late during convalescence from hepatitis B virus infection and is usually accompanied by the disappearance of HBsAg from serum [Hoofnagle, 1981]. This is the neutralizing antibody for HBV and generally persists for life in the majority of patients, but it is absent in people who become HBsAg carriers [Hoofnagle, 1981]. In some extrahepatic manifestations of viral hepatitis such as transient serum-sickness-like syndrome, acute necrotizing vasculitis and glomerulonephritis, the pathogenesis appears to be through the formation of circulating HBsAg/anti-HBs immune complexes [Gocke et al., 1970; Onion et al., 1971; Myers et al., 1973].

The kinetics of the humoral immune response to HBsAg have been studied in great depth in the murine model. Immunization with HBsAg/p24 produces a group specific (anti-HBs/a) and a subtype specific (anti-HBs/d or y) response, depending on the virus subtype [Milich et al., 1983]. Further work has identified high ($H\text{-}2^{d,q}$), intermediate ($H\text{-}2^{a,b,k}$) and nonresponder strains ($H\text{-}2^{f,s}$) [Milich et al., 1984]. It has also been demonstrated that these responses against HBsAg are regulated by at least two immune response genes mapping in the I-A and I-C subregions of H-2 murine locus [Milich et al., 1984]. The immune response gene control of anti-HBs production in vivo, is mediated through the T cell recognition of S protein determinants and Immune response gene encoded molecules on the antigen presenting cells [Milich et al., 1985a].

The major S protein bears B-cell determinants and one of these is located between residues 120-150 in the hydrophillic area of the molecule and represents the HBsAg 'a' epitope [Bhatnagar et al., 1982; Brown et al., 1984; Howard et al., 1988]. As this epitope is common to all HBV subtypes, a subunit vaccine against HBV can protect against infection with all HBV isolates. The vaccine is prepared from plasma of healthy chronic HBV carriers and consists of 22 nm purified HBsAg particles [Szmuness et al., 1980; Maupas et al., 1981;

Zuckerman, 1981]. Although the safety of this first generation vaccine has been demonstrated, alternative methods for vaccine production are needed. This is because of limitations in the availability of human serum and the expensive inactivation and purification procedures involved in the process. The second generation HBV vaccines have been prepared by recombinant DNA techniques in yeast (Saccharomyces cerevisae) and have been found to be safe and immunogenic and protect against HBV infection [Valenzuela et al., 1982; Miyanohara, 1983]. Although the currently licenced vaccines are safe and efficacious, attempts to prepare less expensive and more immunogenic vaccines are continuously being explored. This includes recombinant vaccinia viruses, use of hybrid surface and core antigen particles and chemically synthesized polypeptide vaccines [reviwed by Zuckerman and Harrison, 1990].

Some reports have described the presence of an HBV surface variant that has a single amino acid change at residue 145 of the S gene. This has resulted in a change of amino acid glycine to arginine in the 'a' determinant of HBsAg [Carman et al., 1990; Harrison et al., 1991; McMahon et al., 1992]. As a consequence, this mutant cannot be neutralized by anti-HBs response produced by immunization, and has implications regarding the design of future HBV vaccines.

1.A.5.b.(4). Immune Response to preS Proteins

In an uncomplicated acute HBV infection, the appearance of preS antibodies (anti-preS1 and anti-preS2) has been considered to be a marker of HBV clearance and recovery [Budkowska et al., 1986; Alberti et al., 1988] and absence of the preS antibodies and persistence of preS antigens has been associated with evolution of chronic liver disease [Budkowska et al., 1986].

In acute, self limited infection, anti-preS antibodies display a biphasic pattern [Budkowska et al., 1990]. The early antibodies are detected at the time of clinical signs of

acute disease when HBsAg and HBeAg are present but HBV DNA is not detectable in the serum. Anti-preS levels fall and then rise as late antibody is made during the recovery phase, after development of anti-HBe but before anti-HBs [Budkowska et al., 1990]. However, an anti-preS response has also been detected in some patients developing chronic liver disease. This phenomenon has been explained by a suggestion that anti-preS antibodies may be continuously produced during chronic HBV infection but remain undetectable in individuals with high levels of viraemia [Budkowska et al., 1992]. Therefore, the appearance of anti-preS antibodies may be the result of decreased levels of virion in the serum or alternatively a sign of transient improvement in antiviral immunity [Budkowska et al., 1992]. The anti-preS responses, when complemented by adequate T-cell immunity, can lead to viral clearance and resolution of liver disease [Budkowska et al., 1990].

The preS2 region is more immunogenic than the S region of HBsAg as has been shown by, greater magnitude of antibody response, minimum antigen immunizing dose for T cell activation and earlier onset of antibody production in vivo [Milich et al., 1985b]. In mice, immunization with HBsAg/gp33 induces antibody production which is also H-2 restricted but the hierarchy of response differs from that to the S region [Milich et al., 1985b]. By using synthetic peptides derived from the preS2 region and denoting the amino terminus of the preS2 region as residue 120, it has been shown that synthetic peptide p120-145 can bind human antibodies produced during HBV infection [Neurath et al., 1984]. Additionally, immunization with HBsAg/gp33 can bypass nonresponse to the HBsAg/p24 in nonresponder mouse strains. This suggests that although the nonresponder strains lack an S-specific T cell response, they must possess preS-specific T cells that can help B cell clones specific for S as well as preS region determinants [Milich, 1988].

The carboxyl terminal region of preS2 has at least 17 T cell recognition sites, concentrated in the region represented by residues 148-174 of both the d and y subtypes, and the relative abundance of these may explain the enhanced

immunogenicity of the preS2 region compared to the S region [Milich et al., 1990]. This region may not be the universal T cell recognition site but the short 27 residue region has been recognized by T cells of eight H-2 strains studied so far [Milich et al., 1990]. It can be inferred from this that the same region will be the T cell recognition site in the human immune response, corresponding to the MHC phenotype of the individual. In addition, this preS2 region [148-174] may also be used as a synthetic carrier for non-HBV B cell epitopes, thus providing T helper cell functions.

The immune responsiveness of the preS1 region has also been examined in detail in the murine model. The immune response to HBsAg/p39 is dependent on H-2 linked genes, but again the hierarchy of response is different from S and preS1 regions [Milich et al., 1986]. Immunization with HBsAg/p39 not only produces anti-preS1 antibodies but also elicits the production of anti-preS2 and anti-S antibodies in all strains including the S and preS2 nonresponder strains [Milich et al., 1986]. This suggests that T_H cells recognizing a determinant on one region can provide help to one or multiple B cell clones recognizing a determinant on another region [Milich, 1988]. A number of overlapping preS1 specific B-cell epitopes have been recognized by human and murine sera [Neurath et al., 1985; Milich et al., 1987c].

It has been shown that, during acute hepatitis B infection, {denoting the amino terminus of preS1 as amino acid 1} 28% of patients produce anti-(21-32) and 47% produce anti-(32-47) antibodies. Antibodies with these two anti-preS1 specificities are present in 50% of patients who become virus free and behave as virus neutralizing antibodies. On the other hand, 22% of patients with chronic hepatitis have anti-(94-117) antibody which does not behave as virus neutralizing. This shows that human anti-preS1 response contains several neutralizing and non-neutralizing specificities [Alberti et al., 1990]. On the basis of these findings, it has been suggested that the preS1 region which contains a binding site for the hepatocyte membrane [Neurath et al., 1986], is capable of eliciting an early antibody

response in acute hepatitis and a defect in preS1 neutralizing antibody production may play a role in the process of chronicity as a result of continuous reinfection of hepatocytes by circulating virions [Alberti et al., 1990].

I.A.5.c. Polymerase Protein

The overlapping coding regions for P and C genes are represented in a single pregenomic mRNA but have different reading frames. A similar situation exists in retroviruses in which a polymerase-nucleocapsid fusion protein is formed by ribosomal frameshift or read through during translation of the overlapping region. On the other hand, hepadnaviruses do not use this mechanism but instead initiate translation at an internal AUG codon [Chang et al., 1989].

The largest open reading frame of HBV has been assumed to encode the viral DNA polymerse [Pasek et al., 1979] and contains regions which resemble those of the retroviral reverse transcriptases of the Rous sarcoma virus and Moloney murine leukaemia virus [Toh et al., 1983]. Additionally, sequence similarities between retroviruses RNase H encoding region and P region encoded sequences, have been reported [Schödel et al., 1988]. Antibody to synthetic peptides with sequences derived from P region, have been shown to immunoprecipitate the reverse transcriptase activity found in detergent disrupted HBV virions [Bavand et al., 1989]. The P region may also encode the protein primer linked to the 5' end of the minus DNA strand, as antibody to a synthetic peptide corresponding to the amino terminal P region of DHBV, has been shown to react with this protein primer [Bosh et al., 1988].

If the P ORF is transcribed and translated in its entirety, a 90-92 kD polypeptide would result but Mack et al., [1988] have identified a polypeptide with a molecular mass of 65 kD by immunoblotting, in HBV particles. It has been suggested that the primary translation product is either proteolytically cleaved or translated from a spliced mRNA [Mack et al., 1988]. The expression of the P region product

in HepG2 cells has identified a polypeptide of 90 kD, which has four domains [Radziwill et al., 1990]. Domain I in the amino terminal end encodes the terminal protein primer whereas domain II is non-functional and acts as spacer. Domain III encodes the viral DNA polymerase/reverse transcriptase and domain IV encodes the RNase H activity [Radziwill et al., 1990]. It has been shown that the P region product containing all functional domains is required for encapsidation of pregenomic RNA [Bartenschlager et al., 1990]. A recombinant baculovirus containing the HBV genome has been shown to express two proteins of 93 and 72 kD, recognized by an anti-polymerase monoclonal antibody [McGlynn et al., 1992]. It has been proposed that the 93 kD protein is the terminal genome-linked protein, whereas the 72 kD protein is possibly the reverse transcriptase [McGlynn et al., 1992].

An immune response has been detected against the RNase H domain of the P protein, in 73% and 87% of the patients with acute and chronic HBV infection, respectively [Weimer et al., 1989]. This antibody response is detected early in infection at the time of virus replication and declines with virus clearance [Weimer et al., 1990].

I.A.5.d. x Protein/HBxAg

Candidate mRNAs for the synthesis of x protein have not been detected in HBV infected liver tissue but a 0.7 kb transcript has been detected in cells containing HBV sequences in an adenovirus recombinant [Saito et al., 1986]. Although the x protein can be translated from any of the HBV mRNAs, an X region specific transcript of 0.65 kb has been detected in the nucleus of liver cells infected with WHV [Kaneko and Miller, 1988]. In addition, a 0.9 kb RNA species has also been detected in the nucleus. As this transcript represents less than 1% of the total virus specific RNA, it is possible that the X gene promoter sequence taken out of its natural location can produce large amount of the X transcript but

during active virus replication it is under stringent control [Kaneko and Miller, 1988].

The fourth and the smallest (X) ORF encodes the smallest protein of 154 amino acids. The HBxAg can activate transcription which is under the control of heterologous enhancer or regulatory sequences of human ß-interferon gene [Twu and Schloemer, 1987], human immunodeficiency virus type 1 (HIV-1) [Seto et al., 1988; Twu and Robinson, 1989; Twu et al., 1989a and b], simian virus 40 (SV40) [Twu and Robinson, 1989; Spandau and Lee, 1988] and Rous sarcoma viruses [Spandau and Lee, 1988]. Study of the HBxAg responsive element has indicated that it may trans-activate viral and cellular genes through transcriptional regulatory factors like NF-kB, AP-1 and AP-2 [Twu et al., 1989a; Seto et al., 1990]. Viruses such as HIV-1 and SV40 contain transcriptional enhancer sequences (kB-like sequences) that bind and respond to NF-kB factors but none of the genes of these viruses can encode or activate these factors [Twu et al., 1989a and b]. It has now been shown that HIV-1 kB-like sequence is activated by HBxAg and this suggests that the protein might play a role in regulating transcription of a gene under the control of kB-like enhancer during HBV infection [Twu et al., 1989a and b]. These findings also raise the possibility that HBV could activate HIV-1 replication in cells infected with both HBV and HIV-1 [Seto et al., 1988; Twu et al., 1989a].

The heterogeneity of transcriptional regulatory elements which are responsive to HBxAg trans-activation, suggest that the protein may act by a general mechanism that is not sequence specific [Wu et al., 1990]. This notion may be supported by findings that the HBxAg possesses a serine/threonine protein kinase activity which appears to be associated with transcriptional activation by the x protein. As the protein kinase domain bears no homology with the known protein kinases it could be an example of a novel protein kinase [Wu et al., 1990].

Deletions, frameshift mutations and point mutations have been introduced into the X ORF. Three regions of HBxAg have been identified, which are essential for trans-activating

function. These are amino acid residues 46-52, 61-69 and 132-139 and all three regions are conserved among hepadnavirus x proteins [Arii et al., 1992]. Two groups of residues (61-69 and 132-139) have been shown to be homologous to the serine protease inhibitors and amino acid substitutions in these cause a significant reduction in trans-activating function of the x protein [Arii et al., 1992]. However, amino acids 5-27 and last 12 amino acids in the carboxyl terminal region make no contribution to trans-activation.

The HBxAg is expressed in the livers of some patients infected with HBV [Moriarty et al., 1985] and antibody reactive to synthetic peptides representing sequences from the x protein, have detected the protein in HBV infected livers and in hepatocellular carcinoma tissues [Kay et al., 1985; Moriarty et al., 1985]. But the amount of x protein produced during HBV infection is significantly less than the core and the surface antigens [Moriarty et al., 1985; Kay et al., 1985].

The x protein is considered to be immunogenic as anti-x response has been detected in serum of some HBV infected patients [Kay et al., 1985].

I.A.6. HBV Transcription

Four different classes of mRNAs have been identified in HBV infected tissues so far and experimental evidence indicates that the transcripts are produced using the host cell transcription unit [Will et al., 1987; Chisari et al., 1989]. The transcripts are unidirectional, overlapping and have heterogenous 5' ends. All the transcripts have the same 3' ends and terminate at the same polyadenylation site [Will et al., 1987; Ganem and Varmus, 1987; Kaneko and Miller, 1988; Chisari et al., 1989]. These are 3.5 (discussed below), 2.4 (see section I.A.5.b), 2.1 (section I.A.5.b) and 0.9 kb (section I.A.5.d) RNAs.

The 3.5 kb RNAs function as templates for the synthesis of viral DNA (pregenomic RNA) and also serve as mRNAs for P

and C gene products [Enders et al., 1985]. The 5' ends of 3.5 kb transcripts are heterogenous and it has been oberved that the shortest molecules serve as templates for reverse transcription whereas the other transcripts serve as mRNAs for P and C gene products [Enders et al., 1987; Will et al., 1987]. The synthesis of both types of RNAs is under the control of C promoter [Rall et al., 1983] which is located within 150 nucleotides upstream of the RNA start site [Yaginuma and Koike, 1989]. The 5' end of the pregenomic RNA is located 5-6 bp downstream of the preC initiation codon in HBV and GSHV [Enders et al., 1985; Will et al., 1987]. The RNA species span the full genome length and have between 120 [Will et al., 1987] and 160 [Enders et al., 1985] terminally redundant nucleotides. The presence of terminal redundancy indicates that during the synthesis of these transcripts, RNA polymerase II must read through the polyadenylation site (transcription termination sequence) that is located within the nucleocapsid ORF, in order to produce these transcripts. If polyadenylation occurs on the first pass then this would result in very short transcripts and these have not been detected in HBV infected tissues [Enders et al., 1985]. This also points out that template for the transcript appears to be the closed circular DNA which would allow the synthesis of a transcript with a terminal redundancy [Ganem and Varmus, 1987]. As the closed circular DNA is the first form of DNA detected after infection and the only form found in the nucleus, it is the best candidate for viral transcription during virus replication [Tuttleman et al., 1986a and b].

I.A.6.a. Regulation of Transcription

Genetic elements that play a role in regulation of HBV transcription include promoters, enhancers, GRE sequences, some viral gene products and cellular factors.

The activities of HBV promoters have been investigated by linking specific regions of the HBV genome containing promoter sequences [Antonucci and Rutter, 1989] or the entire HBV

genome [Raney et al., 1990] to a reporter gene, for example, CAT or firefly luciferase (LUC) genes, and transfecting and expressing the constructs in cultured cells.

The cell type specificity of the four HBV promoters has been examined in context of the complete viral genome in differentiated hepatoma cell lines (HepG2, Hep3B and PLC/PRF/5), a dedifferentiated hepatoma cell line (HepG2.1) and non-hepatoma cell lines (Hela S3 and NIH 3T3) [Raney et al., 1990]. It has been shown that the S and X promoters are the strongest in all the cell lines examined, the C promoter has strong activity only in differentiated hepatoma cell lines whereas preS1 promoter has weakest activity (X=S>C>preS1) [Raney et al., 1990]. In another experimental system, which has not utilized complete HBV genome, a different order of promoter strength has been observed (C>X>S>preS1) [Antonucci and Rutter, 1989]. These two orders are significantly different from the relative amounts of transcripts observed in vivo (S>C>preS1>X) [Will et al., 1987] (for a general description of promoters, see section I.A.4.a).

The increased activity of HBV promoters in the presence of enhancer sequences suggests a pivotal role for these sequences in HBV transcription and has been described above (section I.A.4.b) [Shaul et al., 1985; Antonucci and Rutter, 1989; Honigwachs et al., 1989]. The GRE sequence has also been shown to regulate the transcriptional activity of HBV genes and has been described (section I.A.4.c) [Tur-kaspa et al., 1986 and 1988].

The transcriptional trans-activating property of the X gene product has been discussed in section I.A.5.d. A protein synthesized from 3' truncated preS2/S sequences integrated in a human hepatoma cell line (huH-4) can also act as a transcriptional trans-activator [Kekule et al., 1990]. As the full length preS2/S gene does not show trans-activation activity, it has been postulated that the rearrangements during viral DNA integration lead to generation of carboxyl terminal truncated preS2/S gene products that have the trans-activation potential [Kekule et al., 1990].

Cellular factors or DNA binding proteins are involved in

regulation of HBV transcription and several that bind to specific sequences in the HBV genome have been described [Ben-Levy et al., 1989; Yaginuma and Kioke, 1989; Raney et al., 1990]. It has been observed that the activity of the preS1 promoter is significantly higher in differentiated hepatoma cells than dedifferentiated cells. There is a specific sequence element in the promoter region, which binds the liver specific transcription factor called hepatocyte nuclear factor 1 (HNF1) and it has been suggested that the dedifferentiated cells lack the HNF1 polypeptide resulting in reduced transcription from this promoter [Raney et al., 1990]. The enhancer/X promoter region contains several binding sites for cellular factors [Ben-Levy et al., 1989] and the C promoter has been shown to contain a region that interacts with a binding factor present in cell extracts of differentiated hepatoma cells [Yaginuma and Kioke, 1989].

I.A.7. HBV Replication

Studies of hepadnavirus genome replication have been made difficult without the availability of cell culture systems and the present understanding of the process is based upon studies carried out on the liver tissue of infected animals. The development of fetal duck liver cell culture system that can be infected with DHBV has been an important advance for studies of virus replication [Tuttleman et al., 1986a].

I.A.7.a. Virus Entry

The major target organ for HBV is liver. However, cells other than hepatocytes, such as peripheral blood lymphocytes and monocytes, may become infected with the virus. The cell receptor binding site has been assigned to the preS1 region (amino acids 21-47) of the HBV envelope protein [Neurath et al., 1986]. A search for the cell receptor for HBV has revealed that Interleukin-6 (IL-6) contains recognition sites

for the preS sequence and mediates HBV-cellular interactions [Neurath et al., 1992]. Thus, HBV belongs to a group of viruses that utilize cytokines or cytokine receptors for replication.

I.A.7.b. Covalently Closed Circular DNA

The appearance of covalently closed circular (CCC) DNA in the nuclei of liver cells within 6-16 hours of infection with DHBV, is one of the earliest events in replication of hepadnaviruses [Tagawa et al., 1986]. As the synthesis of this DNA species precedes the expression of other viral RNA and DNA intermediates, it is believed that the virion DNA is directly converted into CCC DNA [Miller and Robinson, 1984; Tagawa et al., 1986]. This conversion must involve 1) completion of the plus strand of the open circular form of the virion DNA, 2) removal of the terminal protein covalently attached to the 5' end of minus strand and oligoribonucleotide primer on the 5' end of the plus strand, 3) removal of the terminal redundancy of the minus strand and 4) ligation between the ends of each DNA strand. The exact mechanism of how these reactions are carried out is not clear yet.

In vitro, infection of uninfected hepatocyte cultures with DHBV has shown that a 50 fold amplification of CCC DNA occurs during an early stage in infection before virus production [Tuttleman et al., 1986b]. Amplification produces a pool of transcriptional templates in the cell without the need for semiconservative replication or multiple rounds of infection [Tuttleman et al., 1986b]. The data suggest that this amplification is through an intracellular pathway prior to virus release rather than through reinfection of cells by progeny virus [Tuttleman et al., 1986b]. In persistently infected hepatocytes in vivo, CCC DNA is limited to low copy numbers suggesting that amplification during natural infection is limited by a specific control mechanism. The process of amplification may account for the ability of hepadnavirus infected cells to produce virus particles in the absence of

integrated viral DNA [Tuttleman et al., 1986b].

I.A.7.c. Packaging of Pregenomic RNA

Selective encapsidation of hepadnaviral pregenomic RNA into nucleocapsids requires specific interaction between the core protein, P gene product and pregenomic RNA. In addition, encapsidation has been shown to be a selective process using only the shortest of the 3.5 kb transcripts which are packaged into core particles [Enders et al., 1987; Lavine et al., 1989]. It now appears that the packaging of longer transcripts may be suppressed as a consequence of the translation of the preC gene. HBV and WHV variants with mutations in the preC initiation codon allow packaging of longer 3.5 kb mRNAs suggesting that translational usage of RNA can determine whether it is packaged into nucleocapsids [Junker et al., 1987].

A signal consisting of 137 nt has been identified near the 5' end of the pregenomic RNA and has been termed encapsidation sequence or ϵ [Junker-Niepmann et al., 1990]. The sequence is considered sufficient and essential for RNA encapsidation and is highly conserved between mammalian hepadnaviruses, particularly the first 80 nt. Fusion of the encapsidation sequence to a foreign RNA also results in its encapsidation into core particles suggesting that RNA with this sequence would be packaged [Junker-Niepmann et al., 1990]. In the larger, preC mRNAs the signal is present but is made inactive by some unexplained mechanism. The distinguishing feature must be present in short 5' terminal extension present in preC mRNAs. The translational start site for the preC mRNA is about 40 nucleotide upstream of the encapsidation signal and it is suggested that the proteins bound to this signal in a preassembly complex would not interfere with translation. But on the other hand, ribosomes involved in translation of the preC region could encounter and presumably destroy the preassembly complex preventing encapsidation of preC mRNA [Nassal et al., 1990; Junker-

Niepmann et al., 1990]. Therefore, translational inactivation of the encapsidation signal by ribosomes on the preC mRNA would allow hepadnaviruses to make use of the short genomic transcripts both for translation and as a pregenome and at the same time would allow the exclusive use of preC mRNA to be used for HBeAg translation [Nassal et al., 1990].

The polymerase gene product also plays an essential role in the encapsidation reaction, as results obtained with point mutations, deletions, and insertions throughout the P gene demonstrate that all functional domains are required for encapsidation [Bartenschlager et al., 1990]. No known enzymatic activities are involved in the process suggesting that the P protein is required as a structural component [Bartenschlager et al., 1990]. A single missense mutation occurring naturally in the in the carboxyl terminus of the P gene product in DHBV, presumably in the RNase H domain, results in a packaging deficiency. This supports the view that multiple regions of HBV polymerase protein are required for RNA packaging [Chen et al., 1992].

I.A.7.d. Synthesis of Minus Strand DNA (Reverse Transcription)

The pregenomic RNA is the template for the synthesis of minus strand DNA. The synthesis is probably initiated within the DR1 sequence at the 3' end of the pregenomic RNA's terminal redundancy (termed R, see fig 4). The synthesis proceeds to the 5' end of the RNA template resulting in a minus strand DNA with a 9-10 nt terminal redundancy (termed r, see fig 4) [Seeger et al., 1986; Will et al., 1987]. It is possible that the initiation of minus strand DNA could occur at the 5' end of the RNA template but this would require a template switch to the 3' end of the same or another RNA molecule [Ganem and Varmus, 1987].

The mechanism by which minus strand synthesis is primed is unclear. A terminal protein (TP) attached by a covalent linkage to the 5' end of the nascent minus strands, has been

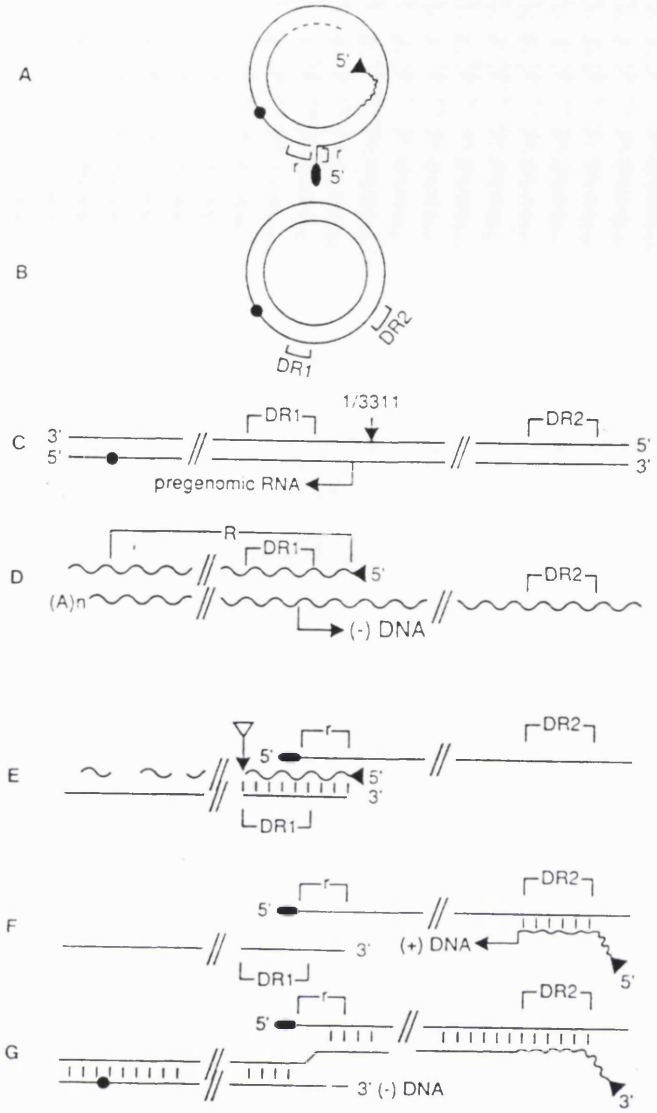


FIG 4: Replication strategy of HBV.

Relaxed circular virion DNA (A) is converted into CCC DNA (B), during a new round of infection. The CCC DNA has a copy each of DR1 and DR2 (C), that serves as the template for pregenomic RNA. The pregenomic RNA (wavy lines) initiates upstream of DR1 and terminates at the polyadenylation signal (represented by a solid circle) downstream of DR1, thus creating terminal redundancy 'R'. The cap structure at the 5' end of the RNA is represented by a solid triangle (D). The minus strand DNA synthesis begins at the copy of DR1 close to the 3' end of the pregenomic RNA (D) and proceeds to the 5' end of RNA template, producing a terminal redundancy 'r'. The terminal protein is depicted as a solid oval symbol. The RNA is degraded by RNAaseH activity (E). The plus strand DNA synthesis initiates downstream of DR2 and is primed by oligoribonucleotide at the 5' end of pregenomic RNA (F). Plus strand synthesis proceeds to the 5' end of the minus strand, where a template switch occurs to the 3' end of the minus strand. The template switch is facilitated by 'r' region at the 5' and 3' ends of the minus strand (G). Consequently, a relaxed circular configuration of DNA results (A) [from Seeger et al., 1991].

isolated from core particles in DHBV-infected livers and it has therefore been suggested that this protein primes the synthesis of minus DNA strands [Gerlich and Robinson, 1980; Molnar-Kimber et al., 1983]. During minus strand DNA synthesis, the RNA in RNA-DNA hybrids is progressively hydrolysed by an RNase H-like activity. This notion has been supported by construction of HBV variants with mutations in the P gene, which interfere with RNase H activity [Radziwill et al., 1990].

I.A.7.e. Synthesis of Plus Strand DNA

The 5' end of the plus strand is a 17-18 nucleotide RNA oligomer whcih is derived from the 5' terminal fragment of the RNA pregenome and contains the DR1 sequence [Lien et al., 1986; Seeger et al., 1986]. The initiation site for synthesis of the plus strand has been mapped to the first nucleotide downstream of DR2, and synthesis proceeds to the 5' end of the minus strand [Seeger et al., 1986; Will et al., 1987]. This suggests that the RNA fragment containing the primer is dissociated from the completed 3' end of the minus strand and transported to the 5' end of the minus strand where it base pairs with the DR2 sequence for initiation of plus strand synthesis. This model has been confirmed by the findings of Seeger et al., [1986] who have introduced mutations into the DR sequences and shown that a mutation in DR1 leads to expression of viral genomes which carry the same mutation in the RNA primer attached to the plus strand DNA. When the 3' end of the elongating plus strand reaches the 5' end of the minus strand template, there is a template switch to the 3' end of the minus strand. As a consequence, the 3' end of the plus strand, after dissociation from the minus strand, contains a sequence complementary to the terminally redundant segment 'r' of the minus strand (fig 4). This sequence in the plus strand base pairs with the 'r' at the 3' end of the minus strand and helps in recirculariztion of the molecule and continued elongation of the plus strand [Seeger et al., 1986;

Ganem and Varmus, 1987; Will et al., 1987] (fig 4).

I.A.7.f. Virus Assembly and Release

These processes are not well characterized for HBV assembly but evidence obtained from biogenesis of 22 nm subviral particles {discussed in section I.A.5.b.(1)} has thrown light on the formation of HBV particles [Eble et al., 1986 and 1987].

preS1 proteins are essential components of HBV particles and filaments but not of HBsAg 22 nm particles [Heermann et al., 1984]. It has been shown that HBV mutants affecting myristylation of preS1 proteins are competent for virion assembly and secretion but it is not possible to test their infectivity [Bruss and Ganem, 1991b]. On the other hand, similar DHBV mutants are not infectious in the animal host suggesting that myristylation might play an important role in adsorption, entry and uncoating of virion [Bruss and Ganem, 1991b; Ganem, 1991]. Based on these observations, it is proposed that the preS1 protein anchored in the membrane might interact with the nucleocapsids and result in a conformational change in the protein that is important for this process.

I.A.8. Pathological Effects of HBV Infection

I.A.8.a. Hepatocellular Injury

Hepadnavirus infection may cause a variety of clinical syndromes with or without underlying hepatic disease to a carrier state or to a more severe form of acute hepatitis that can rarely become fulminant. The spectrum can give rise to cirrhosis and chronic hepatitis that may be self limited or progressive and occasionally fatal [Robbins et al., 1985].

Clinical manifestations of acute hepatitis B are determined by the immunological response of the host, as the virus itself is not cytopathogenic. In immunocompetent

individuals, the immune response to infection results in liver cell necrosis followed by an inflammatory response, thus producing the clinical picture of acute hepatitis. Virus interaction with the hepatocyte cell membrane results in internalization and replication. Viral proteins are expressed at the surface of hepatocyte membrane, thus initiating immune reaction which is vital for elimination of the virus. T lymphocytes also attack infected hepatocytes by interaction with specific HLA class I antigens and viral proteins (HBcAg and HBeAg) expressed at the cell surface. Consequently, with cytolysis, the intracellular virus particles are released and are neutralized by circulating antibodies before being taken up and destroyed by macrophages. Histologically, necrosis and panlobular infiltration with lymphocytes and monocytes is accompanied by elevation of serum aminotransferases. Occasionally, during acute viral hepatitis, more extensive necrosis of the liver leading to severe impairment of hepatic function produces fulminant hepatitis [Robbins et al., 1985; Pignatelli et al., 1987; Walter and Israel, 1988; Hollinger, 1990].

If virus elimination cannot be achieved by these mechanisms, and virus replication persists, chronic liver disease follows acute viral hepatitis in about 2-10% of immunocompetent adult hepatitis B patients. The course of chronic liver disease is not uniform and may remain quiescent for years or progress to cirrhosis and death within a short period of time. The histologic classification consists of chronic persistent hepatitis, chronic active hepatitis and chronic lobular hepatitis with or without cirrhosis. However, in newborns who have been infected perinatally, up to 98% can follow the chronic course but most of these patients remain asymptomatic [Robbins et al., 1985; Hollinger, 1990; Zuckerman and Harrison, 1990].

I.A.8.b. Hepatocellular Carcinoma (HCC)

I.A.8.b.(1). Epidemiological Correlation

An association of viral hepatitis and HCC was postulated before the identification of any of the viruses causing viral hepatitis [Steiner and Davies, 1957]. Although HCC is an unusual malignancy in many parts of the world, it occurs commonly in sub-Saharan Africa, southeast and eastern Asia, Greece and Italy [Szmuness, 1978]. Geographic areas with the highest incidence are also the areas where HBV infection is common and the annual incidence of HCC correlates with HBV carrier rate of the region. A prospective study of more than 22,000 male civil servants in Taiwan, has shown that the incidence of HCC is about 200 times higher in HBsAg positive individuals than HBsAg negative controls [Beasley et al., 1981; Beasley and Hwang, 1990].

There is a stronger association of HCC with other mammalian hepadnaviruses such as WHV [Popper et al., 1987]. In one study, more than 90% of woodchucks experimentally infected at birth with WHV and maintained in captivity, have developed HCC after 2-3 years of persistent infection and this is the highest reported incidence of HCC following insult with a carcinogenic agent. Analysis of food provided to the woodchuck colony has not revealed presence of any aflatoxin or other carcinogens. Liver histology in these animals shows a picture of active hepatitis with inflammation and regeneration but no cirrhosis [Popper et al., 1987].

I.A.8.b.(2). Integration of HBV DNA and Topoisomerase I

The first documentation of presence of integrated HBV DNA has been in the continuously growing cell line PLC/PRF/5, established from HCC tissue of an individual positive for HBsAg [Alexander et al., 1976]. Since this report, about 40 integrations have been studied with the observation that the pattern of integration is heterogeneous and virus-cell

junctions are scattered throughout the cellular genome. In 18 out of 40 integrations, however the viral integration site has been in the region between DR1 and DR2 suggesting that the properties of this region make it 'recombination proficient' [Dejean et al., 1984; reviewed by Sherker and Marion, 1991]. It has been proposed that the presence of an unpaired DNA strand near DR1 [Shih et al., 1987] or the presence of linear DNA molecules containing activated ends, may help in integration of DNA [Nagaya et al., 1987; Tokino et al., 1987].

Since hepadnaviruses do not contain an integrase gene, cellular enzymes would be needed for viral integration. Topoisomerase I (topo I), is a nuclear enzyme which allows movement of transcription and replication complexes along DNA. As a part of this mechanism, it generates transient nicks in genomic DNA and may remain covalently bound to DNA via 3' phosphoryl bond while the DNA swivels [Champoux, 1977]. If a 5'OH acceptor end is provided, these 3' bound topo I molecules are capable of linking heterologous DNA fragments [Halligan et al., 1982]. This mechanism may be involved in some cases of viral integration as several cleavage sites for the enzyme have been identified in the DR1 and DR2 region of HBV and WHV [reviewed by Rogler, 1991]. However, as majority of virus-cell junctions do not contain the DR1 and DR2 region, other mechanisms of integration would exist as well.

The pattern of integration in individual HCC's is generally identical within the tumour from cell to cell, indicating a clonal origin but more than one type of clonal tumour may coexist in the same liver [Chen et al., 1989]. Recurrent HCC's may show the original integration pattern or develop a new one [Chen et al., 1989].

I.A.8.b.(3). Insertional Mutagenesis

The discovery of integrated hepadnaviral DNA in cellular genes has led to speculation that integration could be responsible for activation of cellular protooncogenes resulting in hepatocellular transformation [Dejean et al.,

1986; De The et al, 1987; Wang et al., 1990].

Analysis of an integration in a human HCC has shown interruption of the coding region of a previously unidentified gene bearing resemblance to DNA-binding domain of steroid and estrogen hormone receptor genes and v-erb-A oncogene families [Dejean et al., 1986]. Another integration in the intron of cyclin A gene has resulted in enhanced expression of this gene [Wang et al., 1990]. Thus, HBV insertion in genes which are essential for cell growth and differentiation, suggest that HBV may act occasionally as an insertional mutagen. Evidence supporting this view has also come from woodchucks infected with WHV, as activation of myc oncogene family (c-myc and n-myc) has occurred in one third of infected woodchucks leading to HCCs [Moroy et al., 1986; Hsu et al., 1988; Fourel et al., 1990]. In one HCC, WHV sequences containing an enhancer element have integrated in the untranslated exon 3 of the c-myc gene about 90 bp downstream of gene coding sequences. This has resulted in enhanced expression of the c-myc viral cotranscript, under the control of c-myc promoter [Hsu et al., 1988]. In a second WHV HCC, rearranged viral sequences have integrated 621 bp upstream of c-myc exon 1, producing high levels of normal c-myc mRNA. In both cases, viral enhancer insertion and disruption of normal transcription or posttranslational control of c-myc genes appears to be involved in c-myc activation and possibly contributes to genesis of HCC [Hsu et al., 1988]. WHV DNA integration can occur in two n-myc genes (n-myc1 and n-myc2) in about 20% of WHV HCCs [Fourel et al., 1990]. This results in production of chimeric mRNAs in which the 3' untranslated region of n-myc is replaced by WHV sequences containing the viral enhancer. These results suggest that activation of myc genes by insertion of hepadnaviral DNA is now emerging as a common event leading to WHV HCCs [Fourel et al., 1990]. But except for myc gene activation by WHV, viral integrations have not been found to play a causal role in generation of other hepadnavirus associated HCCs.

I.A.8.b.(4). Chromosomal Deletion and Rearrangement at Viral Integration Site

Four different patterns of hepadnavirus DNA integration have been proposed, depending on the end specificity and strand polarity [Shih et al., 1987]. These four groups include integration with one virus-cell junction at DR1, with viral DNA either extending downstream into C gene (group I) or upstream into X gene (group II) or similar integrations with one virus-cell junction at DR2 with viral sequences extending into the C gene (group III) or X gene (group IV). Therefore, the preferred sites for virus-virus DNA rearrangements and virus-cellular junction are the DR sequences and the cohesive overlap region [Shih et al., 1987; Tokino et al., 1987]. Out of four integrants analyzed by one group, two have been found to have large inverted repeating structures, each consisting of the HBV genome along with flanking cellular sequences [Tokino et al., 1987]. It was suggested that these structures must have arisen by duplication of the primary integrant including the flanking cellular DNA, followed by recombination within the viral DNA. In two other integrants, HBV sequences are flanked by cellular DNA from two different chromosomes and these must be the products of cellular DNA translocation using the integrated HBV DNA as the switch point [Tokino et al., 1987]. Another group has described an HBV induced translocation in a single integration HCC, in chromosomes 17 and 18 [Hino et al., 1986]. Deletions of cellular DNA have also been found to occur at most hepadnavirus integration sites. Out of 14 integrations analyzed, microdeletions in cellular DNA have been observed in 11 whereas 2 show macrodeletions suggesting that integration can result in chromosomal defects [reviewed by Rogler, 1991].

I.A.8.b.(5). Role of Hepadnavirus Proteins in HCC

The function of X protein as a transcriptional transactivator of hepadnavirus and heterologous promoters and

other functions which could play a role in hepatocarcinogenesis have been discussed above [section I.A.5.d].

If HBV X-dependent transactivation is a general mechanism of hepadnavirus-associated oncogenesis, most integrated DNA sequences should contain the X gene and show a transactivating function. The X gene is present, and more importantly, expressed in at least 80-90% of integrants [Wollersheim et al., 1988]. In some of these cases the X gene is expressed with flanking cellular sequences as a fusion protein and these X-cellular fusion proteins have been shown to maintain their transactivating potential in the integrated state [Wollersheim et al., 1988].

The X gene, under its own regulatory elements and HBV enhancer, has been placed in the germline of mice and the transgenic animals have shown progressive histopathological changes in the liver leading to the development of malignant carcinoma, within 8-10 months [Kim et al., 1991]. This suggests that the X protein, acting as a transcriptional transactivator of viral genes may also be involved in the alteration of host gene expression and development of hepatocellular carcinoma [Kim et al., 1991]. However, in another report, transgenic mice carrying the X ORF under the control of human alpha 1 antitrypsin regulatory region have remained normal for up to two years of observation period [Lee et al., 1990].

But not all HCCs seem to harbour a functional X ORF. On the other hand it has been noticed that PreS/S is the only gene which has been found integrated in almost every HBV related HCC analyzed so far [Kekule et al., 1990]. A transcriptional transactivator activity has been identified in the 3' truncated preS2/S region of an integrated HBV DNA obtained from a human hepatoma cell line huH-4. It has been shown that 3' truncated sequence in the integrated form can stimulate c-myc gene P2 promoter in trans, as c-myc is expressed by huH-4 cell line. This suggests that accidental 3' truncations of preS2/S gene could be a causative factor in hepatocarcinogenesis [Kekule et al., 1990].

I.A.9. Perspectives

HBV infection is considered to be one of the most important chronic virus infections in the world which has also been implicated in the development of HCC. Effective strategies for its eradication have been employed including vaccination. The experience with first and second generation of HBV vaccines has not only proved that they are effective and safe but have also emphasized the potential importance of particulate form of HBsAg. This has encouraged the use of HBsAg particle for presentation of the foreign epitopes to the immune system. Based on the evidence obtained from this system, HBcAg has also been used for presentation of epitopes from other viruses and it is more effective than HBsAg in this aspect. This system of epitope presentation is now being developed with fruitful results.

I.B. CYTOMEGALOVIRUS (CMV)

I.B.1. Introduction

CMV infections were first reported in the early twentieth century when cellular changes in the form of intranuclear inclusions were described in tissues obtained from still born fetuses, and hence the name cytomegalic inclusion disease (CID) was coined even before the causative agent was isolated. The experimental evidence supporting a viral etiology for CID came from studies by Cole and Kuttner [1926], when they showed that guinea pig salivary gland extract was infectious for younger animals, after being passed through a Berkefeld N filter. Following the successful propagation of murine CMV in mouse embryonic fibroblast cultures [Smith, 1954], human CMV (HCMV) was isolated by three laboratories [Smith, 1956; Rowe et al., 1956; Weller et al., 1956]. The term 'cytomegalovirus' was coined by Weller et al., [1956], reflecting the histopathological changes associated with these viruses.

I.B.2. Virus Classification

CMV belongs to the family Herpesviridae and is placed in subfamily of betaherpresviruses [Roizman and Batterson, 1985]. The betaherpesviruses share common molecular and pathogenic characteristics and have two genera: Cytomegalovirus and Muromegalovirus or murine CMV [Roizman and Batterson, 1985].

I.B.3. Virus Morphology

CMV is one of the largest animal viruses with a diameter of 200 nm. The 64 nm core contains the viral DNA, enclosed by 110 nm icosahedral capsid which is made up of 162 capsomeres. The capsid is surrounded by a poorly demarcated area called the tegument [Wright et al., 1964; Alford and Britt, 1985].

I.B.4. Other Forms of the Virus

In addition to the complete infectious viral particle which contains DNA, capsid, matrix protein and envelope, two other forms of the virus are produced when CMV is propagated in cell culture. These are, (i) a non-infectious enveloped particle (NIEP) which is an empty capsid surrounded by a lipid envelope and is similar to the virion in structure and composition and (ii) the dense body which is an amorphous structure without a nucleocapsid or DNA and is composed primarily of protein [Wright et al., 1964; Smith and De Harven, 1973; Smith and De Harven, 1974].

I.B.5. Viral Life Cycle and Growth in vitro

In vitro, human fibroblasts are the only cells that are permissive for CMV replication, allowing the virus to grow slowly in cell cultures. In vivo, CMV can be recovered from infected epithelial and endothelial cells. In vitro studies indicate that CMV penetrates the cell either by fusion of the viral envelope with cell membrane or through phagocytosis [Smith and De Harven, 1974]. Both fusion and phagocytosis occur within few minutes of penetration of the virus after which naked capsids are observed in the cytoplasm of the infected cells. The capsids migrate towards the nucleus and become located near nuclear pores within five minutes of penetration [Smith and De Harven, 1974]. The capsids become coated with a fibrillar material and have a well defined

morphology until the eclipse phase of visible viral particles is observed between 1-1½ days p.i. [Smith and De Harven, 1974].

Viral DNA synthesis is initiated at approximately 15 hours p.i. [Stinski, 1978] and at 72 hours p.i. infectious virus is found intracellularly and is released shortly thereafter [McAllister et al., 1963]. Encapsidation occurs in the nucleus and the virus envelope is acquired by budding through the inner nuclear membrane to the golgi apparatus. Enveloped virions are found in the cytoplasm in vesicles, and latter fuse with cellular membranes to allow the virion to be released. Dense bodies also mature in a similar way in the golgi apparatus and are released from infected cells [Griffiths, 1990].

During late times after infection, CMV also induces the production of IgG-Fc membrane receptors in the cytoplasm of infected fibroblasts [Rahman et al., 1976]. The importance of this to the virus is unknown at the moment but it has been suggested that in vivo this might allow opsonized bacteria to gain access to the cells which they cannot normally infect [Griffiths, 1990].

I.B.6. Genomic Organization

The linear double stranded DNA of 229 kb in length, has a molecular weight of 147×10^6 and is 50% larger than the genome of HSV [Geelen et al., 1978; Alford and Britt, 1985; Chee et al., 1990]. The genome is divided into two segments, designated as short (S) and long (L) comprising 18% and 82% of the genome respectively, by a pair of inverted repeats [DeMarchi, 1981]. Each segment consists of a unique (U) set of sequences, termed U_S and U_L, which are bracketed by inverted repeats (IR_S and IR_L) or terminal repeats (TR_S and TR_L) [Westrate et al., 1980] (see fig 5). As a result of this arrangement both S and L segments can invert relative to one another and can occur in either orientation producing four isomeric conformations of DNA, which are present in equimolar

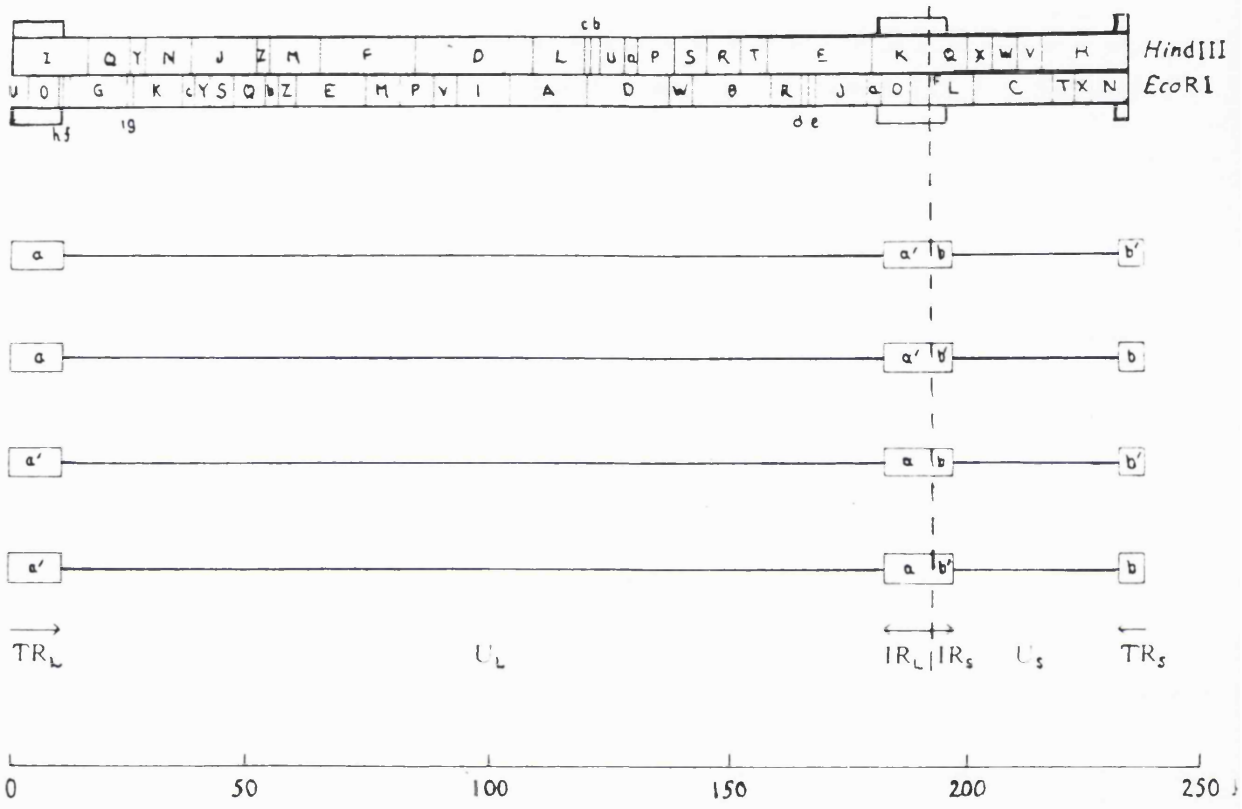


FIG 5: Schematic representation of isomeric forms of CMV DNA.

The restriction maps for the enzymes HindIII and EcoRI are given in the upper section of the diagram. L, long segment; S, short segment; U, unique segment; I, internal repeat; T, terminal repeat [from Mach et al., 1989].

amounts in virion DNA preparations [Westrate et al., 1980; DeMarchi, 1981].

Physical maps of CMV DNA of various laboratory (AD169, Towne, Davis, UW-1) and wild type strains have been constructed by digesting DNA with various restriction enzymes [Westrate et al., 1980; DeMarchi, 1981; Chandler and McDougall, 1986]. Distinct differences in restriction enzyme cleavage patterns are observed, and this restriction site polymorphism can serve as a convenient marker for the identification of different CMV strains [Chandler and McDougall, 1986]. However, despite the presence of restriction enzyme polymorphism, the gross sequence is conserved for CMV strains, as has been shown by identical southern blot hybridization of several clinical isolates, with cloned DNA probes from the laboratory strain AD169 [Chou et al., 1984].

I.B.7. Gene Expression

The replicative cycle has been divided into three interdependent and sequential phases of viral gene expression, based on the synthesis of three different classes of CMV-specific proteins during each phase. These are immediate early (IE) {0-2 hours}, early (E) {2-24 hours} and late (L) {after 24 hours} phases and the resulting proteins are designated as alpha (α), beta (ß) and gamma (γ) respectively [Honess and Roizman, 1974; DeMarchi et al., 1980; DeMarchi, 1981; Rasmussen, 1990]. The α proteins control the transcription of mRNA for the second group of ß proteins, which in turn allow the DNA synthesis to proceed and also regulate the production of the γ proteins. The proteins synthesized during IE and early phases are generally associated with the regulation of viral replication whereas the late proteins constitute the structural elements of the virus. Specific metabolic inhibitors have been used at various stages of genome expression and have helped in understanding of replication strategy [Honess and Roizman,

1974; Stinski, 1978].

I.B.7.a. IE Phase of Gene Expression

In the IE phase, transcription occurs in the absence of protein synthesis or viral DNA replication. After entry of the viral DNA into permissive cells, IE genes are the first to be transcribed in the absence of protein synthesis as viral mRNA can be synthesized in the presence of cyclohexamide [DeMarchi, 1981; Jahn et al., 1984] or anisomycin [Stinski et al., 1983] both of which are inhibitors of protein synthesis. Although in the IE phase, transcription is restricted to a limited number of viral genes [DeMarchi, 1981], several transcripts are produced abundantly [Stinski et al., 1983; Jahn et al., 1984]. IE viral transcripts originate from the U_L segment of CMV genome within a region of approximately 20 kb that corresponds to HindIII E fragment of the AD169 strain and XbaI E and N fragments in the Towne strain [Mach et al., 1989]. In contrast, similar IE transcripts of herpes simplex virus (HSV) originate from the region containing the repeat sequences [DeMarchi, 1981].

The IE region has been further divided into four transcriptional units; IE1, IE2, IE3 and IE4 [Stinski et al., 1983; Jahn et al., 1984] (see fig 6). The IE1 region, which is referred to as major IE gene, produces a spliced RNA transcript of 1.95 kb which originates from a region of approximately 2.8 kb within the XbaI E DNA fragment [Stenberg et al., 1984]. This transcript has been shown to be a spliced molecule consisting of a 3' terminal exon of 1341 nt and three small exon sequences of 185, 88 and 121 nt [Stenberg et al., 1984]. A single ORF is present in the second exon of the gene and has a coding capacity for a protein of 491 amino acids and predicted molecular weight of 64,000 [Stenberg et al., 1984] which is modified after translation into a polypeptide of 75 kD [Rasmussen, 1990]. Phosphorylation of this protein has been suggested to account for the size difference seen in CMV Towne, Davis and AD169 strains [Gibson, 1981a]. The IE2

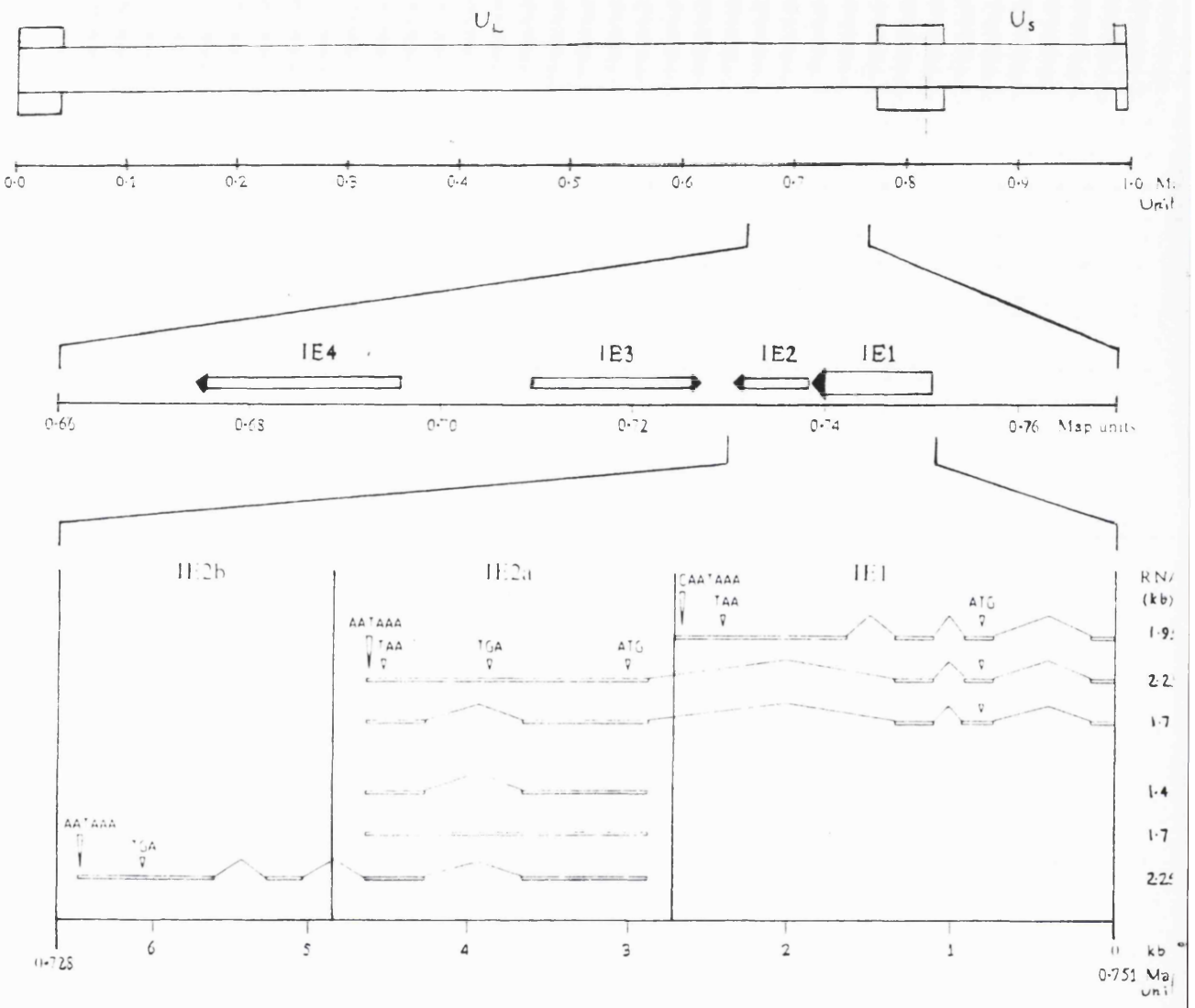


FIG 6: Organization of CMV IE genes.

The upper section of the figure shows the genomic localization of four major IE transcription units. The lower section shows the mRNA size and predicted ORFs of IE1 and IE2 region in detail, with initiation and termination signals and polyadenylation signals indicated. The thin lines indicate introns and open boxes represent mRNA exons [from Mach et al., 1989].

region comprises approximately 2 kb of DNA which is adjacent to IE1 region [Stenberg et al., 1985]. Several RNA transcripts of 1.1 to 2.25 kb originate from this region and encode several proteins with molecular weight of 16.5 to 56 kD [Stinski et al., 1983], originating from a DNA region of approximately 2 kb [Stenberg et al., 1985]. The IE2 region has been further divided into IE2a and IE2b (fig 6).

I.B.7.b. Early and Late Phases of Gene Expression

The early phase of gene expression is dependent on IE gene products. Hence the genes can be transcribed in the presence of inhibitors of viral DNA synthesis such as phosphonoacetic acid and phosphonoformic acid. Although early transcription can be detected throughout most of the viral genome, the early transcripts in highest abundance originate from the repeat sequences (IR_L and TR_L) of the CMV genome [Wathen and Stinski, 1982; Hutchinson et al., 1986]. Three classes of poly(A$^+$) RNA of 2.7, 2.0 and 1.2 kb have been identified [Hutchinson et al., 1986]. The 2.7 kb unspliced RNA is transcribed at both early and late times p.i. [Hutchinson et al., 1986]. A potential ORF encoding a polypeptide of 170 amino acids can be identified within the sequence of the gene encoding this RNA, but attempts to identify a protein have failed [Mach et al., 1989]. The 1.2 kb unspliced RNA is relatively low in abundance at early times but accumulates at late times. The least abundant class is the 2.0 kb RNA which is only expressed at early times [Hutchinson et al., 1986]. Another early gene codes for multiple transcripts of 2.2 kb which have a complex spliced structure, and have been shown to encode several phosphoproteins of 84, 50, 43 and 34 kD [Wright et al., 1988].

Another important early CMV gene is the DNA polymerase gene which is transcribed as an unspliced 4.7 kb mRNA and codes for a protein of 140 kD [Kouzarides et al., 1987]. The sequence of the polymerase gene shows homology to the predicted EBV and HSV polymerases both on the DNA and amino

acid level [Kouzarides et al., 1987].

The late genes which are primarily structural in nature require virus DNA synthesis for their expression [Mach et al., 1989]. The proteins are described below, in detail.

I.B.8. Structural Proteins of the CMV

There are distinct structural proteins that can be identified during different stages of CMV replication cycle and the proteins present on the extracellular forms of the virus are not exactly the same as those found on the intracellular forms.

I.B.8.a. Structural Proteins Associated with Intracellular Forms of the Virus

Virus particles have been classified into four types based on the intracellular location, sedimentation properties in sucrose gradients, protein composition and infectivity and have been given alphabetical designations by analogy with HSV [Gibson, 1981b]. The simplest are the A capsids which have three protein species of 145, 34 and 28 kD and each of the intracellular capsid forms contain these proteins. The 145 kD protein makes up 90% of A capsid mass and is the major structural component of the capsid [Gibson, 1981b; Gibson, 1983]. The 28 kD protein is the least abundant of the capsid proteins but maintains the structural integrity of the particle, since the virion nucleocapsid which lacks this protein can easily be disrupted with detergents. The B capsids have a 37 kD protein which distinguishes these capsids from A capsids, and may be involved in the DNA packaging and nucleocapsid envelopment. The C capsids are complex in structure as compared to A and B capsids and have 205 and 66 kD proteins. The latter protein might serve as an interface between the nucleocapsid and the outer envelope proteins. The infectious virion has at least seven additional proteins that

are not found in the A, B and C capsids and therefore are considered likely to be the constituents of the outer envelope structure [Gibson, 1981b; Gibson, 1983].

I.B.8.b. Structural Proteins Associated with the Extracellular Forms of the Virus

(1) Matrix Proteins

The region between the nucleocapsid and the envelope, termed tegument contains the three predominant matrix phosphoproteins: the lower matrix protein (LM) of 64-69 kD, the upper matrix protein (UM) of 69-71 kD and the basic phosphoprotein (BPP) of 149-150 kD [Gibson, 1983; Roby and Gibson, 1986]. In addition, the virion also contains nine minor species of phosphoproteins ranging in size from 122 kD to 24 kD [Roby and Gibson, 1986]. NIEP's contain the same three species of proteins and another major component in the form of a 36 kD assembly protein. Approximately 95% mass of the dense body mass is comprised of LM protein and the other two major phosphorylated proteins present in the virion and NIEP's i.e., BPP and UM are either absent or present only in trace amounts [Roby and Gibson, 1986]. Among the predominant phosphoproteins (UM, LM, BPP and assembly protein), BPP is the most highly phosphorylated both *in vivo* and *in vitro* [Gibson, 1983; Roby and Gibson, 1986].

The LM protein is one of the most abundant proteins synthesized during the infectious cycle. Although the immune response to LM protein is highly variable and antibodies against LM protein are not always detected by Western blot analyses, the polypeptide is frequently recognized by CMV positive human sera [Landini *et al.*, 1985]. The UM protein is not as abundant as the LM protein in the infected cells and there is no immunologic cross reactivity between the two proteins [Gibson, 1983]. The BPP (pp150) is the polypeptide that is frequently reactive in immunoblotting analysis with human antisera positive for CMV antibodies and is one of the

most immunogenic polypeptides [Landini et al., 1985; Scholl et al., 1988]. Human sera with high levels of IgG antibody (that are also positive for IgM class) have been shown to react with pp150 [Landini et al., 1985]. Several defined regions of pp150 have been expressed as ß-galactosidase fusion proteins in prokaryotic system and have been tested for immunoreactivity [Scholl et al., 1988]. Human sera so far tested recognize each of the different hybrid fragments irrespective of the computer predicted antigenicity [Scholl et al., 1988].

(2) Major Capsid or Nucleocapsid Protein (MCP)

The MCP is a non-phosphorylated protein of 150 kD which comigrates with BPP (also 150 kD) on an SDS PAGE gel [Gibson, 1983]. But it can be resolved by two dimensional gel electrophoresis or by using modified polyacrylamide gel cross linked with higher amounts of methylene-bisacrylamide [Gibson, 1983].

In one report MCP has been recognized by all human sera positive for CMV [Landini et al., 1985]. A region of the MCP ORF has been expressed as a ß-galactosidase fusion protein in E.coli and has been used to raise antibodies in rabbits. The obtained antisera have reacted with the MCP of purified CMV virion in Western blot analysis [Chee et al., 1989]. In addition, a monoclonal antibody against the human MCP can also detect the fusion protein [Chee et al., 1989].

(3) 28 kD Phosphoprotein

This structural phosphoprotein is present in the cytoplasm of the infected cells during the late phase of the replication cycle and is also found on extracellular viral particles [Rasmussen, 1990]. The protein is acquired in the cytoplasm and localizes on the viral capsids but is presumably different from the 28 kD protein found on the intracellular

capsid forms [Gibson, 1981b].

Immunoblot analysis reveals that the protein is reactive with majority of human sera positive for CMV and is a highly immunogenic polypeptide like pp150 [Landini et al., 1985]. In keeping with the other structural proteins, parts of this polypeptide have also been expressed as fusion proteins with ß-galactosidase in E.coli [Meyer et al., 1988]. Although it is not a target for neutralizing antibodies, pp28 is recognized by human sera [Meyer et al., 1988].

(4) 67-68 kD Phosphoprotein (Protein Kinase)

Extracellular virion, dense bodies and NIEP's recovered from the medium of CMV-infected cells possess protein kinase activity, which has been associated with an abundantly produced protein of 67-68 kD which can be immunoprecipitated with a monoclonal antibody [Roby and Gibson, 1986]. This protein has been distinguished from LM protein which is also 64-69 kD [Roby and Gibson, 1986].

I.B.8.c. Envelope Glycoproteins

The glycoproteins on the viral envelope are assuming increasing importance as antigens because of their humoral and cellular immunogenic potential. Systematic characterization of the glycoproteins has revealed a complex electrophoretic profile [Pereira et al., 1984] that can be grouped into three distinct families designated as gcI, gcII and gcIII by Gretch et al., [1988a]. These glycoproteins exist as disulphide linked complexes [Gretch et al., 1988a] and are described in this section (also see section I.B.9).

(1) gcI Family

The gene encoding this family of proteins has been mapped

[Mach et al., 1986]. A monospecific rabbit antiserum against gp58 (a major immunogenic glycoprotein of the family) has been used to screen a cDNA library, constructed from poly(A+) RNA of CMV infected cells. A clone synthesizing part of gp58 has allowed localization of the coding region within HindIII F fragment of the genome [Mach et al., 1986]. The nucleotide sequencing of this region has identified an ORF with characteristics typical of a membrane glycoprotein and coding capacity for a polypeptide of 906 amino acids and a molecular weight of 102 kD [Cranage et al., 1986]. The translation product of the gp58 ORF shows extensive homology to glycoproteins of other human herpersviruses, namely, gB of HSV, BALF4 reading frame product of EBV and gpII of VZV [Cranage et al., 1986].

The gcI complex of proteins [Gretch et al., 1988a] has also been referred to as gA by Pereira et al., [1984], gB by Cranage et al., [1986] and gp 55-116 by Britt and Auger [1986]. The gcI family consists of three complexes with approximate molecular masses of 250-300, 190 and 160 kD, under nonreducing conditions and 93-130 and 55 kD under reducing conditions [Gretch et al., 1988a]. These glycoprotein complexes have a complicated structure and have multiple conformational and linear epitopes.

The initial steps in the synthetic pathway of gp 55-116 complex involve addition of the carbohydrate side chains to the nonglycosylated 105 kD precursor [Britt and Vugler, 1989]. This precursor protein is glycosylated to give rise to gp150 initially and then gp165-170. Finally, gp58 is generated from the latter precursor by proteolytic cleavage and represents the carboxyl terminal part of the processed polypeptide [Britt and Auger, 1986; Britt and Vugler, 1989].

Monoclonal and polyclonal antibodies that recognize gp58 have been generated from murine or isolated from human sources and have been invaluable in mapping of the protein [Britt et al., 1984; Pereira et al., 1984; Landini et al., 1985]. Following immunoprecipitation of the protein with these antibodies, SDS PAGE under reducing conditions detects two proteins. In addition to gp58 which is present as a discrete

band, a diffuse signal of 116-150 kD is also detected. However, using SDS PAGE under non-reducing conditions, a complex of 250-300 kD is present [Jahn and Mach, 1990].

The overlapping fragments of the carboxyl terminal part of the gp58 ORF have been expressed in E.coli as a fusion protein with ß-galactosidase and a panel of four monoclonal antibodies reactive with gp58 have been tested for the recognition of these hybrid proteins [Utz et al., 1989]. An important epitope of gp58 has been identified using two monoclonal antibodies 7-17 and 27-287. The monoclonal antibodies recognize a sequence between amino acids 608-625 of the primary translation product of gp58 [Britt et al., 1984; Utz et al., 1989; Jahn and Mach, 1990]. This has been a surprising finding as antibody 7-17 can neutralize infectious virus, whereas antibody 27-287 does not exhibit neutralizing activity. Both antibodies inhibit each other in a competitive binding assay with intact virus as antigen and moreover antibody 27-287 is able to inhibit the complement independent neutralizing activity of antibody 7-17 [Utz et al., 1989]. These results indicate that there are two non-conformational epitopes, that are proximal to one another and which can induce antibodies with different biologic activities. The biological relevance of antibody inhibition of neutralizing antibodies is unknown but it has been observed that non-neutralizing antibodies may inhibit the complete neutralization of number of viruses and may account for the pathogenesis of several persistent viral infections [Jahn and Mach, 1990]. Another study has shown that the carboxyl terminal half of the glycoprotein contains two neutralizing domains mapping within a segment of 219 amino acids, between residue 461 and 680 [Banks et al., 1989]. The proximity of the two domains suggests that they are assembled into a discontinuous domain and that both are required to form the neutralizing epitopes mapping in this portion of the glycoprotein [Banks et al., 1989]. In addition, some monoclonal antibodies can neutralize CMV in the presence of complement but they do not react with gp58 in Western blots suggesting that the epitopes recognized by these monoclonal

antibodies are probably conformational and are denatured by techniques involved in immunoblotting [Rasmussen et al., 1985].

(2) gcII Family (gp47-52)

The gene encoding gp47-52 has been cloned and expressed in human fibroblast cells [Gretch et al., 1988b]. The gcII family consists of six different species of proteins ranging from 93-300 kD, under non-reducing SDS-PAGE. The major species under reducing conditions is gp47-52 but additional species of higher molecular mass have been occasionally observed. Biochemical and immunological analysis of the latter have shown that they are related to gp47-52 and it has been proposed that they represent incompletely reduced complexes [Gretch et al., 1988a]. The protein family has been immunoprecipitated using both monoclonal antibodies and human convalescent phase serum [Gretch et al., 1988a and b].

(3) gcIII Family (gp86)

This major glycoprotein family has been described as gcIII by Gretch et al., [1988a] and gH by Cranage et al., [1988]. gH is encoded within HindIII L fragment of CMV genome Cranage et al., 1988]. The predicted primary translation product has the properties of a transmembrane glycoprotein and shows homology with glycoprotein H of HSV type 1 and the homologous proteins in EBV and VZV. The gene product has been expressed using a vaccinia virus recombinant and immunoprecipitated as an 86 kD protein [Cranage et al., 1988].

The gp86 has an epitope that is a target for in vitro neutralization of virus infectivity, as shown by complement-independent activity seen with a gp86 specific monoclonal antibody [Cranage et al., 1988]. When immunoaffinity purified protein is injected into animals, complement independent neutralizing antibodies are produced [Rasmussen et al., 1985].

In addition to being a target for virus neutralizing antibody, gp86 may be involved in either intracellular spread or initiation of virus infectivity [Rasmussen, 1990].

I.B.9. Pathological Effects of CMV Infection

Cytomegalovirus is an ubiquitous member of the Herpesvirus group, that can infect the human host vertically or horizontally and can be transmitted by either route during primary infection, reinfection or reactivation. Primary infection of an immunocompetent host is usually mild or asymptomatic, and the virus establishes a latent state which may persist throughout life without sequelae. Being an opportunistic pathogen, primary infection or reactivation in an immunocompromised host, such as organ transplant recipients or patients with acquired immunodeficiency syndrome, can lead to severe disseminated disease with life threatening consequences. In addition, congenital infection can lead to birth abnormalities ranging from learning disorders to severe psychomotor retardation and death [reviewed by Glenn, 1981; Griffiths, 1990]. The clinical outcome with CMV infection clearly justifies an effort to develop a suitable vaccine.

I.B.10. CMV Vaccines

I.B.10.a. Live Attenuated Vaccine

The first approach to CMV vaccination was to use a live attenuated vaccine prepared from Towne strain which was isolated from a congenitally infected infant and then passaged 125 times in human fibroblasts [Plotkin et al., 1976]. The data obtained from clinical trials conducted in normal healthy volunteers has shown that the vaccine induces CMV-specific antibody and cellular immunity and protects healthy volunteers against a challenge (10-100 plaque forming units) of low passage Toledo strain of CMV. However, in renal transplant

patients, vaccine cannot prevent superinfection but can ameliorate the outcome of infection [Fleisher et al., 1982; Plotkin et al., 1985; Plotkin et al., 1989]. This indicates that the outcome of CMV infection is determined by the immune status of the individual and the dose of the infecting virus.

The sera obtained from Towne vaccinees have been used to identify viral proteins that are important in protective immunity. Antibodies that recognize the gA/gB (gcI) protein complex have been detected in virus neutralizing sera but not in the sera without neutralizing activity [Gönczöl and Plotkin, 1990]. Sera of Towne vaccinees do not recognize or recognize poorly the gp47-52 (gcII) proteins [Kari et al., 1986; Gretch et al., 1988a; Liu et al., 1988]. The reasons for reduced recognition of this class of proteins are not clear. Injection of purified gp86 (gcIII) into animals results in production of complement independent neutralizing antibodies [Rasmussen et al., 1985]. On the other hand, sera obtained from Towne vaccinees do not neutralize Towne or Toledo strains in the absence of complement but can immunoprecipitate gp86 in the infected cell lysate [Gönczöl and Plotkin, 1990]. It seems that the relative abundance of gp86 in mature virion or envelope preparation is less than the infected cell lysate, since it can be detected only by sera with high neutralization titre in immunoblot assays using virion or envelope preparation as antigen.

I.B.10.b. Subunit Vaccine

A subunit vaccine provides the immunogen to the immune system without the need for introduction of complete virus genome into the host. Although the attenuation of the Towne virus seems to prevent virus latency in the immunized individuals, concern still remains about using a complete live CMV for vaccination [Gönczöl and Plotkin, 1990].

The immunogenicity of gA/gB protein complex has been emphasized above and it has been shown that immunoaffinity

purified gA/gB protein complex can induce neutralizing antibody and a cellular immune response in experimental animals [Gönczöl et al., 1986]. Immunization of seronegative and seropositive volunteers with gA/gB preparation results in similar neutralizing antibody and cellular immune responses. Neutralizing antibodies that develop after repeated injections of gA/gB preparation can neutralize AD169, Towne and Toledo strains of CMV [Gönczöl et al., 1990].

The gene encoding gB has been cloned from the Towne strain and inserted into adenovirus type 5, downstream of the E3 promoter. The recombinant adenovirus expressed antigenically related proteins of 58, 30, 25 and 23 kD in A549 and MRC-5 cells [Marshall et al., 1990]. Hamsters infected intranasally with live recombinant virus developed protein specific and CMV neutralizing antibody responses, demonstrating the potential usefulness of a subunit vaccine [Marshall et al., 1990].

I.B.11. Future Prospects

The mechanism by which the immune system controls the CMV infection is incompletely understood but a thorough understanding of this aspect may allow for a better control of infection in a susceptible host. The immunologic approach to the identification of viral gene products with monoclonal antibodies has been very useful and has shown that at least three glycoproteins are present on the viral envelope that are targets for virus neutralizing antibody. The analysis of these glycoproteins is a prerequisite for a better understanding of the immunological response following CMV infection and the development of a potential subunit vaccine.

CHAPTER II

EXPRESSION OF HBV PROTEINS IN A EUKARYOTIC SYSTEM (BACULOVIRUS EXPRESSION VECTOR SYSTEM)

II.A. INTRODUCTION

II.A.1. Baculovirus Expression Vector System (BEVS)

The BEVS is based on insect viruses (baculoviruses), which belong to the family Baculoviridae and possess a double stranded, covalently closed circular DNA ranging from 80 to 220 kb with a molecular weight of 10^8 [Summers and Anderson, 1973; Bud and Kelly, 1977]. The baculoviruses are enveloped and the DNA is packaged into rod shaped nucleocapsids which, in infected cells, can be occluded in large protein crystals, referred to as polyhedra [Harrap, 1972]. The baculovirus classification is based on the presence or absence of polyhedra which surround the nucleocapsids and also on the number of nucleocapsids present in each occlusion. The subgroup A viruses, nuclear polyhedrosis viruses (NPV's), produce virions which have a single nucleocapsid per envelope (SNPV) or one to many nucleocapsids per envelope (MNPV). Both types of NPV form occlusions in the nucleus of infected cells and many virions can be embedded in each occlusion. Subgroup B viruses are called granulosis viruses (GV) and have one nucleocapsid per envelope and a single virion per occlusion. Subgroup C viruses are non-occluded viruses (NOV) and have one nucleocapsid per envelope [Matthews, 1979]. More than 400 baculoviruses have been described so far and these have been isolated from several invertebrate hosts, for example, the orders Lepidoptera (butterflies and moths), Hymenoptera (bees and wasps), Diptera (flies) and Coleoptera (beetles) [reviewed by Miller, 1988].

The prototype virus which has been extensively studied and developed into an efficient eukaryotic expression vector system is Autographa californica multiple nuclear polyhedrosis virus (AcMNPV or AcNPV) [Smith et al., 1983a and b].

II.A.2. Molecular Biology of AcMNPV

During AcMNPV infection two forms of progeny are produced: Extracellular viral particles (ECV) and Occluded viral particles (OV) [Summers and Volkman, 1976]. The OV are embedded in polyhedra which are made up of polyhedrin protein [Rohrmann, 1986]. Polyhedra are crystalline proteinaceous structures 0.1-1.0 μm in diameter which are detectable in the nuclei of infected insect cells, 18-24 hours p.i. and form a characteristic cytopathology. These continue to accumulate until infected cells lyse [Rohrmann, 1986].

The virus embedded in polyhedra can survive in nature for years or even decades and is protected from environmental conditions that would otherwise rapidly inactivate ECV. When foliage contaminated with polyhedra is ingested by feeding larvae, the alkaline environment of the larval midgut solubilizes the polyhedra releasing the virions into the lumen. A generalized infection progresses when the released virus invades and replicates in the midgut epithelial cells and secondary infection spreads to other insect tissues by ECV. The viral DNA is uncoated and replication occurs leading to formation of ECV which are released from the cells by budding (see fig 7) [Summers and Volkman, 1976; Summers and Smith, 1987; Luckow and Summers, 1988].

The mechanism by which virions are stabilized within polyhedra is unclear, but it is known that at least three proteins which are polyhedrin protein, p10 protein and polyhedron envelope protein (PE), contribute towards their structure [Kuzio et al., 1984; Rohrmann, 1986; Russell and Rohrmann, 1990]. The most abundant and well characterized of these three proteins is the 30 kD polyhedrin protein which forms the matrix of the polyhedra [Rohrmann, 1986]. The second is the p10 protein [Kuzio et al., 1984] and it has been shown that mutants lacking the p10 gene are fragile and disintegrate, suggesting that this protein plays an important role in the structure of polyhedra [Williams et al., 1989].

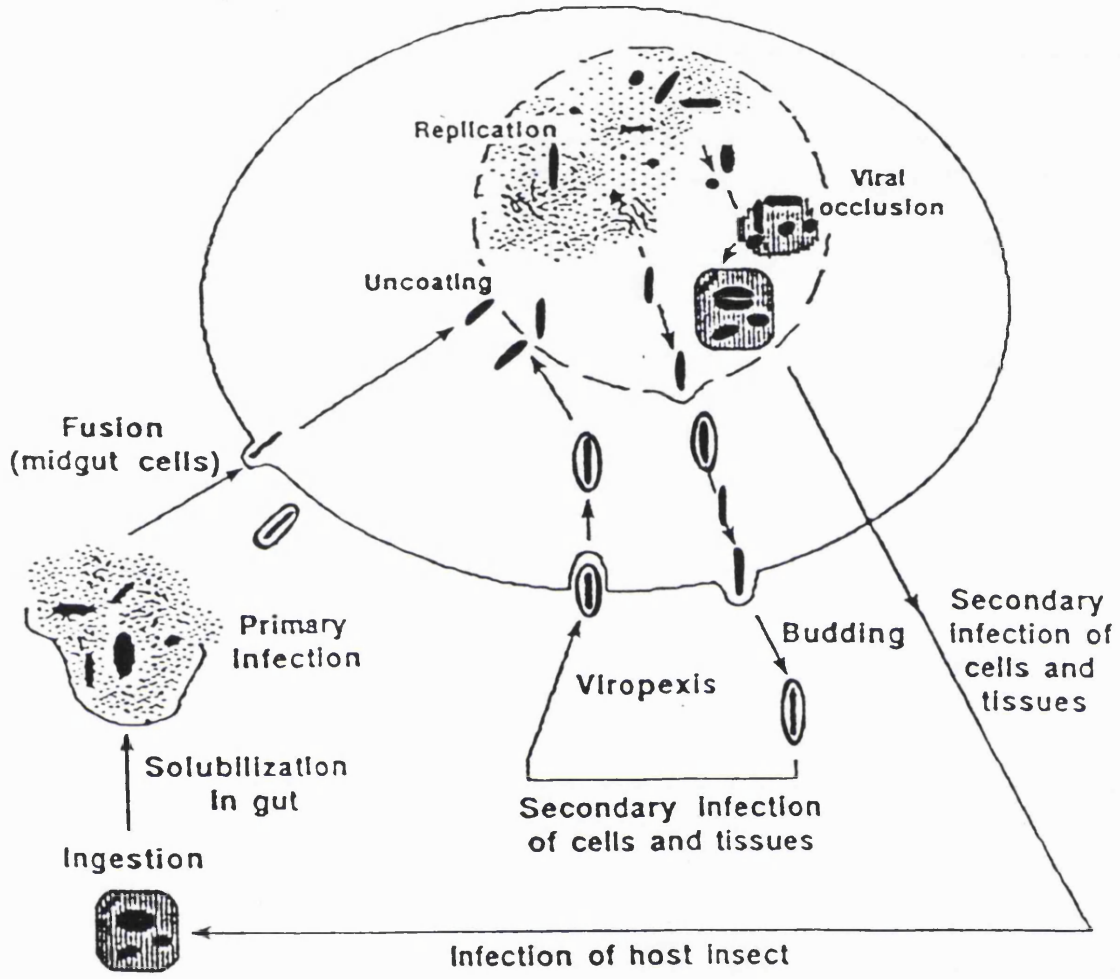


FIG 7: Baculovirus Life Cycle.

A susceptible insect ingests food containing polyhedra which lyse in the alkaline environment of the insect midgut releasing the infectious virus. The virus penetrates the gut cell and enters the nucleus where it is uncoated. Viral DNA replication can be detected within 6 hours and by 12-18 hours p.i. extracellular virus buds through the gut cell wall and invades surrounding tissues. The virus also produces the polyhedrin protein which surrounds the viral particles in form of viral occlusions and, as a result of this abundant production of the protein, the cells lyse.

The third protein is associated with envelope-like structure which surrounds mature polyhedra and is thus called PE protein. Although PE protein has affinity for the surface of polyhedra and forms an important component of polyhedron envelope, its role in baculovirus life cycle is not yet understood [Russell and Rohrmann, 1990].

II.A.3. Gene Expression and Importance of Polyhedrin Promoter

Four phases of gene expression have been distinguished in AcMNPV-infected Spodoptera frugiperda (Spf) cells: Immediate early, delayed early, late and a very late or occlusion specific phase.

During the early phases (0-6 hours p.i.), a number of genes distributed throughout the genome are expressed. Some of these gene products function as transcriptional transactivators of other early genes [Guarino and Summers, 1986]. The late phase (6-15 hours p.i.), is characterized by replication of viral DNA and production of the core and major capsid proteins. Thus, the first three stages result in the production of infectious particles which infect other cells (either in culture or within insect larvae) and disseminate infection [Carstens et al., 1979; Guarino and Summers, 1986; reviewed by Miller, 1988]. During the final, very late phase of gene expression (15-18 and until 72 hours p.i.), two virus encoded gene products are expressed in abundance. These are the polyhedrin and the p10 proteins [Rohrmann, 1986; Kuzio et al., 1984].

Neither protein is required for the production of virus particles, which means that they can be replaced with foreign coding sequences and this has enabled the development of an expression vector system [Smith et al., 1983a and b; Vlak et al., 1988]. The powerful promoters for these two proteins have been characterized [Matsuura et al., 1987; Qin et al., 1989]. The polyhedrin promoter appears to consist of a 49 nucleotide non-coding leader sequence, just upstream of the

gene encoding polyhedrin protein [Matsuura et al., 1987; Rankin et al., 1988]. The sequences upstream of nucleotide -70 do not contribute [Possee and Howard, 1987], whereas the region between -1 and -8 is extremely important for expression [Matsuura et al., 1987]. The polyhedrin promoter has been used for efficient expression of many foreign sequences, for example, human ß interferon [Smith et al., 1983a], human c-myc protein [Miyamoto et al., 1985], human interleukin 2 [Smith et al., 1985], hepatitis B surface antigen [Kang et al., 1987], hepatitis B core antigen [Lanford et al., 1988], lymphocytic choriomeningitis virus proteins [Matsuura et al., 1987] and hepatitis delta antigen [Kos et al., 1991].

The p10 promoter has also been used to express foreign genes, for example, the E.coli lacZ gene [Vlak et al., 1988].

II.A.4. Construction of Replacement Expression Vectors

The baculovirus genome is too large to permit easy insertion of foreign sequences, therefore a part of the viral genome is selected and manipulated in a bacterial plasmid. In the case of polyhedrin based plasmids, sequences from AcMNPV containing the polyhedrin promoter, varying amounts of 5' and 3' DNA flanking the polyhedrin gene, and polyhedrin transcription termination sequences may be used to construct transfer vectors. There are suitable restriction enzyme sites in these vectors which allow insertion of foreign genes in lieu of the polyhedrin gene [Luckow and Summers, 1988]. The recombinant vector DNA is coprecipitated with wild type virus DNA and the mixture is used to transfect Spf cells. Recombination between viral DNA flanking the foreign sequences in the vector and the homologous sequences present in the wild type DNA occurs producing a recombinant (fig 8) [Smith et al., 1985; Luckow and Summers, 1988]. An important consideration in designing a recombinant baculovirus is whether to insert a foreign sequence before or after the polyhedrin start codon. If transfer vectors which have cloning sites downstream of the

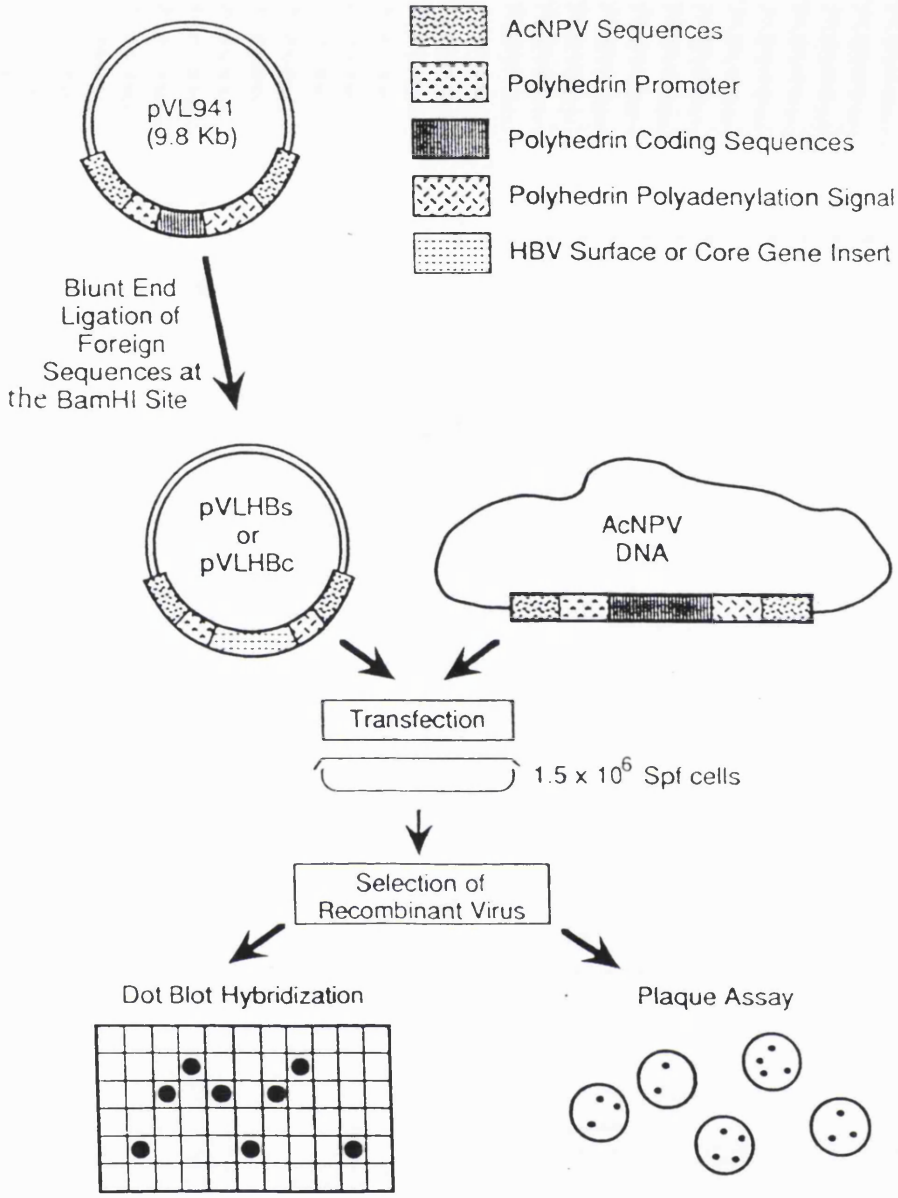


FIG 8: A schematic depicting the procedure for ligation of a foreign gene (for example, HBcAg or HBsAg gene) into a transfer vector (pVL941) and screening for a recombinant baculovirus.

The fragment of interest is inserted into a transfer vector in the correct orientation, in the BamHI site just downstream of the polyhedrin promoter. Cultured insect cells are transfected with a mixture of viral and plasmid DNA and the recombinant virus can be detected by the plaque purification procedure or dot blot hybridization as described in the text.

polyhedrin ATG are used, this will result in a fusion product that will contain amino acids from polyhedrin protein. Examples of such vectors include pAc311, pAc360, pVL101 and pVL106 [Luckow and Summers, 1988]. In most cases a fusion is not desirable and native protein is required. For this purpose, pAc373, pAc461, pVL941 and pAcRP series of vectors can be utilized [Smith et al., 1985; Matsuura et al., 1987; Luckow and Summers, 1988].

II.A.5. Identification of Recombinant Baculoviruses

II.A.5.a. Visual Screening Method

Plaques can be visualized macroscopically on monolayers of Spf cells infected with baculoviruses [Brown and Faulkner, 1977]. Wild type plaques with an occlusion positive morphology appear refractile with a white or yellow colour. Recombinant plaques which are the result of insertional inactivation or deletion of the polyhedrin gene have occlusion negative morphology and appear less refractile than the wild type plaques [Brown and Faulkner, 1977; Summers and Smith, 1987]. Thus the occlusion negative plaque morphology provides a subjective way of detecting and separating recombinant baculovirus and wild type baculoviruses.

II.A.5.b. Hybridization Method

Several difficulties can be experienced during discrimination between occlusion positive and negative plaques. These have been outlined by Fung et al., [1988] and depend on i) variable properties of plaques, ii) resolution of dissecting microscope, iii) presence of high level of recombinant virus and the experience of the worker. The problems associated with the plaque purification method can be overcome by using limiting dilution of the medium obtained

after transfection step and identifying recombinant viruses by hybridization of foreign DNA sequences with a radioactively labelled probe [Fung et al., 1988].

II.A.6. Biological Activity and Posttranslational Modifications of the Recombinant Proteins

II.A.6.a. Glycosylation

The most common type of glycosylation in mammalian system is the N-linked glycosylation in the ER, where a common oligosaccharide is attached to an asparagine. Oligosaccharide side chains may have variety of biological functions such as maintenance of structure and stability, targeting of proteins to different cellular compartments and mediating recognition in cell adhesion and tissue organization [Rademacher et al., 1988].

A variety of baculovirus produced mammalian gene products are known to be glycosylated, although their sizes are smaller than the corresponding molecules synthesized in mammalian cells. This has been shown for influenza virus haemagglutinin [Kuroda et al., 1989], vesicular stomatitis virus complete glycoprotein [Bailey et al., 1989] and lymphocytic choriomeningitis virus glycoprotein precursor [Matsuura et al., 1987] and has been attributed to differences in glycosylation pathways. However, in other situations such as HBV surface antigen [Lanford et al., 1989], HIV glycoprotein [Rusche et al., 1987] and Rift valley fever glycoproteins [Schmaljohn et al., 1989], normal sized polypeptides have been produced.

II.A.6.b. Phosphorylation

Some nuclear proteins have been phosphorylated in insect

cells, for example, HTLV-1 p40^{x} [Jeang et al., 1987], human c-myc protein [Miyamoto et al., 1985] and the Kruppel gene product of Drosophilia [Ollo and Maniatis 1987]. However, it is not known whether phosphorylation occurs in an identical position to that observed in natural cell.

II.A.6.c. Signal Sequence Cleavage and Proteolysis

Insect cells can recognize and cleave mammalian signal sequences, thus directing the proteins to the ER. During this process, the signal peptide is cleaved from the amino terminal end giving rise to the active product. Signals for human ó and ß-interferons [Maeda et al., 1985; Smith et al., 1983a], human interleukin 2 [Smith et al., 1985] and mouse interleukin 3 [Miyajima et al., 1987] are recognized and cleaved. In view of this, it seems likely that mammalian signal sequences are recognized and cleaved in the normal fashion in insect cells.

In addition to signal sequence cleavage, other proteolytic events like cleavage of proteins into their subunits, have been observed in virus-infected cells. This has been shown for fowl plague influenza virus haemagglutinin (HA) protein which is cleaved into the two subunits, however the cleavage is more efficient in insect larvae as compared to the cells [Kuroda et al., 1989].

II.A.6.d. Formation of Tertiary and Quaternary Structures

The HA glycoprotein of influenza virus and vesicular stomatitis virus glycoprotein, assemble as trimers in the insect cells [Kuroda et al., 1989; Bailey et al., 1989]. HBV surface antigen expression in insect cells has also been characterized. The 22 nm particles are synthesized and may be located intracellularly [Lanford et al., 1989] or secreted into the medium [Kang et al., 1987]. In the latter study, use

of spinner cultures and consequent cell lysis rather than secretion can explain the presence of particles in the medium.

In a recent study, assembly of the VP3 and VP7 proteins of bluetongue virus into core like particles has been observed [French and Roy, 1990]. This has been achieved by coexpressing VP3 and VP7 proteins in a multiple gene expression baculovirus vector [French and Roy, 1990]. The genome of P3/Leon/37 strain of poliovirus type 3 between nt 743 to 7363 has been expressed to produce a non-infectious virion [Urakawa et al., 1989].

II.A.7. Experimental Objectives

The objective was to develop experimental systems capable of expressing HBcAg and HBsAg from recombinant baculoviruses. Using the HBcAg system as a control expression, the next step would be to express a HBcAg-HCMV(gp58) recombinant baculovirus and then compare its expression with the prokaroytic sysyem.

II.B. MATERIAL AND METHODS

II.B.1. Plasmids

Three plasmids used for expression of HBcAg and HBsAg in the BEVS, are described below.

II.B.1.a. Plasmid pVLHBc

This plasmid (constructed by Dr T J Harrison) was a derivative of the expression vector pVL941 (Luckow and Summers, 1988) that contained a 0.56 kb DNA fragment encoding HBcAg, cloned in the unique BamHI site of pVL941. The DNA fragment was obtained by digesting a plasmid pL3A (which had HBV adw2 DNA {Vaudin et al., 1988} cloned in the multiple cloning site of pUC18; constructed by F.D'Mello {personal communication}) with StyI. The fragment was end-filled using the Klenow enzyme and blunt end ligated into BamHI digested and similarly end-filled pVL941 [fig 9]. The orientation of the fragment was determined using appropriate restriction enzymes.

II.B.1.b. Plasmid pVLHBs

This plasmid was constructed in a similar fashion to pVLHBc, but contained a 1 kb DNA fragment encoding HBsAg. The fragment was obtained by digesting pL3A with OxaNI and HpaI and end-filling it with Klenow enzyme. It was blunt end ligated into the BamHI site of pVL941 and the orientation was determined [see fig 10A and 11].

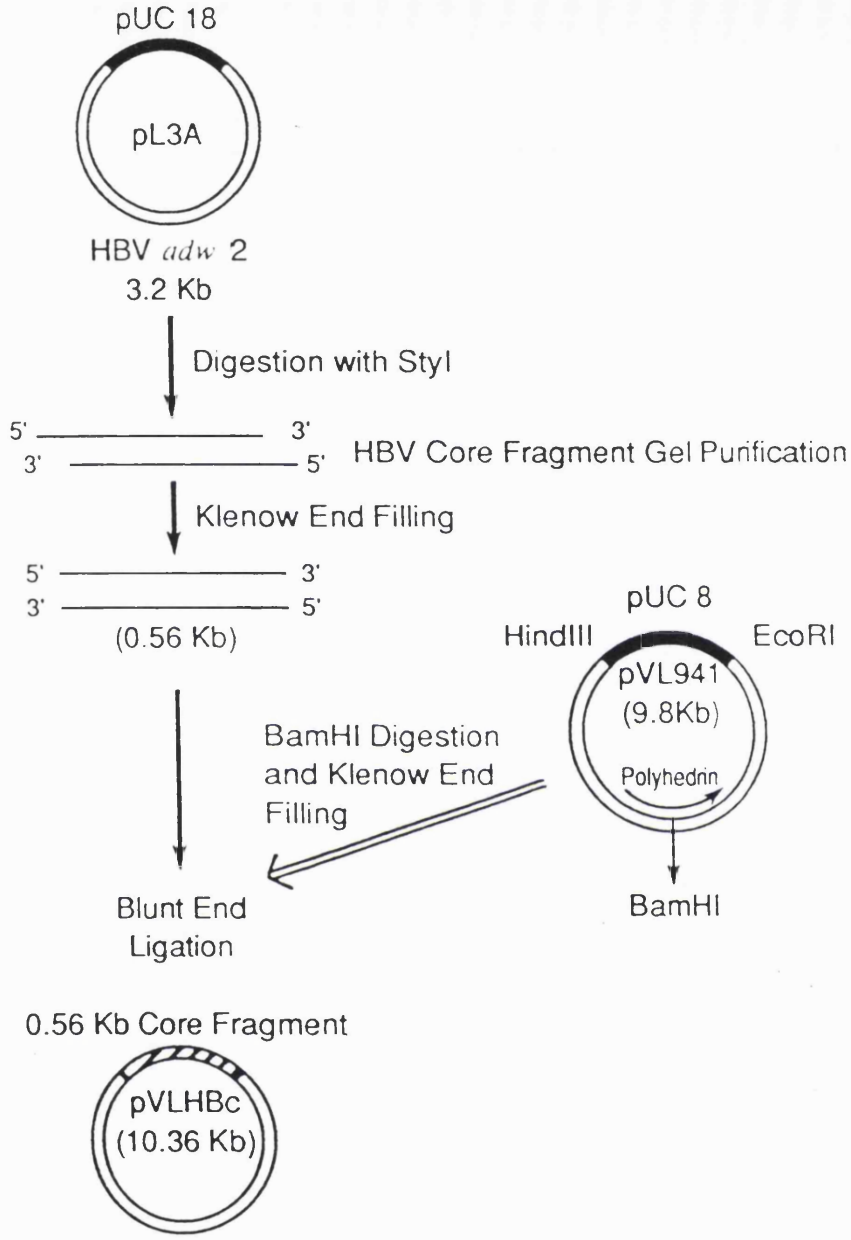


FIG 9: Plasmid pVLHBc.

The HBcAg encoding fragment has been excised from plasmid pL3A using StyI. (see fig 11 for localization of StyI sites, on a linear restriction enzyme map of HBV adw2 DNA).

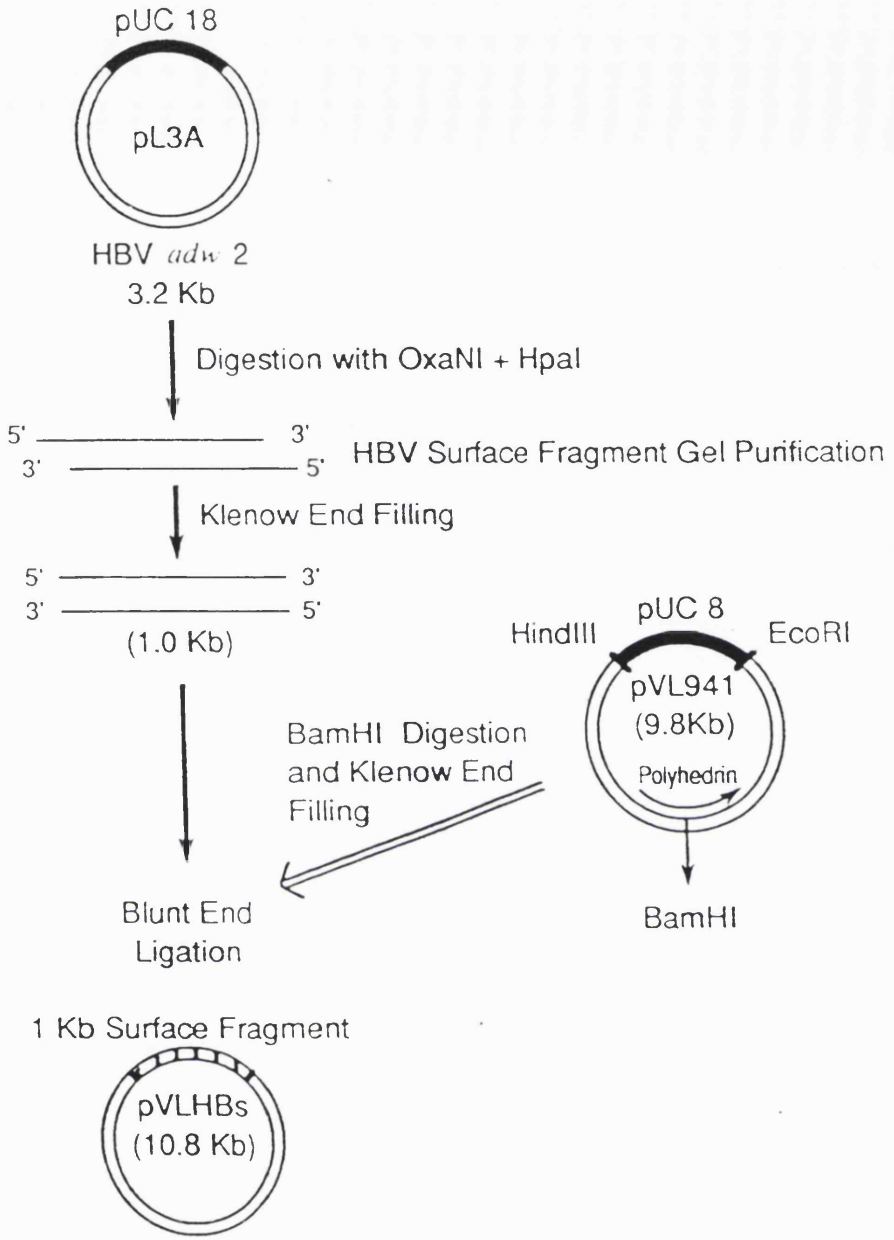


FIG 10 (A): Plasmid pVLHBs.

The source plasmid that has been used for excising HBsAg encoding fragment with OxaNI and HpaI enzymes, is pL3A (compare with fig 10B in which the HBsAg encoding fragment has been taken from another plasmid pMSG.sAg). See fig 11 for localization of OxaNI and HpaI enzyme sites on a linear restriction enzyme map of HBV adw2 DNA.

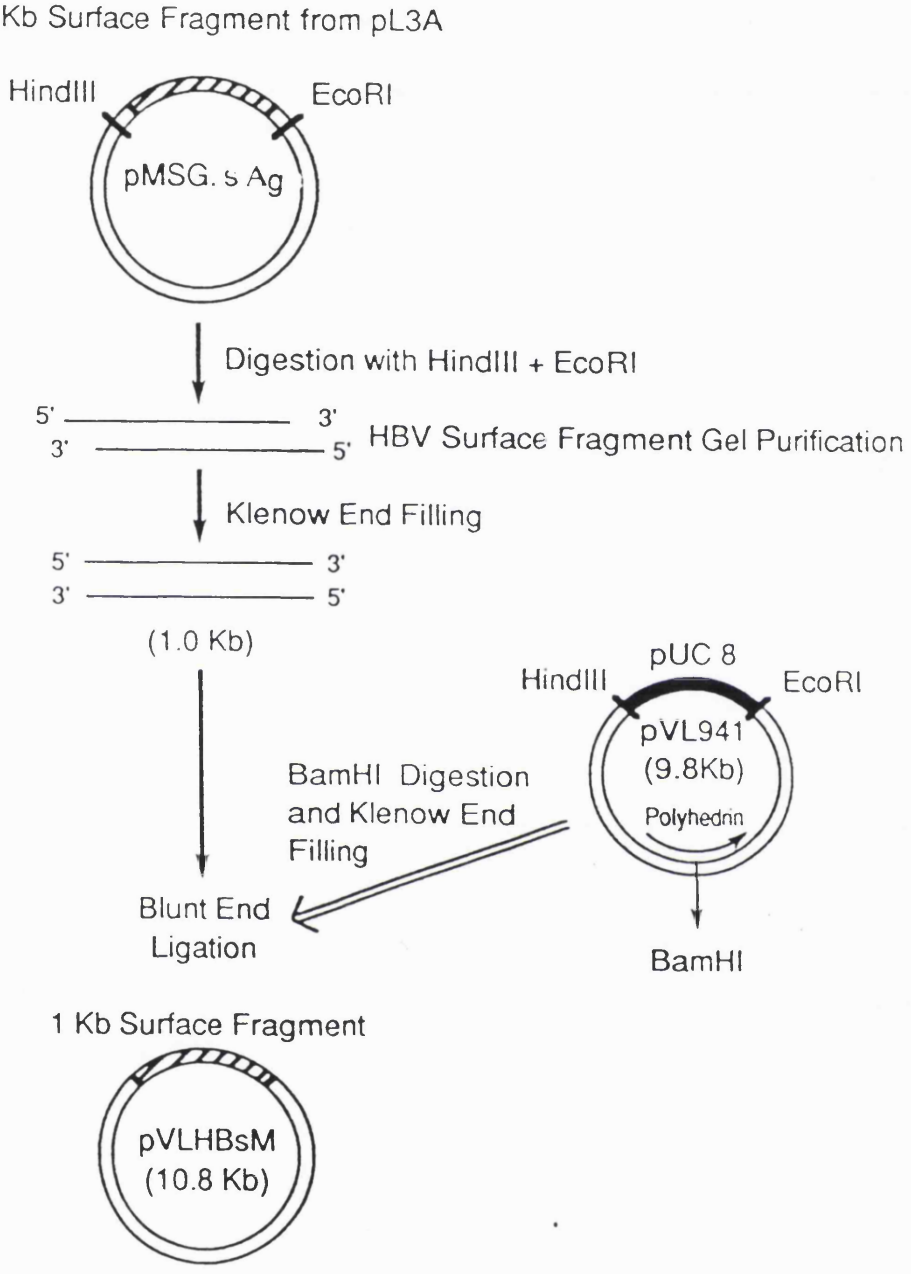


FIG 10 (B): Plasmid pVLHBs(M).

The HBsAg encoding fragment was excised from pMSG.sAg using HindIII and EcoRI.

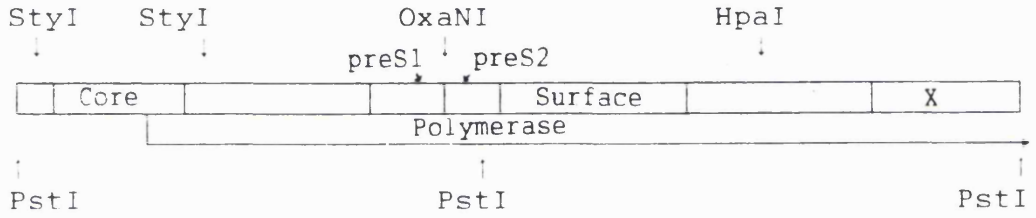


FIG 11: A linear restriction enzyme map of HBV adw2 DNA (Vaudin et al., 1988), showing the relevant enzyme sites.

II.B.1.c. Plasmid pVLHBs(M)

This plasmid also contained the same 1 kb DNA fragment encoding HBsAg as above, but the construction was slightly different. The fragment was not obtained from pL3A but from another plasmid pMSG.sAg (F.D'Mello, personal communication) which contained HBsAg fragment, cloned in the polycloning site of the vector pMSG [Sambrook et al., 1989]. Digestion of pMSG.sAg with HindIII and EcoRI resulted in a HBsAg encoding fragment which contained longer 5' and 3' non-coding ends. The fragment was end-filled with Klenow enzyme and blunt end ligated into BamHI site of pVL941 [fig 10B and 11].

II.B.2. Insect Cell Culture [Summers and Smith, 1987]

II.B.2.a. General Culture Techniques

Spodoptera frugiperda (Spf) cells were grown in 25 and 80 cm^2 tissue culture flasks, and 35 mm dishes in TC100 medium (Gibco, BRL). The medium was supplemented with 10% foetal calf serum and 50 µg/ml gentamicin sulphate and used within one month of reconstitution. Before use, the medium was equilibrated to room temperature.

When medium was added to the flask, it was allowed to flow down the side avoiding the cell monolayer. While removing the liquid, the flask was tilted at an angle and medium removed without touching the cells.

When medium was added to the dishes, it was added dropwise to the centre and allowed to flow to the edges. While removing, the dish was tilted at an angle. If agarose was added, it was added at the edge of the plate.

II.B.2.b. Monolayer Culture and Cell Staining

The Spf cells (a gift from Dr V.Emery), in tissue culture

flasks in the TC100 medium, were incubated at 28°C and subcultured at a 1:4 ratio every 2 to 3 days or when the cells formed a confluent monolayer.

The cell viability was checked by adding trypan blue (0.4% in PBS) to an equal volume of the cell suspension. After 5 minutes, the cells were examined under light microscope on a Neubar chamber.

II.B.2.c. Freezing, Storage and Removal of Spf Cells

The cells forming a confluent monolayer were suspended in TC100 medium and then pelleted by centrifugation at 1500 rpm for 10 minutes, and resuspended in fresh TC100 medium at a density of $4x10^6$ cells/ml. The cell suspension was diluted with an equal volume of TC100 medium containing 10% filter sterilized DMSO, and transferred to sterile freezing vials on ice for 30 minutes. The vials were then stored at -20°C for few hours before being placed in liquid nitrogen.

After storage, the cells were rapidly thawed by placing the vials in a 37°C water bath. After decontaminating the outside of the vial using methyl alcohol, fresh cold TC100 medium was mixed with cells in the vial and the suspension was transferred to 25 cm^2 tissue culture flask. The cells were allowed to attach for one hour at room temperature, and 3-4 hours at 28°C. The old medium was replaced with fresh after this incubation, and the cells were resuspended and transferred to 80 cm^2 flask after 2 to 3 days.

II.B.3. Infection of Cells with Autographa californica Nuclear Polyhedrosis Virus (AcNPV) [Summers and Smith, 1987]

II.B.3.a. AcNPV

AcNPV was supplied in the form of an infected cell supernatant by Dr V.Emery.

II.B.3.b. Cell Infection

Viable Spf cells, at a density of $9x10^6$ cells/80 cm^2 flask were allowed to attach for 1-2 hours at room temperature. The medium was replaced with 1 ml of virus inoculum and the flask was incubated at room temperature for 1 hour. The inoculum was replaced with 10 ml of fresh TC100 medium, and the flask incubated for 2-4 days at 28°C. The cells were examined daily for the presence of polyhedra and were harvested when 90% or more were infected with polyhedra. The infected medium was centrifuged for 10-15 minutes at 1000 x g to pellet the cells, and the supernatant was stored at 4°C.

II.B.4. Purification of Extracellular AcNPV DNA [Smith and Summers, 1982] (modification of protocol by V.Emery)

Spf cells were seeded in five 80 cm^2 flasks and infected with medium containing AcNPV as described above. After 48-72 hours, the medium was centrifuged at 1000 x g for 10 minutes to remove the cells. The supernatant was centrifuged at 100,000 x g for 60 minutes at 4°C, and the virus pellet was resuspended in 1 ml of TE pH8.0. The virus was layered on a step gradient consisting of equal volumes of 10% and 50% sucrose (w/v) in TE pH8.0, and centrifuged in a swing-out rotor at 100,000 x g for 90 minutes at 4°C. The band containing the virus was visualized against a black background and collected with a pastette. This was diluted with TE pH8.0 to 50 ml, and centrifuged in a fixed angle rotor for 60 minutes at 4°C. The pellet was resuspended in 0.5 ml of TE pH8.0 and kept at 4°C for 12-18 hours.

After overnight storage, 0.6 ml of lysis buffer A (10% w/v sodium N-lauryl sarcosinate, 10 mM EDTA) was added to the gradient purified virus and incubated at 60°C for 20-30 minutes and then layered onto 54% (w/v) CsCl in TE pH8.0 containing EtBr (0.1 ml EtBr (10 mg/ml) per 10 ml CsCl). The

virus was centrifuged at 200,000 x g for 18 hours at 20°C. The gradient was viewed with a UV lamp and the viral DNA band was collected. EtBr was extracted 5x with water saturated butanol, and solution of the viral DNA was dialysed against three changes of sterile TE pH8.0 within 48 hours. The amount of DNA present in the preparation was determined by gel electrophoresis and then stored at 4°C. Virus titer was determined by plaque assay as described in section II.B.6.a.

II.B.5. Co-transfection of Recombinant Plasmid and Wild Type AcNPV DNA for Recombination In Vivo (V.Emery, unpublished protocol)

Spf cells were seeded into 35 mm tissue culture dishes at a density of $2x10^6$ cells/dish in TC100 medium, and allowed to attach for 30 minutes. Meanwhile the following were mixed in 1.5 ml eppendorf tube:

HEPES bufferd saline 2x	0.75 ml
AcNPV DNA	1 µg
Transfer vector DNA	25 µg
Glucose 100 mM	0.1 ml
DW	to 0.95 ml

To the tube, 50 µl of 2.5 M $CaCl_2$ was added dropwise, mixed by vortexing, and left for 30 minutes to coprecipitate. This transfection mix was added to 35 mm dishes above after removing the TC100 medium, and incubated at room temperature for 1 hour. The transfection mix was replaced with fresh TC100 medium, and the dishes were kept in a humidified container at 28°C, for two days. The transfected cells were examined for the presence of polyhedra under a light microscope, to ensure that transfection had been successful. The medium was collected from the dishes and cells were pelleted by centrifugation for 10 minutes at 1500 rpm. The supernatant was stored at 4°C, and used for plaque assays and dot blot experiments, as described below.

II.B.6. Identification of Recombinant AcNPV

II.B.6.a. Morphological Screening / Plaque Assay [Brown and Faulkner, 1977]

Spf cells were seeded in 35 mm dishes at a density of $1.5x10^6$ cells/dish, and allowed to attach for 1-2 hours, at room temperature. Meanwhile, ten fold dilutions were prepared from the transfection yield obtained in section II.B.5, between 10^{-2} and 10^{-3}. The medium in the dishes was replaced with 100 µl of the dilution, and incubated for 1 hour at room temperature. Twenty dishes were set up for each dilution. Whilst the dishes were incubating, 1.5% LMP agarose was prepared by adding equal volumes of TC100 medium (prewarmed to 45°C) and 3% LMP agarose. The latter was melted in a microwave oven, cooled to 45°C, and added to TC100 medium before use.

The virus inoculum was removed from the dishes after 1 hour and 2 ml of agarose overlay was added to each dish. At least 30 minutes were allowed for the overlay to solidify, after which 1 ml of TC100 medium was added to the dishes to prevent drying. All dishes were incubated in a humidified container at 28°C for 4 to 6 days or until plaques could be visualized. {wild type virus titer was determined by using dilutions between 10^{-2} to 10^{-7} and the number of plaque forming units (pfu) calculated by using the formula: pfu/ml = 1/dilution x number of plaques x 1/ml inoculum per plate}

(1). Detection of Viruses following Plaque Assay

The TC100 medium was discarded and the dishes were inverted on a light box partly covered with a blackened x-ray film. All the plaques were circled with a marker pen and then visualized under an inverted microscope at 400x magnification. Some plates were stained with neutral red for 30 minutes in order to estimate plaque number.

The plaques which were polyhedrin negative on microscopy, were picked with sterile glass pipettes and transferred to 0.4 ml of TC100 medium in eppendorf tubes. The agarose plugs were vortexed and stored at 4°C. Dilutions were prepared between 10^{-2} and 10^{-3} and subjected to another plaque assay to identify any recombinants.

II.B.6.b. Non-morphological Screening / Dot Blot Hybridization [Fung et al, 1988]

Spf cells were seeded into 96 well tissue culture plates (Nunclon), at a density of 1.5x 10^4 cell/well, and allowed to attach for 20 to 30 minutes at room temperature. Dilutions were prepared from transfection yield [II.B.5], in case of primary screening, or from supernatant of a positive well depending on the results of previous screening, between 10^{-2} and 10^{-12}. After the cells had attached in the wells, the TC100 medium was replaced with 70 µl of corresponding dilution. Two wells each were used for Spf cells and AcNPV controls, and four wells for vector DNA, in duplicate, as a positive control.

The plate was incubated at 28°C for 5 to 7 days, at the end of which all the wells were examined under inverted microscope for the presence or absence of polyhedra in the cells and scored. If all the cells had polyhedra, the score was 3+, with half the cells infected, the score was 2+, and the occasional presence of polyhedra was scored as 1+.

After scoring, the supernatant from the wells was transferred to a master plate and stored at 4°C, and the cells were lysed with 200 µl of 0.2 M NaOH, by pipetting and incubating for 15 to 30 minutes at room temperature. The suspension was added to the wells of a manifold apparatus, in which a nylon membrane [Hybond N], which had been soaked in 20x SSC for 15 to 20 minutes, had already been arranged. In addition, 400 µl of 2 M NaCl was also added to all the wells before the suspension was aspirated with a vacuum pump. The

membrane was air dried and exposed to UV light for 2 to 3 minutes and prehybridized and hybridized as described [III.B.12]. The membrane was exposed to Kodak XAR film, for 1-5 days depending on the strength of radioactive signal. The supernatant from wells, that gave a positive signal was used for subsequent screening experiments.

II.B.7. Preparation of Recombinant AcNPV DNA from Infected Cells [Summers and Smith, 1989]

Spf cells were infected with recombinant virus as described [II.B.3.b] Two 80 cm^2 flasks each, were used for recombinant, AcNPV and Spf cell controls. After incubation for 2 days at 28°C, the medium was replaced with 5 ml of Lysis buffer B (30 mM Tris HCl pH7.5, 10 mM magnesium acetate, 1% Nonidet P40), and the flasks incubated for 5 minutes. The cell suspension was transferred to 15 ml corex tubes and kept on ice for 5 to 10 minutes. During this time, cells were vortexed 3 to 4 times for 15 seconds and then pelleted by centrifugation at 2000 rpm for 3 minutes. The pellet was resuspended in cold PBS, repelleted and then resuspended in 4.5 ml of extraction buffer. Then 200 µg of proteinase K was added to the suspension and incubated at 50°C for 1 hour, followed by addition of sarcosyl, to a final concentration of 0.5%. Incubation was continued for a further 2 hours, and the viral DNA was extracted twice with phenol-chloroform-isoamylalcohol (25:24:1). The phases were mixed by inverting the tubes several times for 5 minutes and separation was achieved by centrifuging at 1000 rpm for 2 to 3 minutes. The DNA was precipitated by adding 2 volumes of ethanol, and incubated at -80°C for 1 to 2 hours or overnight. The DNA was pelleted by centrifugation at 2400 rpm for 20 minutes. The pellet was washed with 90% alcohol, air dried, and dissolved in 0.1x TE pH8.0 at 65°C for 20 minutes. A small aliquot was loaded on an agarose gel to estimate the amount of DNA.

II.B.8. Radioactive Labelling of Recombinant Proteins

Spf cells were seeded at a density of $2x10^6$/35 mm dish, and allowed to attach for 30 minutes. The medium was replaced with 0.1 ml of recombinant virus stock or control stock containing AcNPV, and cells. All the dishes were set up in duplicate, and left at room temperature for 1 hour, after which the inoculum was replaced with fresh TC100 medium, and incubated at 28°C. After 24 hours, supernatant was removed from one set of dishes and after 48 hours from the second set. In each case the cell monolayer was washed with TC100 medium without methionine, and incubated in 1 ml of the same medium for 1 hour at 28°C. The medium was replaced with fresh TC100 medium without methionine, but supplemented with ^{35}S labelled methionine (10 µCi/dish) in final volume of 0.2 ml. All the dishes were incubated for 2 hours at 28°C.

At the end of the labelling period, cells were collected by pipetting the medium across the monolayer. The cells were pelleted in a microfuge for 30 seconds and resuspended in 150 µl of RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 50 mM Tris HCl pH7.4, 10 mM EDTA, 1% SDS) and stored at -20°C. The supernatant was stored at 4°C.

II.B.9. Analysis of Recombinant Proteins by SDS-PAGE

The following solutions were utilised for making resolving and stacking gels.

Gel components	Stacking gel 5% (10 ml)	Resolving gel 10% (15 ml)
DW	6.8	5.9
Acrylamide 30%	1.7	5.0
TrisHCl pH6.8, 1M	1.25	-
TrisHCl pH8.8, 1.5M	-	3.8
SDS 10%	0.1	0.15
Ammonium persulphate 10%	0.1	0.15
TEMED	0.01	0.006

The resolving gel solution was poured in the gel mould (Atto equipment, Japan), and layered with few ml of DW. The gel was allowed to polymerize for 45 minutes, after which the DW was discarded and replaced with freshly prepared stacking gel solution. The gel was also allowed to polymerize for 45 minutes, after which it was assembled in the tank containing 1x Tris-glycine buffer (25 mM Tris, 250 mM glycine, 0.1% SDS), following manufacturers instructions. The samples prepared in section II.B.8, and the rainbow protein marker were heated at 95°C for 5 minutes with an equal volume of 2x protein dissociation buffer (2.3% SDS, 10% glycerol, 5% 2-MCE, 62.5 mM Tris HCl pH6.8, 0.01% bromophenol blue), before loading. The gel was allowed to run at 150 volts, until the dye front reached the bottom of the gel. The gel was placed in a tray and the stacking gel was excised and discarded. The gel was fixed with 10% freshly made up acetic acid (v/v in DW) for 15 to 20 minutes, followed by SDS-coomassie blue staining for 20 minutes. The gel was destained with acetic acid for 30 minutes or overnight and photographed at this stage and then exposed to an X-ray film.

II.B.10. Dot Blot Immunoassay for Recombinant Proteins [modification of the method described by Young, 1989]

A dot blot experiment was carried out as described in section II.B.6.b, but instead of prehybridization and hybridization steps the membrane was blocked with 100 ml of 10% PBS-MP, at 37°C for 12 hours with shaking. The membrane was washed with PBS and incubated with 1:200 dilution of primary antibody diluted in 1% PBS-MP, for 1-2 hours, at room temperature. Some immunoassays were performed with primary antibody that had been preabsorbed with Spf cells as below [Bradley, 1990]: 200 µl of lightly packed Spf cells were transferred to 1.5 ml eppendorf tube and allowed to stand for few minutes. Excess fluid was removed and the cell pellet was

quickly frozen and then stored at -20°C. When needed, pellet was rehydrated with DW or PBS and briefly centrifuged. Antibody diluted (10^{-1}) or undiluted was added to cell pellet and rocked overnight at 4°C. Absorbed and diluted antibody was recovered by centrifugation.

The membrane was washed three times in 100 ml of 0.3% PBS-Tween for 5 minutes, and then incubated with 1:1000 dilution of secondary antibody conjugated to HRP, in 1% PBS-MP, for 1 hour. The membrane was washed again in 100 ml of 0.3% PBS-Tween two times for 5 minutes each, followed by last wash in PBS without Tween for same time. HRP conjugate was added and colour development was allowed for 30 minutes.

II.B.11. HBV Probes

II.B.11.a. HBV 3.2 kb Probe

The 3.2 kb HBV insert may be removed from plasmid pL3A (section II.B.1.a) by double digesting it with EcoRI and HindIII. Ten µg of pL3A was digested with these two enzymes and then a sample of reaction was electrophoresed through 1% agarose gel to ensure complete digestion. The remaining reaction was loaded on an agarose gel and electrophoresed for an appropriate time. The 3.2 kb fragment was excised from the gel and purified as described in section III.B.6 [page 181]. Fifty ng of HBV DNA was used for each radioactive labelling reaction as described in section III.B.10 [page 183].

II.B.11.b. HBV Subgenomic Probes (gift from Dr Yue Lin)

Five HBV subgenomic fragments (a, b, c, d and e) have been obtained by digesting entire EcoRI-digested HBV genome with BamHI, HpaI and BglII. These fragments have been subcloned into appropriately digested pUC9 [Chen et al., 1988]. The inserts in these plasmids represent the S,

Enhancer I, X, C and preS genes. The plasmids were purified by large scale plasmid preparation and then the appropriate amount was double digested with EcoRI and HindIII. The appropriate fragment was separated from vector DNA by gel electrophoresis and purified as described in section III.B.6. Only two subgenomic fragments 'a' and 'd', which represent surface and core genes were radioactively labelled and used in the experiments.

II.C. RESULTS

II.C.1. Production of Recombinant AcNPV Containing HBV Core and Surface Antigen Genes

II.C.1.a. Preparation of Plasmids and AcNPV DNA for Transfection

Four plasmids were used to produce recombinant AcNPV containing core and surface antigen genes. The plasmids pVL941, pVLHBc and pVLHBs were purified on CsCl/EtBr gradients following alkali lysis of bacterial cells.

The construction of plasmid pVLHBs(M) is described below. Ten ug of plasmid pMSG.sAg was double digested with EcoRI/HindIII and run on an agarose gel. The 1000 bp fragment containing the surface antigen encoding sequences was excised from the gel and purified on a DEAE sephacel column and ethanol precipitated. The DNA was dissolved in 10 µl of TE pH8.0 and the amount was estimated by running an aliquot on an agarose gel. The fragment was made blunt ended by treating 0.5 µg with Klenow enzyme, extracting, precipitating and running 0.5 µl on a gel to estimate the amount of DNA present. At the same time, 10 µg of pVL941 was digested with BamHI and extracted. The DNA was precipitated, made blunt ended by Klenow enzyme, and 0.5 µl was run on a gel. The ligation reaction was set up between 200 ng of pVLHBs(M) and 100 ng of pVL941. After transformation of competent E.coli strain (TG2), the colonies were hybridized with $1x10^6$ cpm of radioactively labelled HBV probe. A strong signal was obtained from only 4 colonies, which were then grown for mini-prep analysis. The DNA was double digested with HindIII/XbaI to determine the orientation of the surface gene fragment.

Five diagnostic fragments of 4270, 3720, 1050, 930 and 830 bp were seen for one clone [fig 12, lane 2] which was gradient purified and used for transfection experiment.

The nucleotide sequence of plasmids pVLHBc, pVLHBs and pVLHBs(M) was confirmed by direct sequencing using BEVS primer (section III.B.16) {results not shown}.

The AcNPV DNA was prepared by infecting $9x10^6$ Spf cells attached to 80 cm^2 flasks in batches of five flasks at one time, with 1 ml of virus inoculum. One batch yielded sufficient DNA for purification by CsCl/EtBr gradient for transfection experiments. The virus titer of $2.5x10^6$ pfu/ml was determined by plaque assay.

II.C.1.b. Transfection

The transfection experiment was carried out as described. The amount of AcNPV and plasmid DNA used in these experiments was optimized by trying various concentrations. It was observed that the maximum efficiency of transfection was achieved by using 25 to 50 µg of plasmid DNA in the presence of 1 µg of AcNPV DNA. In most of the experiments, however, only 25 µg of the plasmid (pVLHBc and pVLHBs) DNA was used.

Several transfection experiments were performed in duplicate with pVLHBc, pVLHBs and pVLHBs(M) and it was observed that all successful transfections were accompanied by a visible fine precipitate. In addition, numerous polyhedra were observed in Spf cells, usually 2-3 days after transfection. Following some transfections, no polyhedra were seen in the cells, although there was no accompanying change in the variables. These experiments were discarded.

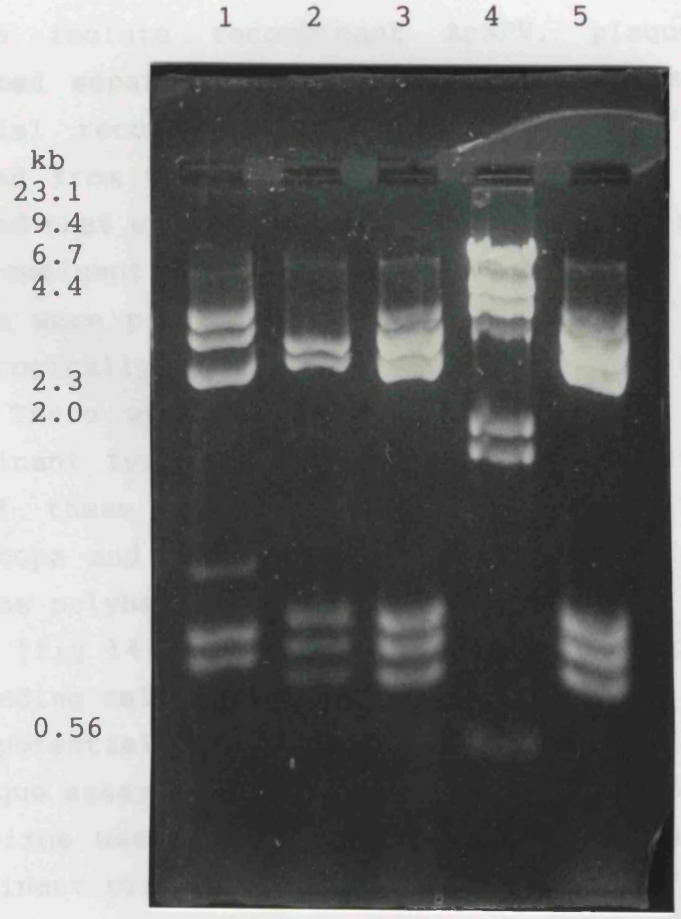


FIG 12: Electrophoresis of HindIII/XbaI double digested DNA, obtained from pVLHBs(M) clones, on a 1% agarose gel.

Lanes 1, 2, 3 & 5: pVLHBs(M) clones 1-4
Lane 4: HindIII digested lambda marker DNA (fragment size shown in kb)

II.C.2. Identification of Recombinant AcNPV

II.C.2.a. Plaque Assay

To isolate recombinant AcNPV, plaque assays were performed separately and at different times for the three potential recombinants. Dilutions of 10^{-2} and 10^{-3} were prepared from the transfection mix obtained above. It was observed that wild type plaques were always present in excess of recombinant plaques. About 200-250 of the wild type plaques were present in the dishes with 10^{-3} dilution, seen macroscopically with or without staining with neutral red [fig 13]. There were only a few plaques that appeared to be recombinant type by this method, usually between 5 and 10. All of these plaques were visualized with an inverted microscope and usually half of these were found to be wild type, as polyhedra were present in the cells surrounding the plaque [fig 14]. The plaques which had no polyhedra in the surrounding cells were picked up and vortexed in TC100 medium. These potential recombinants were evaluated in a second round of plaque assays, with the same dilutions as above. Only wild type virus was isolated from the second round, for all the recombinant plaques, studied in several sets of experiments.

In another set of plaque assays, dilutions between 10^{-4} and 10^{-6} were tried. It was seen that the number of wild type plaques diminished with increasing dilution, and hardly any recombinant plaques were visualized. The failure to retrieve any baculovirus recombinants by plaque assays, was followed by dot blot analysis of the transfectants.

II.C.2.b. Dot Blot Hybridization Analysis

The transfection mixes obtained from pVLHBc, pVLHBs and pVLHBs(M) were used for this analysis and only one mix was used in each experiment to avoid accidental contamination.

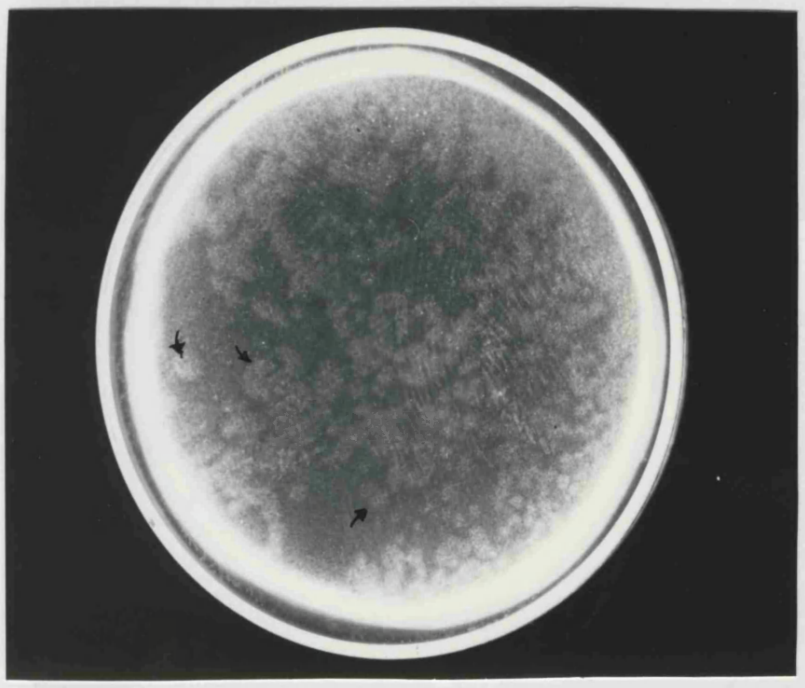
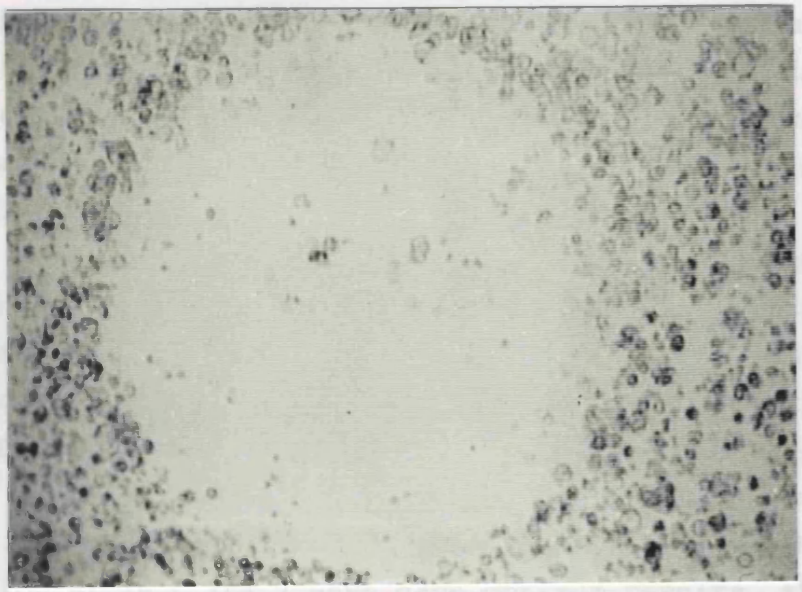


FIG 13: A neutral red stained agarose overlay in a petri dish, showing plaques (marked with an arrow)

(A)



(B)

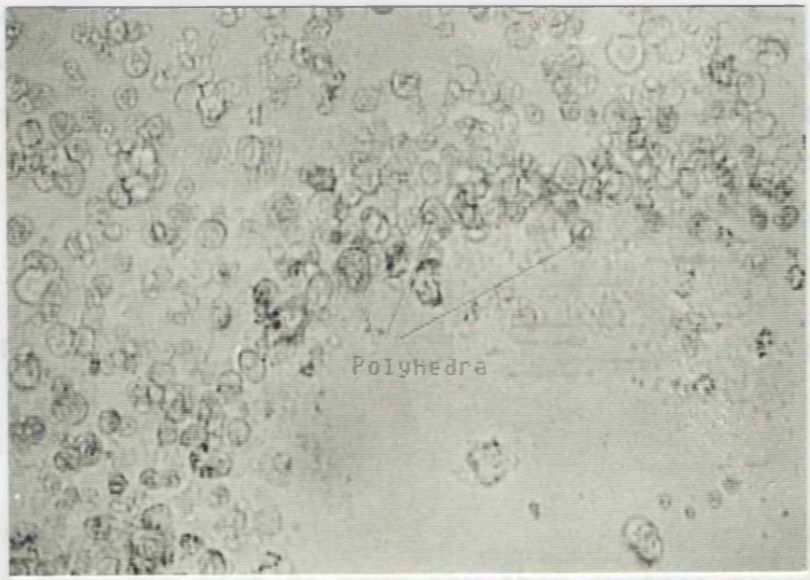


FIG 14: Wild type baculovirus plaques.

Polyhedra can be easily missed under lower magnification (A) but can just be seen at the periphery of the same plaque under higher (400x) magnification (B).

Dilutions were prepared from 10^{-2} and 10^{-5} and 70 µl of each dilution was used to infect $1.5x10^4$ Spf cells attached to the wells of a 96 well plate. Four plates were set up for each mix for the primary screening. In addition, 10 and 20 ng of pVLHBc or pVLHBs DNA was added to wells in duplicate as a positive control, whereas 500 ng of AcNPV DNA alone was added to two wells as a negative control. Two plates were incubated for 5 days and the other two for 7-8 days, at 28°C. At the end of the incubation, before transferring the supernatant from each well to the master plate, the wells were visualized using an inverted microscope. The polyhedra present in the infected cells were scored as 3+ for 10^{-2} and 10^{-3} dilutions, and 2+ for all the other dilutions. The results were the same for all the plates infected with three transfection mixes. From primary screening experiments, it was established that incubation at 28°C for 7 days gave optimum results, therefore all incubations thereafter, were carried out for 7-8 days.

The infected cells were lysed and transferred onto a nylon membrane in a manifold apparatus, and aspirated. The membrane was dried, UV fixed, prehybridized and hybridized with $1x10^7$ cpm of radioactively labelled HBV DNA probe and exposed to an X-ray film for 1-5 days.

II.C.2.b.(1) Screening for AcNPV-HBs Recombinant: AcHBs

Primary Screening

After exposure to an X-ray film, only two signals were obtained from one blot of four for this surface recombinant. The strong signal C2 and faint signal B1 were present in the 10^{-2} dilution [fig 15] and the supernatants from both of the wells were used for secondary screening. In this plate, no positive control signal could be detected [fig 15, wells A3-A6] as no pVLHBs DNA was loaded by mistake.

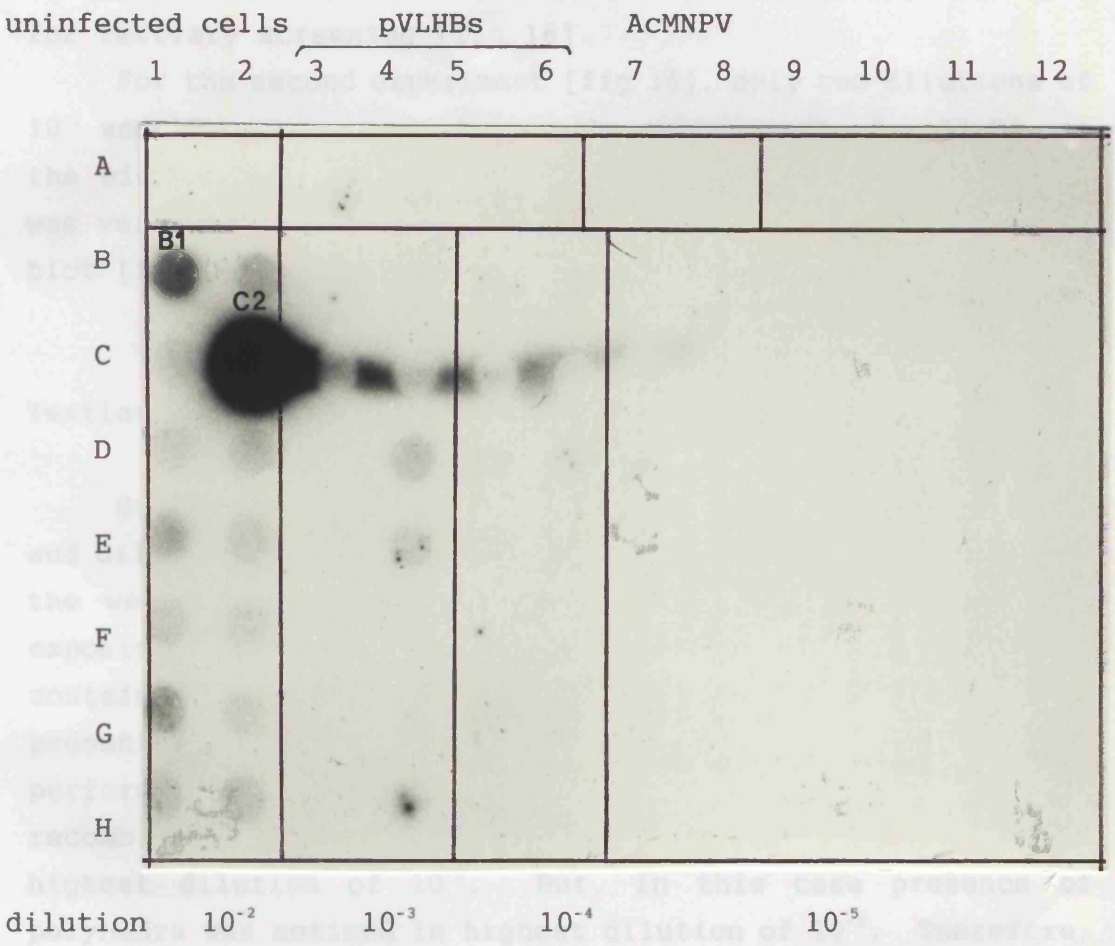
FIG 15: Primary screening for the recombinant baculovirus AcHBs.

Supernatant obtained from primary transfection yield was diluted from 10^{-2} to 10^{-5}.

Wells:

A1-A2	uninfected Spf cells
A3-A6	pVLHBs DNA (Note, DNA was not added to these wells by mistake)
A7-A8	500 ng AcMNPV DNA
A9-A12	empty

(Supernatant from wells B1 and C2 was used for secondary screening)


uninfected cells
pVLHBs
AcMNPV
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
B1
C2
dilution 10^{-2} 10^{-3} 10^{-4} 10^{-5}

Secondary Screening

Supernatants from wells B1 and C2 were diluted and used to infect Spf cells attached to one 96 well plate. For one experiment [fig 16], dilutions were prepared from supernatant of well C2, between 10^{-2} and 10^{-5}. All the wells had polyhedra with either 3+ or 2+ score. After exposure of the blot to an X-ray film, a moderate to strong positive signal was present in all of the wells and the supernatant from well B8 was used for tertiary screening [fig 16].

For the second experiment [fig 16], only two dilutions of 10^{-2} and 10^{-3} were prepared from the supernatant of well B1, as the signal obtained from this well in the primary screening was very faint. No signal was detected after exposure of the blot [fig 16].

Tertiary Screening

Supernatant from well B8 was used for tertiary screening and dilutions were prepared between 10^{-3} and 10^{-8} [fig 17]. All the wells had polyhedra with a score of 2+ or 1+. After exposing the blot, a signal was detected in all the wells containing dilutions 10^{-3} and 10^{-4} and many strong signals were present up to dilution 10^{-8}[fig 17]. In tertiary dot blots performed by Fung et al [1988], no contamination of recombinant virus with the wild type virus was noticed in the highest dilution of 10^{-8}. But, in this case presence of polyhedra was noticed in highest dilution of 10^{-8}. Therefore, supernatant from well C12 was used to perform quaternary screening in order to have recombinants without polyhedra.

Quaternary Screening

For this screening, dilutions were prepared between 10^{-4} and 10^{-12} [fig 18]. Polyhedra were present in many wells

FIG 16: Secondary screening of AcHBs.

One part of the plate was used for dilutions prepared from well C2 and the other was used for dilutions prepared from well B1 [fig 15].

Wells:

A1-A2	uninfected Spf cells
A3-A4	10 ng of pVLHBs DNA
A5-A6	20 ng of pVLHBs DNA
A7-A8	500 ng of AcMNPV DNA
A9-A12	empty

(Supernatant from well B8 was used for tertiary screening)

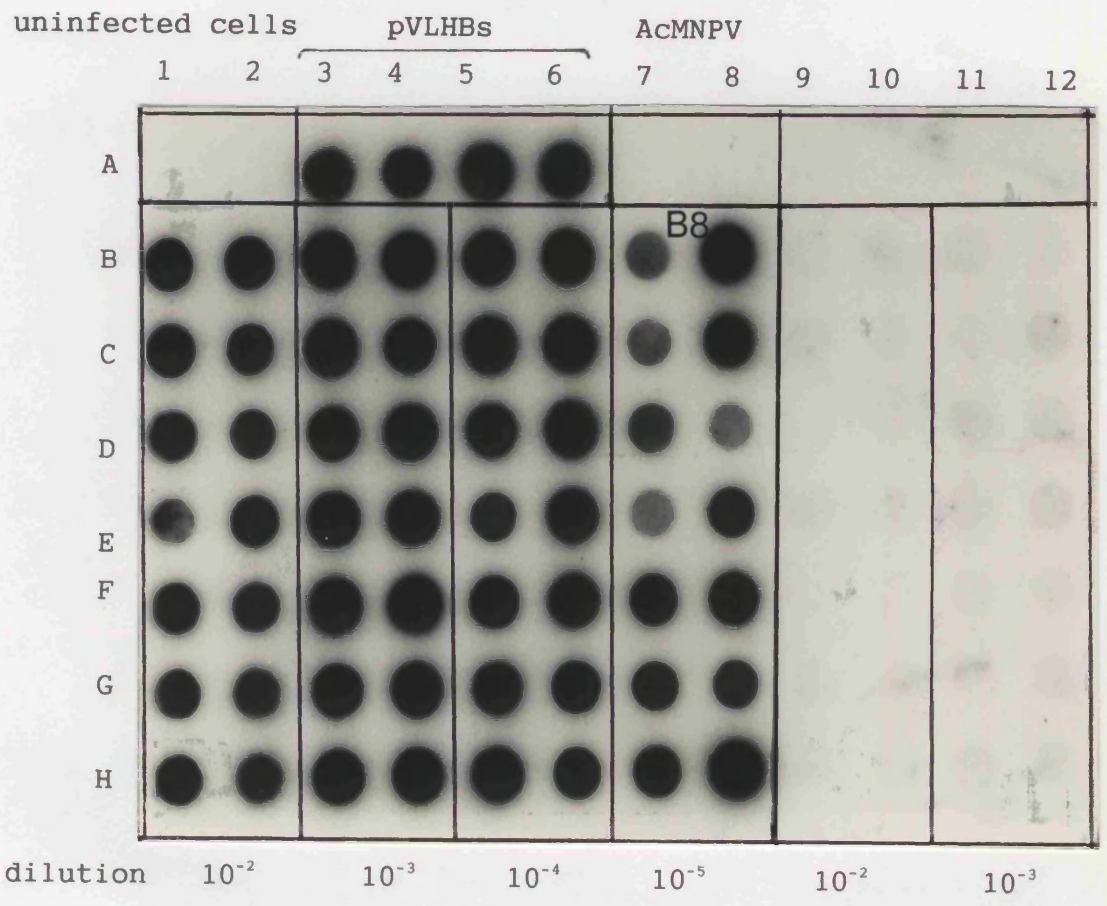

uninfected cells
pVLHBs
AcMNPV
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
B8
dilution
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-2}
10^{-3}

FIG 17: Tertiary screening of AcHBs.

Supernatant from well B8 of secondary screening was used to make dilutions between 10^{-3} and 10^{-8} [fig 16].

Wells:

A1-A2	Uninfected Spf cells
A3-A4	10 ng of pVLHBs DNA
A7-A8	500 ng of AcMNPV DNA
A5-A6	empty
A9-A12	empty

(Supernatant from well C12 was used for quaternary screening)

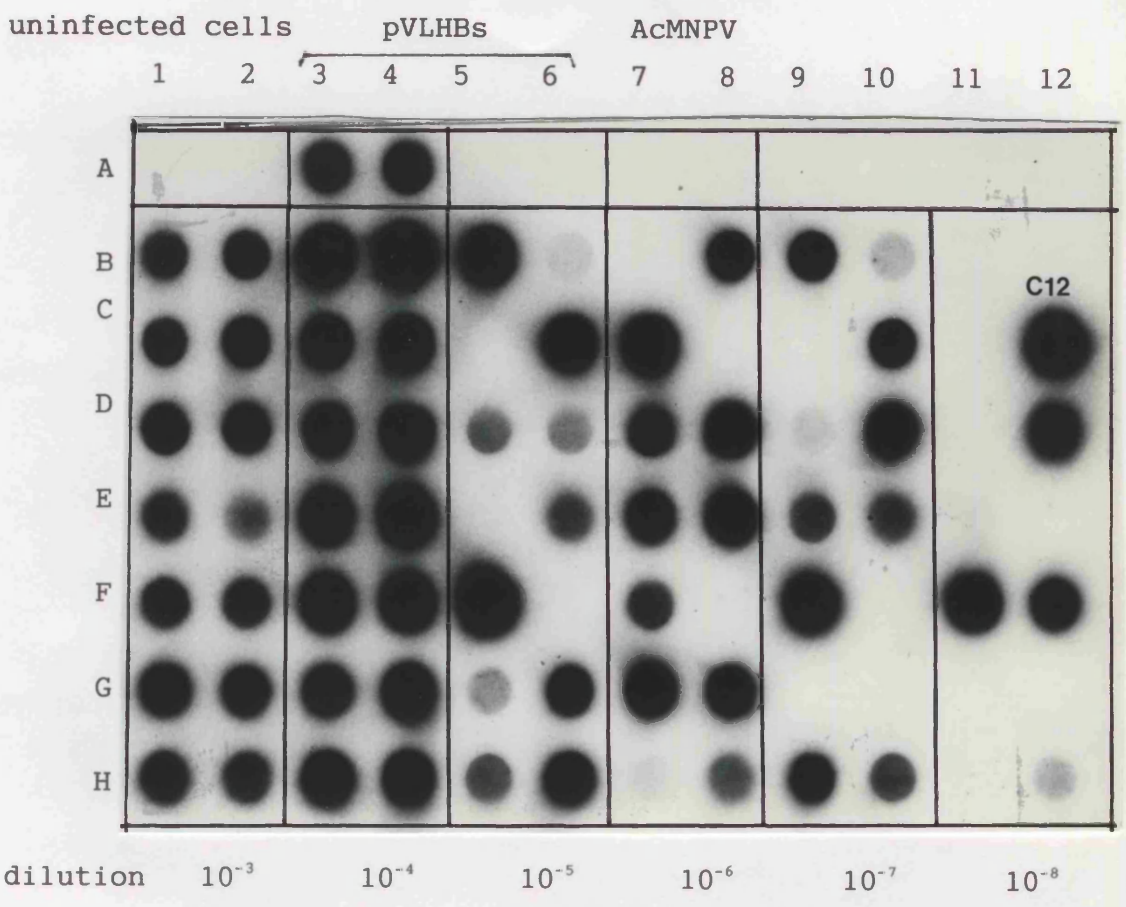

uninfected cells
pVLHBs
AcMNPV
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
C12
dilution 10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} 10^{-8}

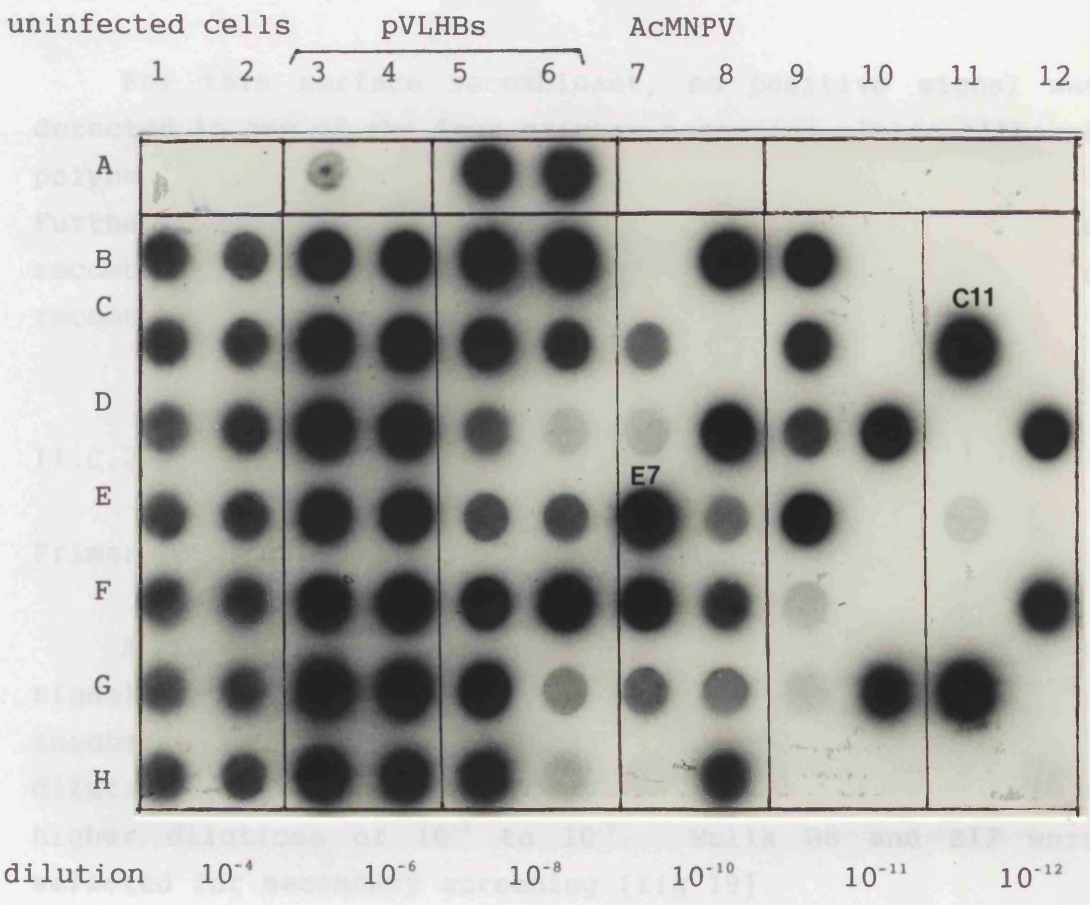
FIG 18: Quaternary screening of AcHBs.

Supernatant from well C12 was used for preparing dilutions between 10^{-4} and 10^{-12} [fig 17].

Wells:

A1-A2	Uninfected Spf cells
A3	1 ng of pVLHBs DNA (no DNA was added to well A4 by mistake)
A5-A6	20 ng of pVLHBs DNA
A7-A8	500 ng of AcMNPV DNA
A9-A12	empty

(Supernatants from wells E7 and C11 were used for further analysis)


uninfected cells
pVLHBs
AcMNPV
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
C11
E7
dilution 10^{-4} 10^{-6} 10^{-8} 10^{-10} 10^{-11} 10^{-12}

containing dilutions between 10^{-4} and 10^{-10} usually with 1+ score. The blot, after exposure showed a positive signal in all the wells up to 10^{-6} and many strong signals were detected in higher dilutions. Supernatants from two wells C11 and E7 which had no polyhedra were used for growth of recombinants for further analysis [fig 18].

II.C.2.b.(2). Screening for AcNPV-HBs(M) Recombinant: AcHBs(M)

For this surface recombinant, no positive signal was detected in any of the four primary screening plates although polyhedra were present in the wells as mentioned earlier. Further dot blot analysis was not carried out for this recombinant, as work had progressed for the other two recombinants under study.

II.C.2.b.(3). Screening for AcNPV-HBc Recombinant: AcHBc

Primary Screening

After exposure of the blot to an X-ray film, positive signals were obtained from one set of plates which had been incubated for 7-8 days. A faint signal was present in the 10^{-2} dilution, whereas many wells were giving a strong signal in higher dilutions of 10^{-3} to 10^{-5}. Wells B8 and E12 were selected for secondary screening [fig 19].

Secondary Screening

Supernatants from wells B8 and E12 of primary screening, were diluted from 10^{-3} to 10^{-7} and used to infect Spf cells attached to wells of 96 well plate in such a way that cells in one half of the plate were infected with dilutions from well

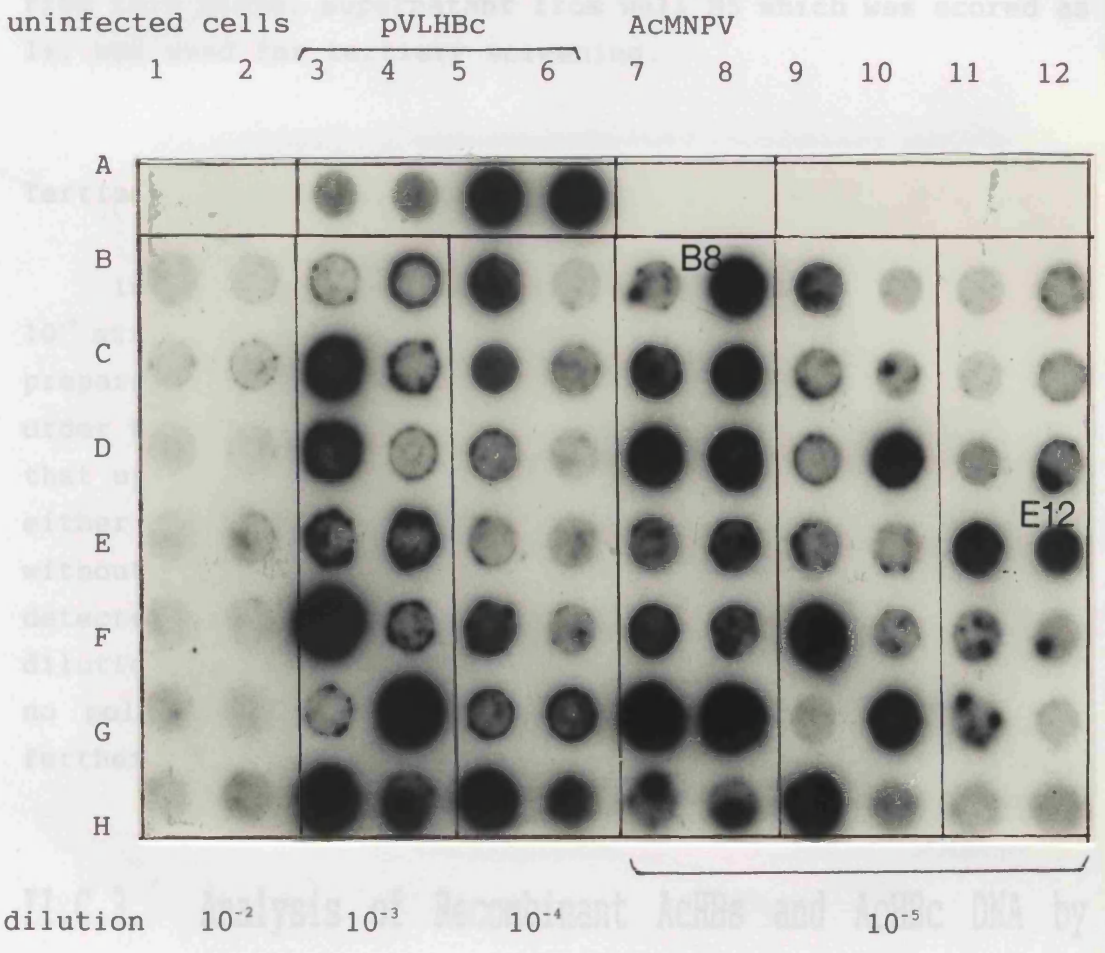
FIG 19: Primary screening for the recombinant baculovirus AcHBc.

Supernatant obtained from primary transfection yield was diluted between 10^{-2} and 10^{-5}.

Wells:

A1-A2	Uninfected Spf cells
A3-A4	1 ng pVLHBc DNA
A5-A6	20 ng pVLHBc DNA
A7-A8	500 ng AcMNPV DNA
A9-A12	empty

(Supernatants from wells B8 and E12 were used for performing the secondary screening)


uninfected cells
pVLHBc
AcMNPV
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
B8
E12
dilution 10^{-2} 10^{-3} 10^{-4} 10^{-5}

B8 and the other half from well E12 [see fig 20]. Polyhedra were present in all the wells with 10^{-3} to 10^{-5} dilutions, and were scored as 3+ and 2+ respectively, whereas wells in 10^{-7} dilution were scored as 2+ or 1+. A faint to moderate signal was detected from the blot after exposure to an X-ray film, from wells containing 10^{-3} dilutions, whereas a strong signal was present in higher dilutions of 10^{-5} and 10^{-7}, for both the experiments [fig 20]. It was observed that all wells gave a positive signal, although weak in the lower dilution of 10^{-3}. From this plate, supernatant from well H5 which was scored as 1+, was used for tertiary screening.

Tertiary Screening

In tertiary screening performed for AcHBs, dilution upto 10^{-9} still contained polyhedra, therefore higher dilutions were prepared for this screening between 10^{-4} and 10^{-12} [fig 21], in order to obtain polyhedrin free recombinants. It was observed that up to the 10^{-6} dilution, wells still had polyhedra with either 2+ or 1+ score, but in higher dilutions some wells were without polyhedra. On exposure of the blot, a signal was detected from many wells which was stronger in higher dilutions. Supernatants from wells D12, F6 and F7 which had no polyhedra, were used for growing the recombinants for further analysis.

II.C.3. Analysis of Recombinant AcHBs and AcHBc DNA by Restriction Enzyme Digestion

This analysis was done in order to confirm genetic recombination event between baculovirus and HBsAg/HBcAg encoding sequences.

FIG 20: Secondary screening of AcHBc.

Supernatants from wells B8 and E12 were used for making dilutions between 10^{-3} and 10^{-7} [fig 19] and one half of the plate was used for each set of dilutions.

Wells:

A1-A2	Uninfected Spf cells
A3 & A5	10 ng pVLHBc DNA
A4 & A6	20 ng pVLHBc DNA
A7-A8	500 ng of AcMNPV DNA
A9-A12	empty

(Supernatant from well H5 was used for tertiary screening)

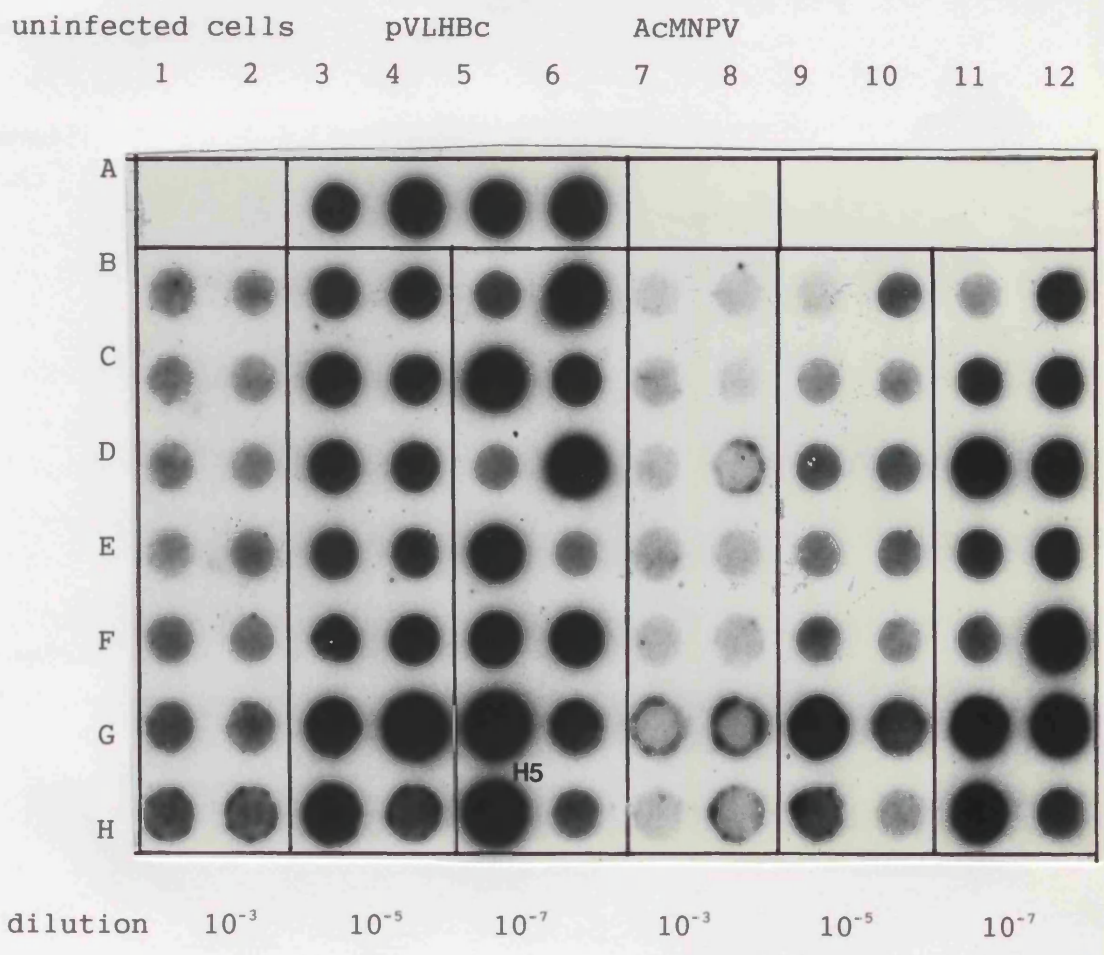

uninfected cells
pVLHBc
AcMNPV
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
H5
dilution 10^{-3} 10^{-5} 10^{-7} 10^{-3} 10^{-5} 10^{-7}

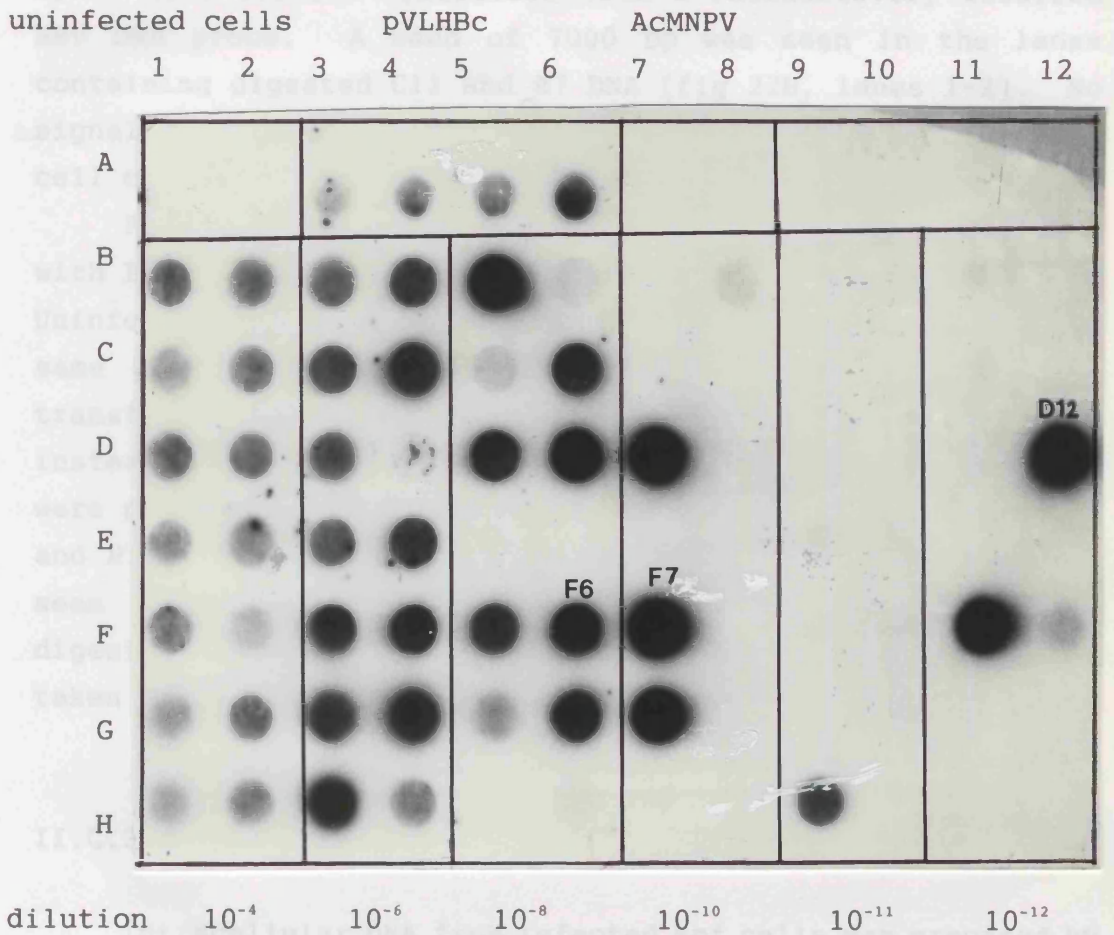
FIG 21: Tertiary Screening of AcHBc.

Supernatant from well H5 was used for making dilutions between 10^{-4} and 10^{-12} [fig 20].

Wells:

A1-A2	Uninfected Spf cells
A3 & A5	1 ng pVLHBc DNA
A4 & A6	5 ng of pVLHBc DNA
A7-A8	500 ng of AcMNPV DNA
A9-A12	empty

(Supernatants from wells D12, F6 and F7 were used for further analysis)


uninfected cells
pVLHBc
AcMNPV
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
D12
F6
F7
dilution 10^{-4} 10^{-6} 10^{-8} 10^{-10} 10^{-11} 10^{-12}

II.C.3.a. AcHBs

Recombinant DNA was prepared from infected Spf cells from stocks grown from two wells C11 and E7 [fig 18], along with uninfected Spf cells and AcMNPV controls. 1-2 µg of DNA from both surface clones, and the controls was digested with EcoRI and run on a 1% agarose gel. Unique low molecular weight bands were visible in all of the lanes except for the cell control sample [fig 22A]. The DNA was transferred on to a nylon membrane and hybridized with a radioactively labelled HBV DNA probe. A band of 7000 bp was seen in the lanes containing digested C11 and E7 DNA [fig 22B, lanes 1-2]. No signal was detected in the lanes containing uninfected Spf cell or AcMNPV DNA [fig 22B, lanes 3-4].

Further analysis of clone C11 was done by digesting DNA with EcoRI, HindIII and TaqI and running on an agarose gel. Uninfected Spf cell and AcMNPV DNA were also loaded on the same gel to serve as controls [fig 23A]. The DNA was transferred and hybridized with surface fragment 'a' probe, instead of entire HBV probe. Two bands of 6000 and 7000 bp were present in the lanes containing EcoRI [fig 22B, lane 1] and HindIII [fig 23B, lane 2] digested C11 DNA. A smear was seen for TaqI digested C11 DNA, probably due to a partial digest. The results suggest that recombination event has taken place between baculovirus and surface genes.

II.C.3.b. AcHBc

Intracellular DNA from infected Spf cells was prepared by growing stocks from three wells D12, F6 and F7 [fig 21] along with uninfected Spf cells and AcMNPV controls. 1-2 µg of DNA from core clones and the controls was digested with EcoRI and was run on a 1% agarose gel. As seen above, unique low molecular weight bands were visible in all of the lanes [fig 24A, lanes 1-4] except for the Spf cell control DNA [lane 5]. The DNA was transferred to a nylon membrane and hybridized with

FIG 22: Electrophoresis of EcoRI digested DNA, prepared from recombinant AcHBs clones, on a 1% agarose gel (A), followed by Southern blot hybridization with a radioactively labelled HBV probe (B).

Lane:	
1	C11 DNA
2	E7 DNA
3	EcoRI digested AcMNPV DNA
4	EcoRI digested Spf cell DNA
5	500 ng undigested pVLHBs DNA
7-8	1 µg of radioactively labelled HindIII digested lambda marker DNA (size of marker DNA fragments is shown in kb)

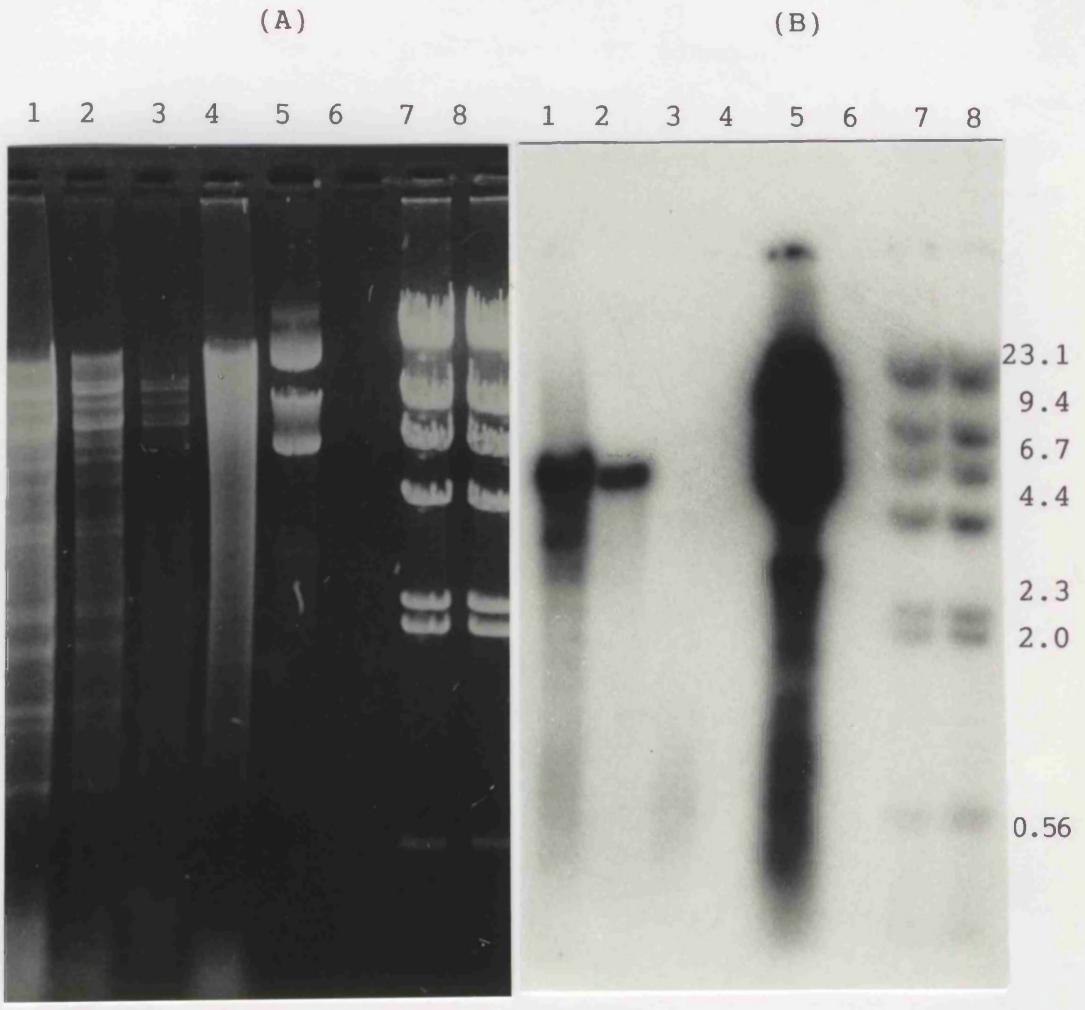

(A)
(B)
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
23.1
9.4
6.7
4.4
2.3
2.0
0.56

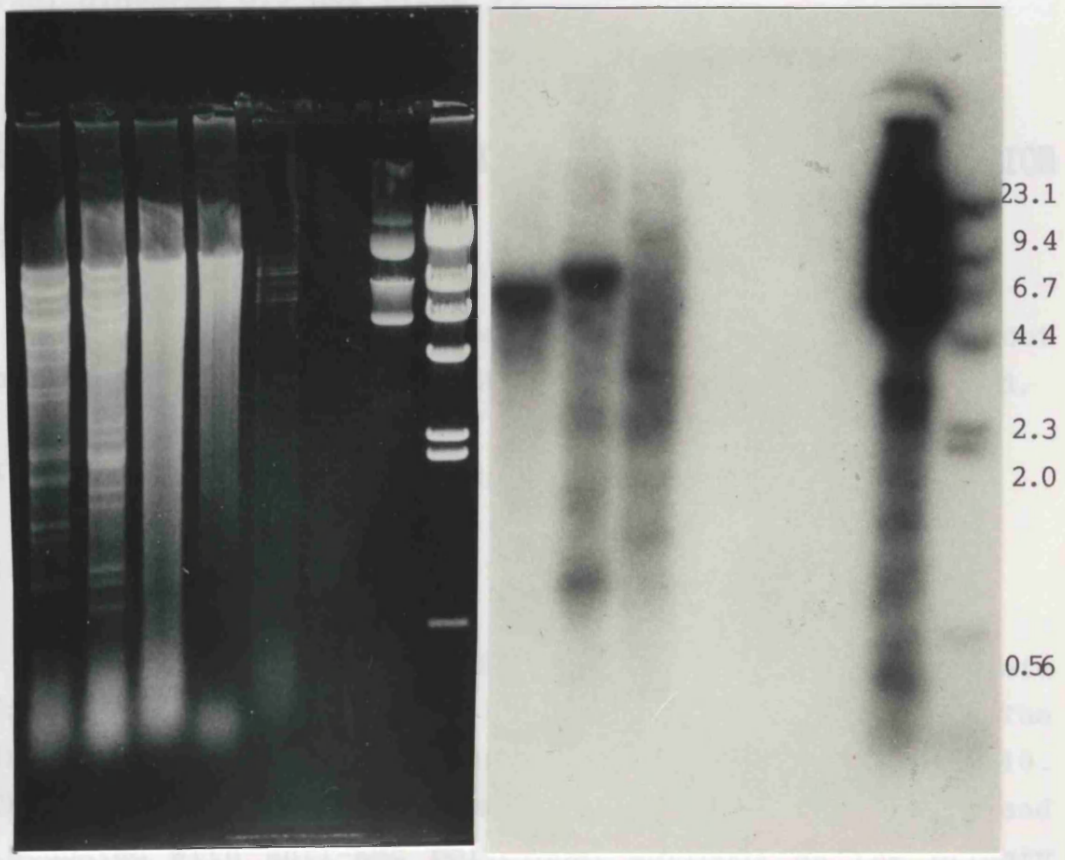
FIG 23: Electrophoresis of EcoRI, HindIII and TaqI digested AcHBs C11 DNA on a 1% agarose gel (A), and Southern blot hybridization with a radioactively labelled HBV subgenomic fragment 'a' probe, containing the S gene (B).

Lane:

1	EcoRI digested C11 DNA
2	HindIII digested C11 DNA
3	TaqI digested C11 DNA
4	EcoRI digested Spf cell DNA
5	EcoRI digested AcMNPV DNA
7	500 ng undigested pVLHBs DNA
8	1 µg of radioactively labelled HindIII digested lambda marker DNA (size of marker DNA fragments is shown in kb)

(A) (B)

1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8



$1x10^7$ cpm of radioactively labelled HBV DNA probe. A band of 6500 bp was present in the lanes containing AcHBc clones [fig 24B, lanes 1-3] whereas no band was present in the lane containing the AcMNPV control sample [lane 5].

The D12 core clone was further analyzed by digesting 1-2 µg of DNA with EcoRI, TaqI and HindIII and running on an agarose gel [fig 25A]. The DNA was transferred and hybridized with a radioactively labelled core fragment `d' probe. Two bands of 6500 bp and 7500 bp were present in the lanes containing the EcoRI [fig 25B, lane 1] and HindIII [lane 2] digested D12 DNA, wheras no definite band could be seen for TaqI digested D12 DNA [lane 3].

II.C.4. Analysis of Expression of HBsAg/HBcAg from Recombinant Baculoviruses

To determine whether HBsAg and HBcAg were expressed, dot blot immunoassay and SDS-PAGE/Immunoblotting was performed.

II.C.4.a. Dot Blot Immunoassay

II.C.4.a.(1). AcHBc

Supernatant from well D12 of the tertiary screening plate [fig 21] was used to prepare dilutions from 10^{-4} and 10^{-12}. The immunoassay was performed as described in section II.B.10. The membrane was prepared and blocked with 10% PBS-MP and incubated with anti-HBc polyclonal antibody as the primary antibody. After washing, it was incubated with goat anti-rabbit antibody conjugated with HRP. The membrane was washed and incubated for 15 minutes with colour development substrate. A faint signal was detected in many wells including two Spf cell control wells, whereas the only strong positive signal was present in five positive control wells containing IPTG-induced cultures of ptacHpaII-R2 and pPV404

FIG 24: Electrophoresis of EcoRI digested DNA, prepared from AcHBc clones, on a 1% agarose gel (A), followed by Southern blot hybridization with a radioactively labelled HBV probe (B).

Lane:

1	F6 DNA
2	F7 DNA
3	D12 DNA
4	EcoRI digested AcMNPV DNA
5	EcoRI digested Spf cell DNA
6	200 ng undigested pVLHBc DNA
7-8	1 µg of radioactively labelled HindIII digested lambda marker DNA (size of marker DNA fragments is shown in kb)

(A) (B)

1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8

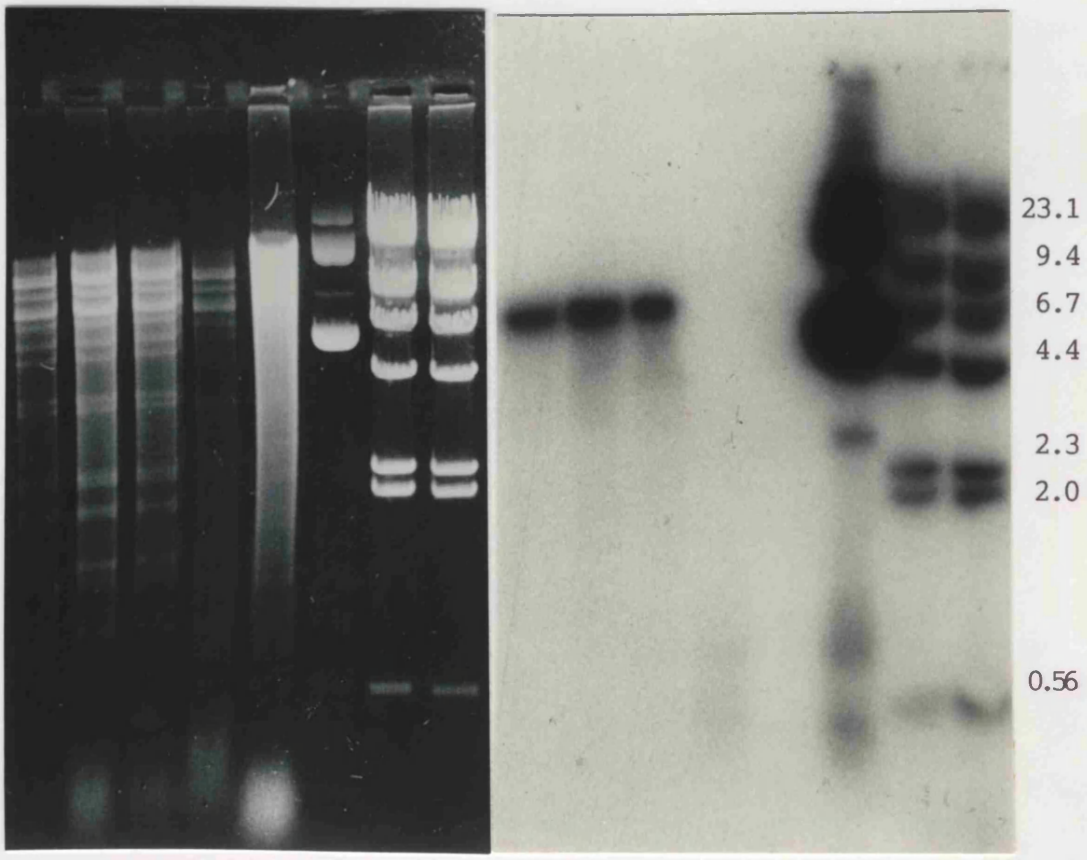
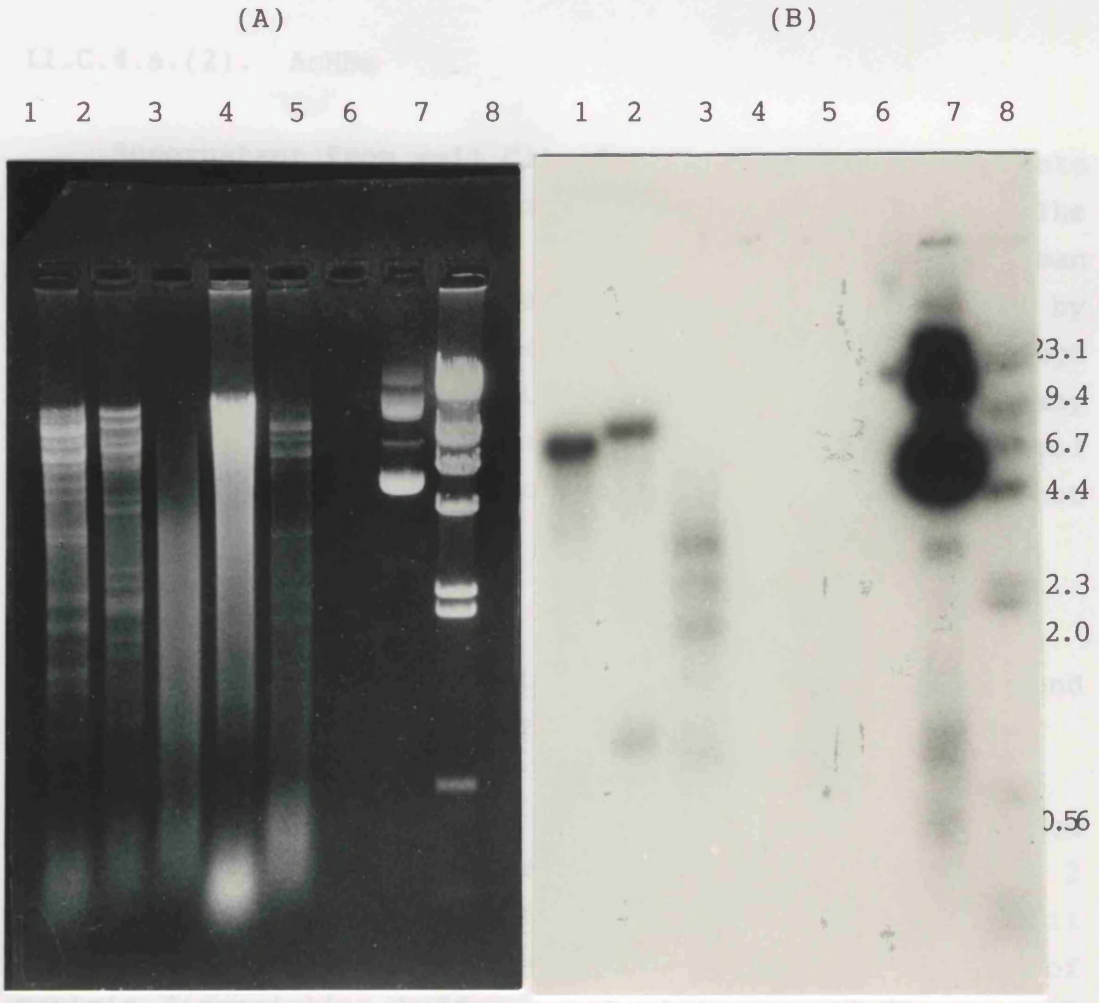


FIG 25: Electrophoresis of EcoRI, HindIII and TaqI digested AcHBc D12 DNA, on a 1% agarose gel (A) and Southern blot hybridization with a radioactively labelled HBV subgenomic fragment 'd' probe, containing the C gene (B).

Lane:

1	EcoRI digested D12 DNA
2	HindIII digested D12 DNA
3	TaqI digested D12 DNA
4	EcoRI digested Spf cell DNA
5	EcoRI digested AcMNPV DNA
7	200 ng pVLHBc DNA
8	1 µg of radioactively labelled HindIII digested lambda marker DNA (size of marker DNA fragments is shown in kb)


(A)
(B)
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
23.1
9.4
6.7
4.4
2.3
2.0
0.56

(chapter III) [fig 26, wells A7-A11]. Similar results were obtained after using antibodies that had been preabsorbed with Spf cells.

One strip from a separate HBc blot was used for screening with anti-HBe monoclonal antibody as a primary antibody. No definite results were obtained with this antibody as well [resuts not shown].

II.C.4.a.(2). AcHBs

Supernatant from well C11 of quaternary screening plate [fig 18] was used for performing dot blot immunoassay. The membrane was prepared as above and incubated with human anti-HBs antibody. The presence of reactivity was detected by using goat anti-human antibody conjugated to HRP. Although antibody reactivity in this immunoassay was stronger in many wells than the core immunoblot, uninfected Spf cell control showed faint a reaction as observed previouly [results not shown].

II.C.4.b. Radioactive Labelling of Recombinant Proteins and SDS-PAGE/Immunoblotting Analysis

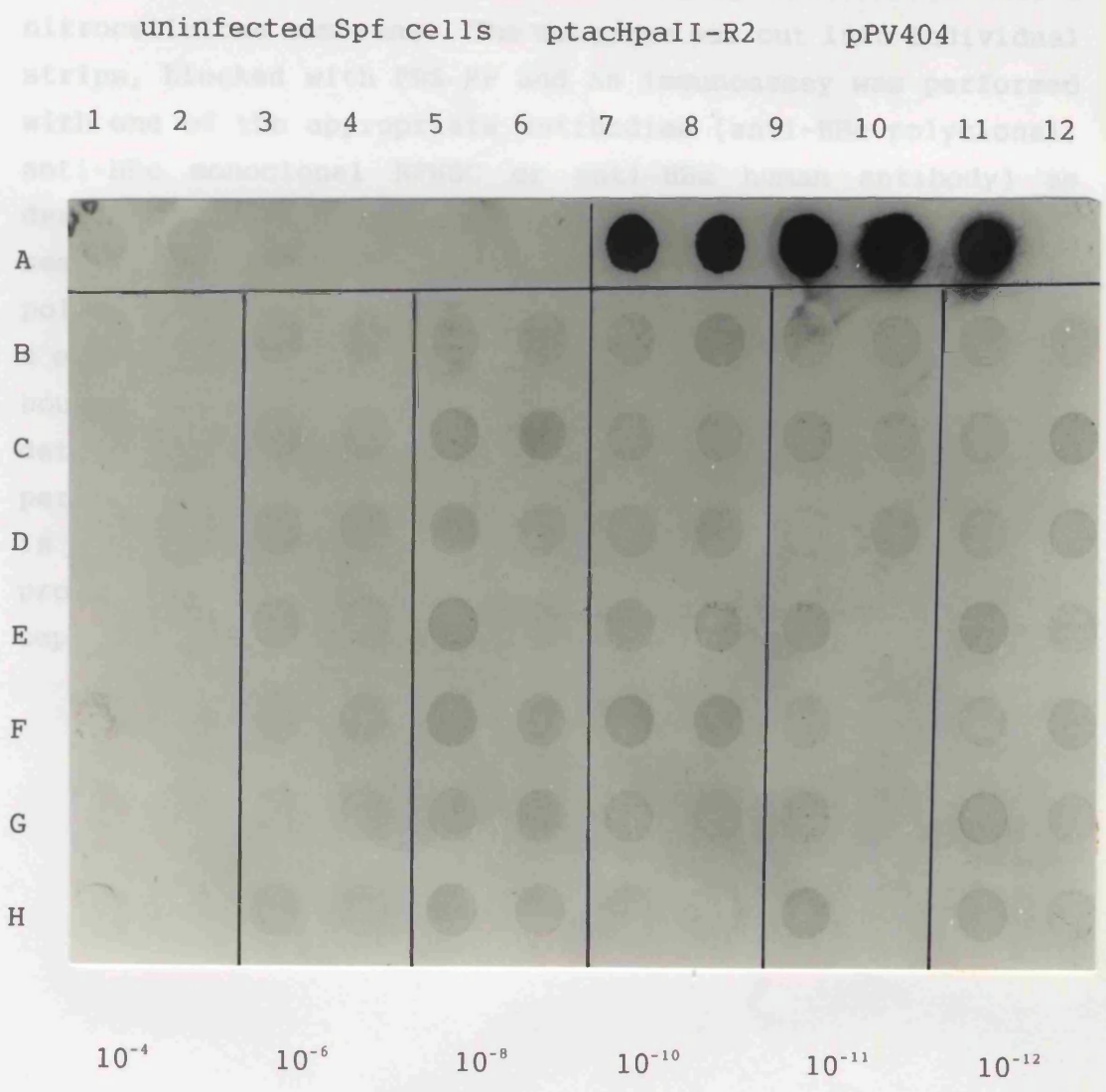
Two core recombinants D12 and F7, and two surface recombinants C11 and E7 were radioactively labelled for 2 hours as described. Ten µl of each of the 24 and 48 hour cell pellet suspension, was heated to 95°C with same amount of protein dissociation buffer and loaded on a 12.5% SDS-PAGE gel, along with control samples. Separate gels were run for the HBc and HBs samples. After staining the HBc gel with coomassie blue, only one conspicuous band of 30 kD was noticed in the lane containing the AcMNPV 48 hour control sample, which was the predicted molecular weight of the the polyderin protein [fig 27A]. No band indicative of the presence of the HBc protein was visualized. Similar results were obtained

FIG 26: Immunoblot analysis of HBc baculovirus recombinants, using rabbit anti-HBc polyclonal antibody.

Wells:

A1-A6	Uninfected Spf cells
A7-A8	10 µl of IPTG-induced culture of ptacHpaII-R2
A9-A11	20 µl of IPTG-induced culture of pPV404
A12	empty

(Wells B to H contain HBc baculovirus recombinants grown from stocks of D12 core clone [fig 21] by making dilutions from 10^{-4} to 10^{-12})


uninfected Spf cells
ptacHpaII-R2
pPV404
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
10^{-4}
10^{-6}
10^{-8}
10^{-10}
10^{-11}
10^{-12}

with the HBs gel [results not shown]. Although both the gels were dried and exposed to an x-ray film for 24 hours, no conclusive results were obtained [fig 27B].

The analysis of HBc and HBs proteins was extended by Western blotting, despite inconclusive results. Several 24 and 48 hour cell pellet samples of HBc (D12), HBs (C11) and control AcMNPV and Spf cell proteins were run on an SDS-PAGE gel as above and were electrophoretically transferred onto a nitrocellulose membrane. The membrane was cut into individual strips, blocked with PBS-MP and an immunoassay was performed with one of the appropriate antibodies (anti-HBc polyclonal, anti-HBc monoclonal RFHBC or anti-HBs human antibody) as described [section III.B.21]. Several non-specific bands were seen when the immunoassay was performed with the anti-HBc polyclonal antibody. When anti-HBc monoclonal RFHBC was used, a specific band of 40-42 kD was present for both the 24 and 48 hour cell pellet samples of HBc D12 [fig 28]. No reaction was detected with anti-HBs human antibody. The analysis was performed for only two clones for each recombinant, but there is a probability that if more clones were tested for protein production, there was a chance of identifying a clone that was capable of producing a detectable level of HBsAg.

FIG 27: Radioactive labelling of AcHBc recombinant and control proteins present in infected Spf cell pellets, with ^{35}S methionine, for 24 and 48 hours.

The recombinant and control proteins were separated by SDS-PAGE and stained with Coomassie blue (A). The gel was also dried and exposed to Kodak XAR film (B).

Lane:

1-2	Uninfected Spf cell proteins obtained after 24 and 48 hours of labelling
3-4	proteins from AcHBc F7 infected Spf cells
5-6	proteins from AcHBc D12 infected Spf cells
7-10	proteins from AcMNPV infected Spf cells
11	empty
12	rainbow protein marker (molecular weight shown in kD)

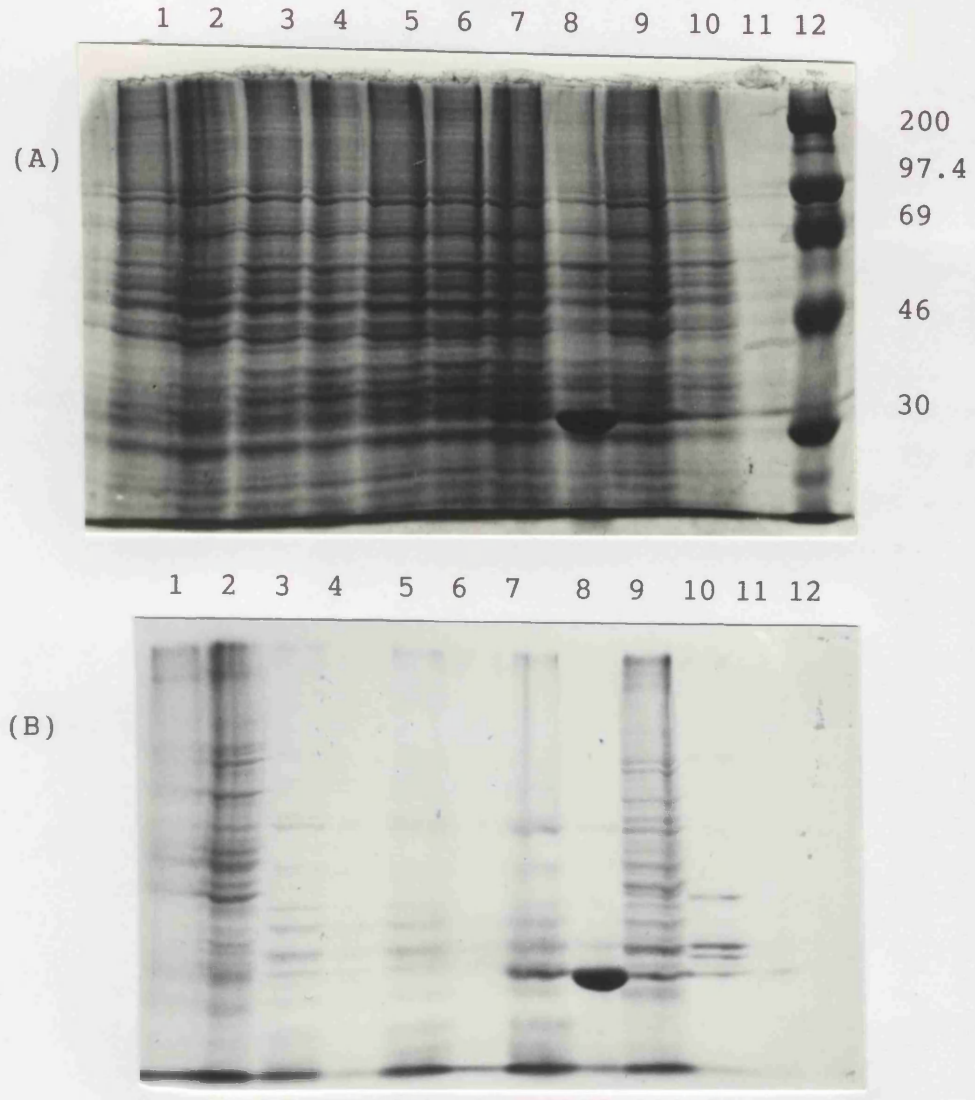
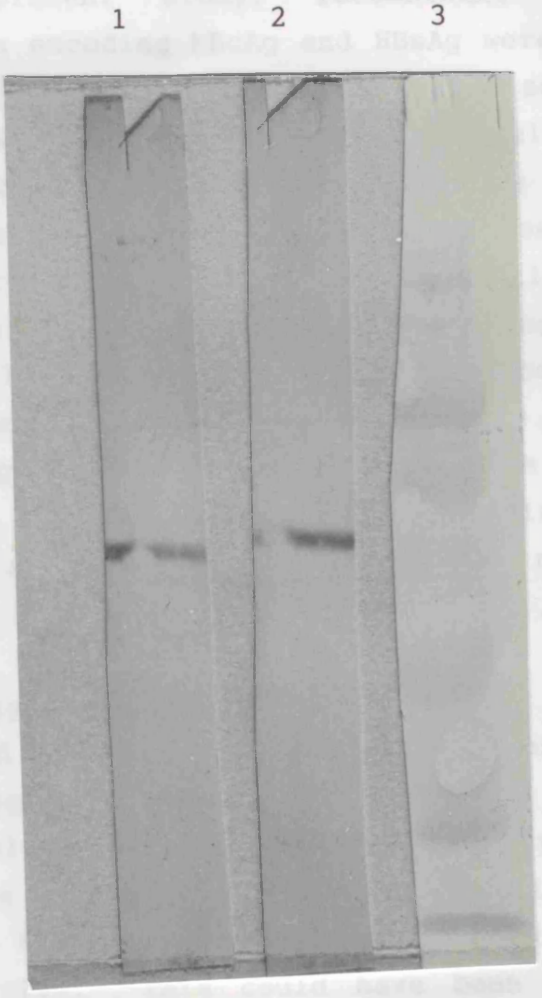

1 2 3 4 5 6 7 8 9 10 11 12
(A)
200
97.4
69
46
30
1 2 3 4 5 6 7 8 9 10 11 12
(B)

FIG 28: Western blot analysis of AcHBc F7 and D12 recombinant proteins, with anti-HBc monoclonal antibody.

The recombinant proteins present in infected Spf cells were separated by SDS-PAGE and transferred onto a nitrocellulose membrane as described in the text.

Strip:

1	F7 AcHBc
2	D12 AcHBc
3	rainbow protein marker (molecular weight shown in kD).


1
2
3
200
97.4
69
46
30
21.5
14.3

II.D. DISCUSSION

In the present study, recombinant baculoviruses containing genes encoding HBcAg and HBsAg were detected and purified. This was achieved by carrying out serial dot blot hybridization analysis of transfection yields, following failure to purify recombinant baculoviruses by the usual method of plaque purification. Although researchers have isolated and purified HBV core and surface antigen expressing recombinant baculoviruses by plaque assays [Kang et al., 1987; Lanford et al., 1988; section II.A], this method has proved to be subjective and laborious (section II.A.5). Especially, visual screening of the recombinant phenotype is a critical step and often a high percentage of potential recombinant plaques picked in the first round turn out not to contain recombinant DNA [Malitschek and Schartl, 1991]. Indeed, this difficulty has been experienced during the course of the present investigations (section II.C.2.a). In addition, results obtained with plaque assays for both surface and core recombinants suggest that the recombination frequency was consistently below 1%. The presence of only one positive clone for pVLHBs [C2, fig 15] and none for pVLHBs(M) surface recombinant, in the primary screen of dot blot hybridization confirms this view. This could have been the result of inefficient transfection or overgrowth of wild type virus (as compared to fewer recombinants), in transfected insect cells [Bradley, 1990]. Because of such problems, several improved methods for easy identification and isolation of recombinant baculoviruses have been published [Fung et al., 1988; Hartig and Cardon, 1991; Malitschek and Schartl, 1991; Manns and Grosse, 1991; Shanafelt, 1991].

The presence of recombinant baculoviruses was confirmed by digesting DNA, obtained from infected insect cells, with

restriction enzymes and analyzing it by Southern hybridization using HBV and subgenomic core and surface probes. This was followed by analysis of expression of HBsAg and HBcAg from both recombinant baculoviruses. In dot blot immunoassays using rabbit anti-HBc polyclonal and human anti-HBs antibodies, nonspecific results were obtained because of background staining. This is a recognized problem and preabsorbtion of antibodies with eukaryotic cells has been recommended for better results [Bradley, 1990]. However, in the present study, no improvement was noticed after using preabsorbed antibodies (section II.C.4.a). Although, monoclonal antibodies were not used for detecting both proteins in dot blot immunoassays, it is speculated that meaningful results could have been obtained by using these antibodies, keeping in view the results obtained with SDS-PAGE/Immunoblotting (below).

In this study, a protein with an estimated molecular mass of 40-42 kD was detected by SDS-PAGE/Immunoblotting, using an anti-HBc monoclonal antibody. Although presence of a core protein of this molecular mass has not been reported, there is a possibility that this could be due to existence of core protein dimers, as observed in Xenopus oocytes injected with a synthetic mRNA encoding the HBV core protein [Zhou et al., 1992; Zhou and Standring, 1992].

In another study, a recombinant baculovirus expression vector was constructed to express HBV core protein [Beames et al., 1991]. A polypeptide with an expected molecular mass of 21 kD was present and this originated from the authentic core initiation codon. In addition, an unexpected polypeptide with a molecular mass of 24 kD was also present. This was demonstrated to be a polyhedrin-core fusion protein, originating from translational initiation at an altered AUU codon of polyhedrin protein [Beames et al., 1991]. Because a similar transfer vector (pVL941), in which the polyhedrin ATG codon had been altered into ATT to prevent formation of polyhedrin fusion protein, was used in the present project, it was interesting to determine if the 40-42 kD protein was a

similar polyhedrin-core fusion protein. A comparison of the nucleotide sequence of vectors p1393-core [see fig 2 and 4 in Beames et al., 1991] and pVLHBc revealed that the core protein obtained in this study could not have been a fusion protein because the core gene was not in-frame with the altered ATT codon [fig 29].

It is now recommended that several recombinant baculovirus clones containing the gene of interest should be tested for protein production [Bradley, 1990]. In this study, failure to detect HBsAg could be related to the fact that only one positive clone was originally detected in the primary screen by dot blot hybridization. Several positive clones were available for core recombinant and there was a greater probability of isolating one that would express the protein of interest. Even for the core recombinant, out of two confirmed clones, only one was able to express core protein. This suggests that it would be ideal, although more laborious, to work with several recombinant clones isolated from a primary screen.

FIG 29: Comparison of DNA sequence at core gene cloning sites in two similar transfer vectors pVLHBc [T.J.Harrison, unpublished] and p1393-core [Beames et al., 1991].

The bases have been grouped to indicate the appropriate ORF. Small letters designate bases that have been altered as a result of site-directed mutagenesis or added during cloning from polylinker or synthetic sequences. (A) Wild type polyhedrin non-translated region with the tranlation initiation site (underlined) and amino terminal region of the protein to +35. (B) Sequence of p1393-core at the cloning site, aligned at position -9. Altered initiation codon of polyhedrin gene (ATT) is underlined along with core ATG. (C) Sequence of pVLHBc, as determined by direct sequencing (section II.C.1.a).

(A)

Wild type polyhedrin

```
-9        +1                                                    +35
CCTATAAAT ATG CCG GAT TAT TCA TAC CGT CCC ACC ATC GGG CG
```

(B)

p1393-core

```
-9         +1                                                   +35
CCTATAAAT ATt CCG GAT TAT TCA TAC CGT CCC ACC ATC GGG CGc

gga tcc gcc CTT GGG TGG CTT TGG GGC ATG GAC
                                    core
```

(C)

pVLHBc

```
-9         +1                                                   +35
CCTATAAAT ATT CCG GAT TAT TCA TAC CGT CCC ACC ATC GGG CGc

gga tcC TTG GGT GGC TTT AGG GCA TGG
                               core
```

CHAPTER III

EXPRESSION OF HBV CHIMERIC PROTEINS IN A PROKARYOTIC SYSTEM (E.COLI)

III.A. INTRODUCTION

Foreign immunogenic epitopes have been expressed on the surface of self assembling particles such as HBsAg [Valenzuela et al., 1985; Delpeyroux et al., 1986 and 1990], HBcAg [Clarke et al., 1987; Francis and Clarke, 1989; Borisova et al., 1989; Stahl and Murray, 1989; Clarke et al., 1990; Francis et al., 1990; Schödel et al., 1990a and b] and yeast transposon virus-like particles (Ty VLP's) [Adams et al., 1987].

The use of HBcAg to present the foreign epitopes to the immune system offers several advantages. It can be expressed in a wide range of systems, including bacterial [Pasek et al., 1979; Cohen and Richmond, 1982; Stahl et al., 1982], insect [Lanford et al., 1988; Takehara et al., 1988], mammalian [Roossinck et al., 1986] and yeast cells [Kniskern et al., 1986; Beesley et al., 1990] and vaccinia viruses [Clarke et al., 1987]. In addition, HBcAg is highly immunogenic because of its ability to function as a T-cell independent and T-cell dependent antigen [Milich and McLachlan, 1986]. Furthermore, both amino [Clarke et al., 1987; Francis and Clarke, 1989; Francis et al., 1990] and carboxyl [Stahl and Murray, 1989; Borisova et al., 1989] termini of HBcAg have been separately used to express several fusion proteins, as described below.

III.A.1. HBcAg Amino Terminal Fusion

The amino terminus of HBcAg has been fused to amino acids 141-160 of the VP1 protein of foot and mouth disease virus (FMDV) serotype O_1. Although the recombinant protein has proved to be toxic for E.coli cells, it has been efficiently expressed using a vaccinia virus expression system to give rise to a highly immunogenic, particulate product [Clarke et al., 1987]. Subsequently, an E.coli expression vector, pBC404 has been developed where the gene expression is driven by the

tac promoter [Francis and Clarke, 1989]. The RNA transcripts that originate from the tac promoter initiate translation at an AUG codon, overlapped in part by a unique EcoRI site. A second unique site for BamHI has been inserted in the precore region, downstream of the EcoRI site, just upstream of the HBcAg initiation codon [Francis and Clarke, 1989; Francis et al., 1990]. Therefore, providing that translational reading frames are preserved, proteins can be produced which are initiated at the EcoRI AUG and are then translated through any heterologous sequences into the HBcAg gene [Francis and Clarke, 1989]. Using this approach, an immunogenic epitope of poliovirus type 1 [Francis and Clarke, 1989] and an epitope of the human rhinovirus type 2 capsid polypeptide VP2 have been expressed in E.coli and have been found to be highly immunogenic [Francis et al., 1990]. Although the majority of HBcAg amino terminal fusions have been expressed in E.coli systems, Beesley et al., [1990] have described the expression in yeast of recombinant core particles containing the FMDV VP1 peptide (amino acids 142-160) or the carboxyl terminus of the human chorionic gonadotrophin ß-subunit (amino acids 109-145). Both recombinants have been shown to present the epitopes at the surface of the core particles [Beesley et al., 1990]. This shows that a foreign epitope can be fused to the amino terminus of HBcAg without affecting particle integrity and the resulting chimeric core particles exhibit the immunogenicity of the foreign epitope.

III.A.2. HBcAg Carboxyl Terminal Fusion

It has been suggested that the integrity of the carboxyl terminus of HBcAg, which is arginine rich, is essential for HBcAg particle assembly [Clarke et al., 1987; Francis and Clarke, 1989]. Recent data has also confirmed the importance of this region for interaction with nucleic acid and pregenome encapsidation [Nassal, 1992]. However, heterologous amino acid sequences of variable length fused to a truncated

carboxyl terminus have been successfully expressed as recombinant core particles in E.coli [Stahl and Murray, 1989; Borisova et al., 1989].

In one report, plasmids expressing the fusion proteins have been derived from a parent plasmid ptacHpaII, in which the expression is driven by the tac promoter. The plasmid is engineered in a way that it contains the initiation codon of the ß-galactosidase gene fused to nucleotide number 7 of HBV sequence {in the numbering system of Pasek et al., [1979]} which encodes the third HBcAg amino acid. The plasmid contains the coding sequence for the first 144 amino acid residues of HBcAg (thereby deleting aa 145-183) and a HindIII site is present at this position to facilitate ligation to other sequences [Stahl and Murray, 1989]. The coding sequences inserted in different constructs include those for the preS1 (aa 1-20 and 1-36), preS2 (aa 109-134) and S (aa 111-156 and 111-165) epitopes of HBV. In addition, synthetic coding sequences for fragments of the transmembrane region (gp41) of the HIV-1 envelope protein have also been inserted [Stahl and Murray, 1989]. These sequences have been expressed in E.coli to give stable products that assemble to form particles morphologically similar to HBcAg. The products exhibit the immunogenic characteristics of both the HBcAg epitopes and the epitopes carried by the additional sequences [Stahl and Murray, 1989].

In summary, foreign sequences of around 40-65 amino acids can be inserted into the carboxyl terminus of HBcAg before the arginine rich region or by replacing the latter, without influencing the assembly of HBcAg particles [Borisova et al., 1989; Stahl and Murray, 1989].

III.A.3. Direct Comparison of HBcAg Amino and Carboxyl Termini

Both the amino and truncated carboxyl termini of HBcAg

have been separately used for fusion of foreign epitopes in independent expression systems (section III.A.1 and III.A.2). Recently, Schödel et al., [1992] have used epitopes derived from preS1 and preS2 sequences of HBV and i) fused these into the amino terminus of HBcAg, ii) inserted internally between HBcAg amino acids 75 and 83 {thereby deleting residues 76-82} or iii) fused to the HBcAg carboxyl terminus amino acid 156. This strategy could answer the question as to which terminus of HBcAg is better suited for insertion of foreign sequences without disrupting particle integrity.

In this study, a HCMV-gp58 epitope comprising 50 amino acids, was used. By analogy with previous reports, 50 amino acid residues would be appropriate for carboxyl terminal fusion and the hybrid polypeptide would assemble into core particles. But, for amino terminal fusion it would far exceed the limit set by other groups and it would be interesting to determine if this addition could still produce core particles. Thus, the objective of this study was to compare the expression in E.coli, of a foreign sequence (a sequence encoding one of the neutralizing epitopes {gp58} of HCMV), inserted into the amino or the truncated carboxyl terminus of HBcAg.

III.B. MATERIALS AND METHODS

III.B.1. Plasmid Constructions

Three plasmids, pPV404, p73HCMV and ptacHpaII-R2 were used for making two separate HBcAg-amino and HBcAg-carboxyl terminal fusions with HCMV-gp58 encoding sequence.

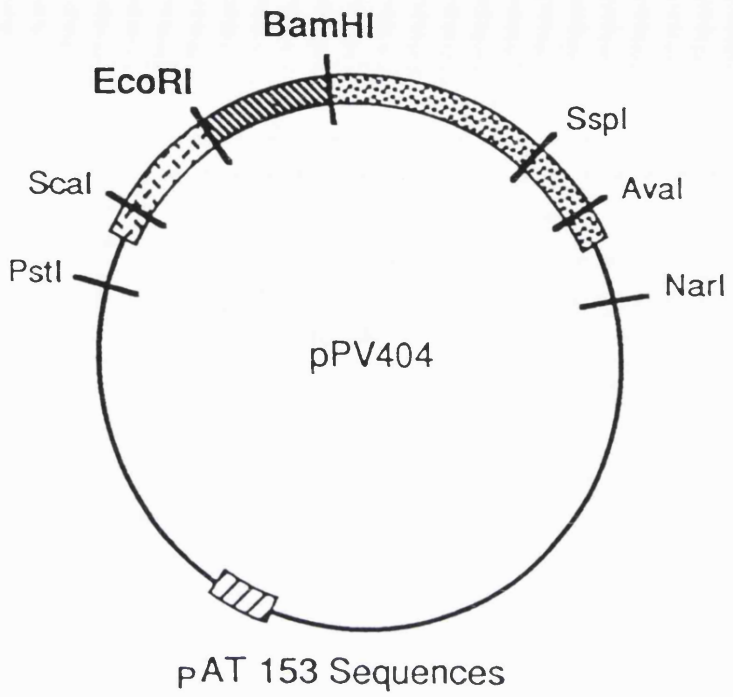
III.B.1.a. Plasmid pPV404 (a gift from Dr B.E.Clarke)

This plasmid is a derivative of the expression vector pBC404 that has been discussed above. pPV404 contains a 39 bp synthetic DNA sequence that encodes an immunogenic epitope derived from poliovirus type 1. This sequence has been inserted as a fragment with EcoRI and BamHI sticky ends at its 5' and 3' ends, into pBC404 with similar sticky ends (see fig 30) [Francis and Clarke, 1989].

The experiment was designed with the idea that the poliovirus sequences would be replaced with the HCMV-gp58 coding DNA sequences containing EcoRI and BamHI sticky ends at its 5' and 3' ends. To obtain the HCMV-gp58 coding DNA fragment, another plasmid p73HCMV was used.

III.B.1.b. Plasmid p73HCMV (a gift from Dr V.Emery)

This plasmid is based on vector pT7T3/18U (Pharmacia) and contains a PCR amplified 149 bp HCMV-gp58 coding sequence, obtained from a clinical isolate. The HCMV-gp58 coding sequence has been cloned in the polycloning site of the vector pT7T3/18U, and the latter contains several restriction enzyme sites including EcoRI and BamHI (see fig 31).



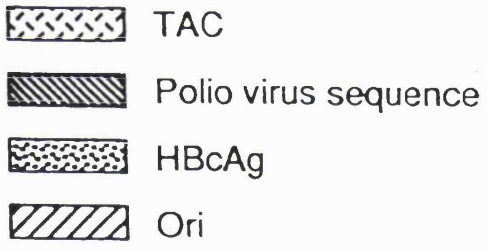


FIG 30: Plasmid pPV404.

The position of some important restriction enzyme sites surrounding the poliovirus and HBcAg encoding sequences are shown. EcoRI (5') and BamHI (3') are the unique enzyme sites that can be used for ligation of foreign sequences (from Francis and Clarke, 1989).

(Ori, origin of replication; TAC; tac promoter)

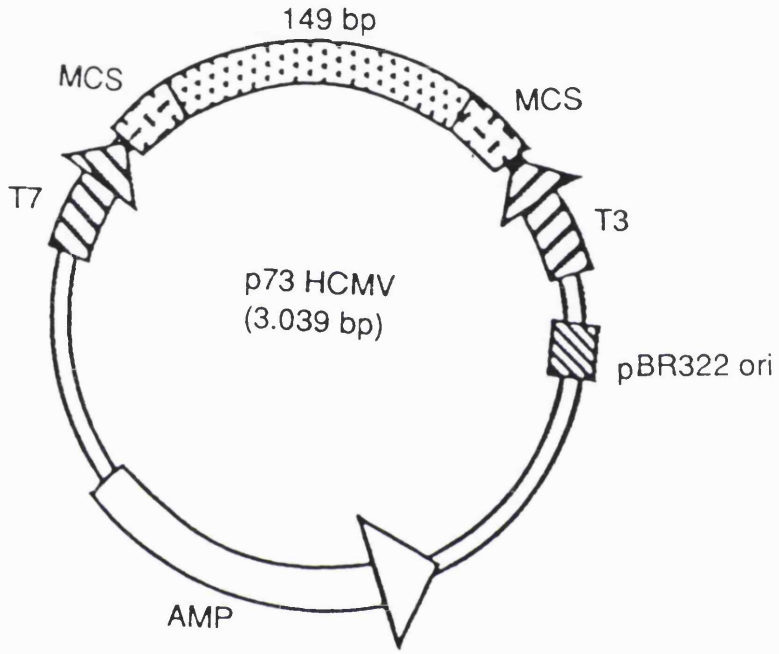


FIG 31: Plasmid p73HCMV.

The plasmid contains the 149 bp sequence encoding HCMV-gp58, cloned in pT7T3/18U.

(Ori, origin of replication; AMP, ampicillin resistance gene; T3&T7, phage promoters; MCS, multiple cloning site)

For this experiment, it would have been convenient to cut the HCMV-gp58 encoding fragment cloned in p73HCMV, with the EcoRI and BamHI (after making certain that the two sites are not present in the HCMV-gp58 sequence) and then ligate it into similar sticky ends of plasmid pPV404. But it was realized that the digestion with these enzymes would result in an HCMV-gp58 fragment in the reverse orientation containing BamHI sticky end at its 5' end, and EcoRI at the 3' end.

This situation could be resolved by PCR amplifying the HCMV-gp58 fragment present in p73HCMV, by two PCR primers that would contain EcoRI and BamHI restriction enzyme sites incorporated at their respective 5' and 3' ends. The digestion of PCR product with the two enzymes would then result in an HCMV-gp58 fragment containing the sticky ends for both the enzymes in the right orientation, which is required for ligation into pPV404. The expression of the recombinant protein could then be analyzed by immunological methods, electron microscopy and SDS-PAGE analysis.

III.B.1.c. Plasmid ptacHpaII-R2 (a gift from Professor K.Murray)

This plasmid has been derived from ptacHpaII, that has been described above. It contains a HindIII site in carboxyl terminus of HBcAg and just downstream of this, contains another unique site for BamHI. These two sites could be used for ligation of foreign sequences with similar sticky ends (see fig 32).

The HCMV-gp58 sequences could be PCR amplified by using another primer pair that would contain HindIII and BamHI restriction enzyme sites, incorporated into their respective 5' and 3' ends. After digestion with appropriate enzymes, the PCR product could be ligated into similarly digested ptacHpaII-R2. The expressed recombinant protein could then be analyzed by same methods as mentioned above.

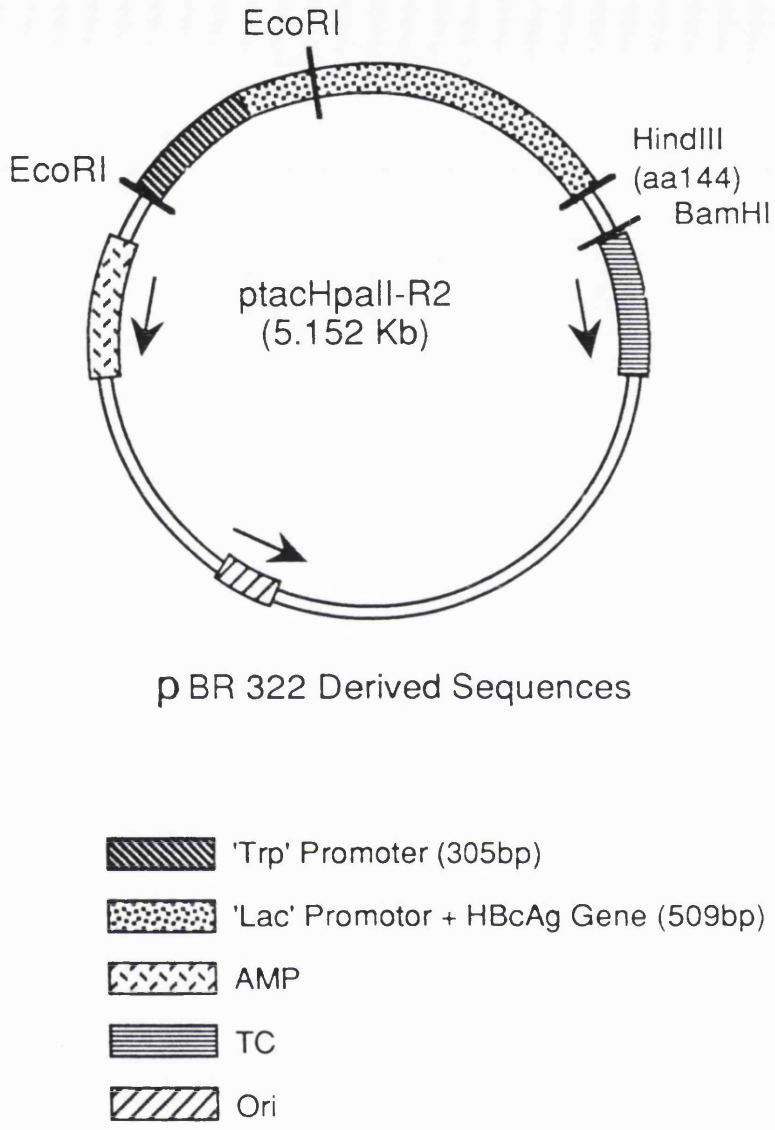


FIG 32: Plasmid ptacHpaII-R2.

The position of HindIII site at aa 144 of HBcAg, which is used for ligation of the 5' end of the foreign sequences, is shown. The unique BamHI site present downstream of HindIII site can be used for ligation of the 3' end of the foreign sequences.

(AMP, ampicillin resistance gene; TC, tetracycline resistance gene; Ori, origin of replication)

The experimental scheme for the construction of amino and carboxyl terminal fusion of HBcAg with HCMV-gp58 has been summarized in fig 33. The predicted nucleotide and protein sequence of part of both amino and carboxyl terminal fusions has also been shown in figs 34 and 35.

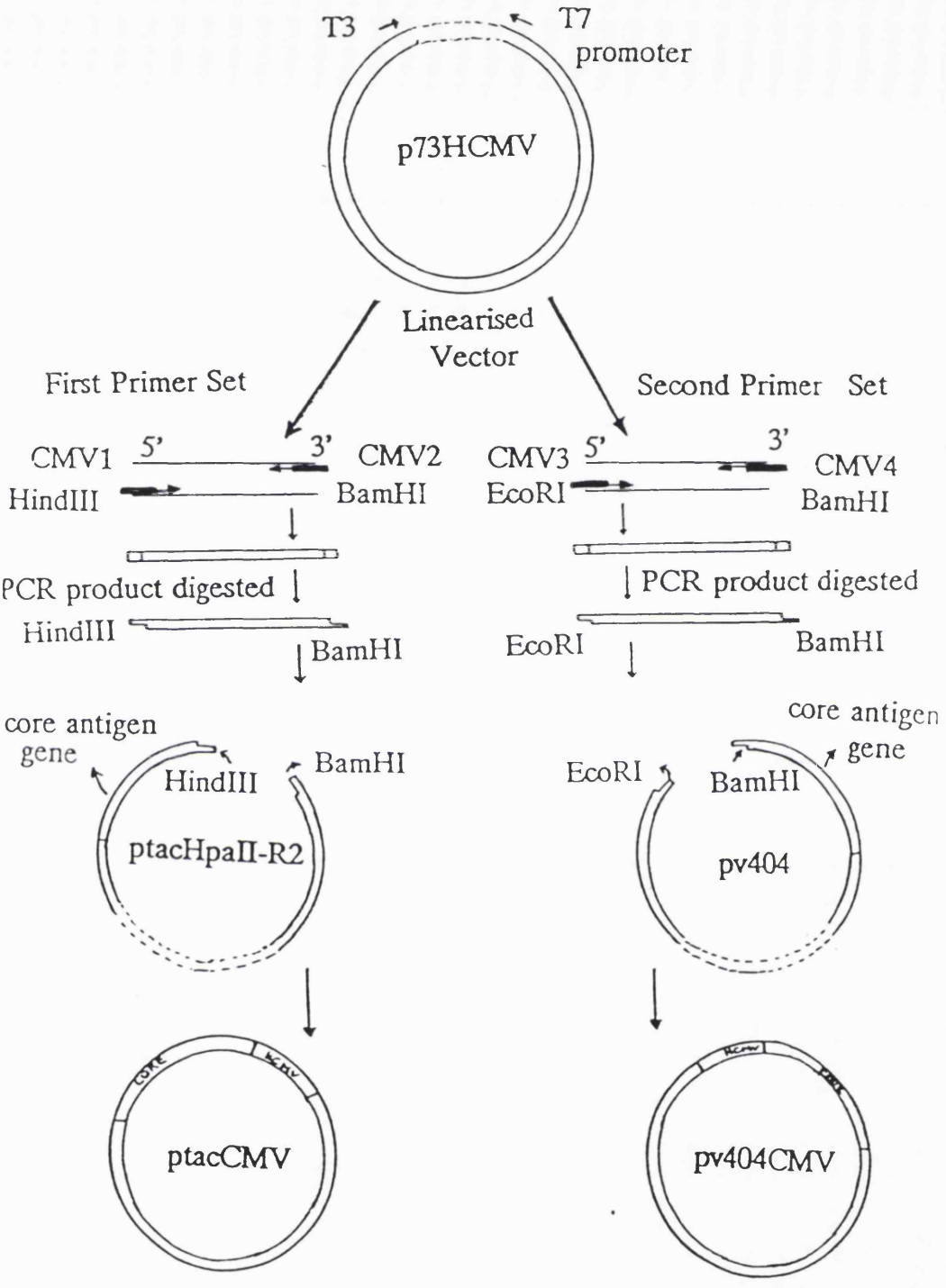


FIG 33: Schematic representation of plasmid constructions of ptacCMV and pv404CMV.

FIG 34: (A) Part of the predicted nucleotide and amino acid sequence of the junction region of the construct containing HBcAg amino terminal fusion with HCMV-gp58 (pv404CMV).

The deduced amino acid sequence is represented by a single letter code, and has been shown in one reading frame only. The unique EcoRI (Eco) and BamHI (Bam) restriction enzyme sites used for constructing the fusion have been marked by an arrow. The preC and C initiation codons have been underlined and the nucleotide sequence of CMV3 and CMV4 primers is double underlined. The nucleotide sequence of the primer used for direct sequencing of the construct is represented as a boxed area.

(A)


preC ↓ Eco↓ primer CMV3
Q E T V M N S N E I L L C N H R T E E C
CAGGAAACAGTTATGAATTCCAACGAAATCCTGTTGGGCAACCACCGCACTGAGGAATGT
70 80 90 100 110 120
GTCCTTTGTCAATACTTAAGGTTGCTTTAGGACAACCCGTTGGTGGCGTGACTCCTTACA
Q L P S L K I F I A G N S A Y E Y V D Y
CAGCTTCCCAGCCTCAAGATCTTCATCGCCGGGAACTCGGCCTACGAGTACGTGGACTAC
130 140 150 160 170 180
GTCGAAGGGTCGGAGTTCTAGAAGTAGCGGCCCTTGAGCCGGATGCTCATGCACCTGATG
↓Bam ↓
L F K R M I D L S S I S T V D P R A L G
CTCTTCAAACGCATGATTGACCTCAGCAGTATCTCCACCGTGGATCCGCGCGCCCTTGGG
190 200 210 220 230 240
GAGAAGTTTGCGTACTAACTGGAGTCGTCATAGAGGTGGCACCTAGGCGCGCGGGAACCC
primer CMV4
W L W G M D I D P Y
TGGCTTTGGGGCATGGACATTGACCCTTAT
250 260 270
ACCGAAACCCCGTACCTGTAACTGGGAATA
Core

(B)

MNSNEILLGNHRTEECQLPSLKIFIAGNSAYEYVDYLFKRMIDLSSISTVDP
RALGWLWGMDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTASALYREALES
PEHCSPHHTALRQAILCWGELMTLATWVGNNLQDPASRDLVVNYVNTNMGLK
IRQLLWFHISCLTFGRETVLEYLVSFGVWIRTPPAYRPPNAPILSTLPETTV
VRRRDRGRSPRRRTPSPRRRRSQSPRRRRSQSRESQC*

FIG 34: (B) Protein sequence of the construct in fig 34A, showing all amino acids representing HBcAg, HCMV-gp58 (underlined) and the junction amino acids (shaded), as single letter codes.

FIG 35: (A) Part of the predicted nucleotide and amino acid sequence of the junction region of the construct containing HBcAg carboxyl terminal fusion with HCMV-gp58 (ptacCMV').

The HindIII and BamHI restriction enzyme sites, used for constructing the fusion, have been underlined. The C termination codon is double underlined and the nucleotide sequence of CMV1 and CMV2 primers is shown as a shaded area.

(A)

```
            HindIII      primer CMV1
 S  T  L  P  I  S  F  N  E  I  L  L  G  N  H  R  T  E  E  C
TCAACGCTTCCGATAAGCTTCAACGAAATCCTGTTGGGCAACCACCGCACTGAGGAATGT
450        460        470        480        490        500
AGTTGCGAAGGCTATTCGAAGTTGCTTTAGGACAACCCGTTGGTGGCGTGACTCCTTACA

 Q  L  P  S  L  K  I  F  I  A  G  N  S  A  Y  E  Y  V  D  Y
CAGCTTCCCAGCCTCAAGATCTTCATCGCCGGGAACTCGGCCTACGAGTACGTGGACTAC
510        520        530        540        550        560
GTCGAAGGGTCGGAGTTCTAGAAGTAGCGGCCCTTGAGCCGGATGCTCATGCACCTGATG

 L  F  K  R  M  I  D  L  S  S  I  S  T  ✡  G  S
CTCTTCAAACGCATGATTGACCTCAGCAGTATCTCCACCTAGGGATCC
570        580        590        600        610
GAGAAGTTTGCGTACTAACTGGAGTCGTCATAGAGGTGGATCCCTAGG
                      primer CMV2   BamHI
```

(B)

MTMITDSLEFHIDPYKEFGATVELLSFLPSDFFPSVRDLLDTAAALYRDAL
ESPEHCSPHHTALRQAILCWGDLMTLATWVGTNLEDPASRDLVVSYVNTNV
GLKFRQLLWFHISCLTFGRETVLEYLVSFGVWIRTPPAYRPPNAPILSTLP
ISFNEILLGNHRTEECQLPSLKIFIAGNSAYEYVDYLFKRMIDLSSIST*GS

FIG 35: (B) Protein sequence of the construct in fig 35A, showing all amino acids representing HBcAg, HCMV-gp58 (underlined) and the junction amino acids (shaded).

III.B.2. Preparation of Competent E.Coli by Calcium Chloride Method [Cohen et al., 1972]

A single isolated colony of E.coli RB791 (Brent and Ptashne, 1981) or TG2 strain (prepared by M.Biggin {Sambrook et al., 1989}, which contains a defective lacZ gene) was inoculated into 5 ml of LB medium and incubated overnight at 37°C in a shaker. One ml of culture was transferred into 100 ml of LB medium, and incubated at 37°C with shaking. When an OD_{600} of 0.4 was reached, 60 ml of the culture was centrifuged at 5000 rpm for 5 minutes at 4°C. The bacterial pellet was resuspended in 30 ml of ice cold $CaCl_2$ Tris buffer (50 mM $CaCl_2$, 10 mM Tris HCl pH8.0) and centrifuged again, as above. The pellet was again resuspended in 30 ml of $CaCl_2$ Tris buffer and left on ice for 30 minutes. The cells were centrifuged as above and then resuspended in 4 ml of $CaCl_2$ Tris buffer. The cells were stored on ice for 30 minutes or more and then used for transformation within 24 hours.

III.B.3. Transformation of Competent E.coli [Cohen et al., 1972]

For transformation, 300 µl of competent cells obtained above were transferred to a tube containing an appropriate amount of plasmid DNA or ligation product in a final volume of 10 µl. In addition, a control tube was also set up without DNA. The tubes were kept on ice for 30 minutes, and then transferred to a 42°C water bath, for 90 seconds.

At the end of this incubation, for ampicillin resistant plasmids, 1 ml of LBG medium (LB medium containing 0.2% glucose) was added to all tubes followed by incubation at 37°C for 60 minutes. An appropriate volume of transformed cells (0.1, 0.2, and 0.3 ml/90mm plate) was added to LB agar plates containing 50 µg/ml of ampicillin (LB-amp) and spread with sterile bent glass rod. The plates were inverted after liquid had absorbed, and incubated at 37°C for 12-18 hours.

After the heat shock step, for M13mp18 vectors, following components were added to ligation product and control eppendorf tubes:

10 µl of 100 mM IPTG

50 µl of 2% X-gal

200 µl of TG2 cells (1:100 dilution of overnight culture)

The contents were mixed and added to 3 ml of soft top agar (0.75% bacto agar in YT broth) which had been maintained in sterile bijoux bottles in a 50°C waterbath. The contents were poured on prewarmed YT agar plates and left at room temperature until the agar had set, then incubated overnight at 37°C.

III.B.4. Plasmid DNA Preparation

III.B.4.a. Large Scale Plasmid Preparation / Lysis by Alkali Method followed by Purification of Plasmid DNA by Equilibrium Centrifugation in CsCl/EtBr Gradient [Birnboim and Doly, 1979; Ish-Horowicz and Burke, 1981 (modification by Sambrook et al., 1989)]

A single colony of the bacterial strain carrying the plasmid was inoculated into 30 ml of LB-amp medium and incubated overnight at 37°C with shaking. Then 25 ml of the culture was used to inoculate 500 ml of LB-amp medium, for overnight incubation. The culture was centrifuged at 4000 rpm for 15 minutes and the pellet resuspended in 100 ml of ice cold STE buffer and centrifuged again, as above. The pellet was resuspended in 10 ml of alkali lysis solution I and 1 ml of freshly prepared lysozyme solution (10 mg/ml in 10 mM Tris HCl pH8.0) was added. After mixing, 20 ml of alkali lysis solution II was added, followed by incubation at room temperature for 10 minutes, after which 15 ml of ice cold alkali lysis solution III was added. The lysate was mixed, left on ice for 10 minutes and centrifuged at 4000 rpm for 15

minutes. The supernatant was filtered through several layers of cheesecloth into a 250 ml beaker and 0.6 volumes of isopropyl alcohol added, followed by incubation at room temperature for 10 minutes. The precipitate was centrifuged at 5000 rpm for 15 minutes, supernatant discarded and the pellet rinsed with 70% ethanol, air dried and dissolved in 12.5 ml of TE pH8.0.

To the solution prepared above, 1.25 ml of EtBr (10 mg/ml) and 13.75 gm of CsCl was added and it was centrifuged at 30,000 rpm for 30 minutes at 20°C in a TFT 50.38 (fixed angle rotor) in the OTD 65B (Dupont). The supernatant was collected in a fresh tube and centrifuged again for 48 hours, as above. The rotor was allowed to stop without braking. The DNA was visualized using a UV transilluminator and the lower band containing the supercoiled plasmid DNA was collected. The DNA was extracted with water saturated butanol, 5 times and dialysed against three changes of TE pH8.0 within 48 hours. The amount of DNA present was estimated by spectrophotometric analysis at 260 nm.

III.B.4.b. Small Scale Preparation (Mini-Prep) / Lysis by Alkali Method [Birnboim and Doly, 1979 (modification by Sambrook et al., 1989)]

A single bacterial colony was transferred to 2 ml of LB-amp medium, and incubated overnight at 37°C with shaking. Then, 1.5 ml of the overnight culture was transferred to an eppendorf tube and centrifuged for 30 seconds at 4°C, whilst the remaining culture was stored at 4°C. The supernatant was removed and the pellet was resuspended in 100 µl of ice cold alkali lysis solution I and 200 µl of freshly prepared alkali lysis solution II, by vortexing. The lysate was kept on ice for few minutes and then 150 µl of ice cold alkali lysis solution III was added, mixed and stored on ice for 5 minutes. The contents were centrifuged for 5 minutes at 4°C in a microfuge and the supernatant was transferred to a fresh tube.

An equal volume of phenol-chloroform was added, the contents were mixed, and centrifuged for 2 minutes at 4°C. The aqueous phase was recovered and two volumes of ethanol were added. The precipitation was allowed for 2 to 5 minutes at room temperature and centrifugation was carried out in a microfuge for 5 minutes at 4°C. The pellet was rinsed with 70% ethanol, air dried for 15 to 30 minutes, and dissolved in 50 µl of TE pH8.0 containing DNAase free pancreatic RNAase (20 µg/ml). The amount of DNA present was estimated by gel electrophoresis.

III.B.4.c. Medium Scale Preparation by Qaigen Column (Diagen, Germany) {protocol was provided with the kit}

An overnight culture of a single bacterial colony containing the plasmid was inoculated into 100 to 150 ml of LB-amp medium and incubated overnight. The cells were pelleted by centrifugation at 5000 rpm for 15 minutes at room temperature, and the pellet was resuspended in 4 ml of buffer P1 (50 mM Tris HCl, 10 mM EDTA, 400 µg/ml of RNAase A {pH8.0}) and 4 ml of buffer P2 (200 mM NaOH, 1% SDS). The contents were incubated at room temperature for 5 minutes, and 4 ml of buffer P3 (2.5 M potassium acetate {pH4.8}), was added. The lysate was centrifuged at 6000 rpm at 4°C for 30 minutes and the supernatant was applied to a Qaigen-pack 100 column, which had been pre-equilibrated with 2 ml of buffer QB (750 mM NaCl, 50 mM MOPS, 15% ethanol {pH7.0}). The column was washed with 2x4 ml of buffer QC (1000 mM NaCl, 50 mM MOPS, 15% ethanol {pH7.0}), and the DNA was eluted with 2 ml of buffer QF (1200 mM NaCl, 50 mM MOPS, 15% ethanol {pH8.0}). The DNA was precipitated with 0.8 volumes of isopropanol, and centrifuged for 30 minutes in a microfuge. The pellet was washed with 70% ethanol and air dried before being dissolved in TE pH8.0.

III.B.5. Restriction Enzyme Digestion

An appropriate amount of plasmid DNA was digested with restriction enzyme in the presence of restriction enzyme buffer (supplied with enzyme as 10x stock) and incubated at 37°C for 1-2 hours, following the supplier's instructions. Whenever a double digestion was set up, the enzyme working in the lower salt concentration was added first to the reaction and incubated. This was followed by addition of the enzyme requiring high salt concentration, along with enzyme buffer and the appropriate salt. An aliquot from each digestion was analyzed on a 1% agarose gel (in 0.5x TBE or 0.5x TAE buffer, containing 50 µg/ml EtBr) to see if complete digestion had taken place. The digested DNA was either extracted with phenol-chloroform and then with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with ethanol, or electrophoresed on a gel and purified as described below.

III.B.6. Isolation and Purification of DNA Fragments from Agarose [Sambrook et al., 1989]

The digested DNA was electrophoresed on a 1% agarose gel in 0.5xTBE with EtBr. The gel was examined on a UV transilluminator and the band containing the desired fragment was excised from the gel with new scalpel blade. The slice was transferred to a dialysis bag, containing 0.5xTBE without EtBr, and electrophoresed for 1 hour at 100 volts. Polarity was reversed for 1 minute after this, and the buffer was collected from the bag.

The buffer containing the fragment was loaded on DEAE sephacel (Pharmacia) column, which was prepared by adding 800 µl of DEAE sephacel suspension to a short pasteur pipette, and washing it sequentially with following buffers:

3 ml TE pH7.5, containing 0.6 M NaCl
3 ml TE pH7.5
3 ml TE pH7.5, containing 0.1 M NaCl

The flow through was collected and reloaded. The DNA fragment was eluted 3 times with 0.5 ml of TE pH7.5, containing 0.6 M NaCl. The fractions were extracted twice with phenol-chloroform, once with phenol-chloroform-isoamylalcohol and precipitated with two volumes of cold ethanol. After storing at -20°C for few hours, the fragment was pelleted for 30 minutes in a microfuge at 4°C and dissolved in TE pH8.0.

III.B.7. Purification of DNA Fragments from Low Melting Point (LMP) Agarose Gel [Wieslander, 1979; Parker and Seed, 1980]

The DNA was electrophoresed in 1% LMP agarose in 1xTAE containing 50 µg/ml of EtBr at 4°C. The band containing the fragment was excised and transferred to an eppendorf tube containing 500 µl of 1xTAE buffer without EtBr. The agarose was melted at 65°C for 5 minutes, and then phenol-chloroform extracted twice to remove the agarose. The DNA was precipitated in the presence of 0.1 volume of 3 M sodium acetate pH 5.3 and two volumes of cold ethanol and kept at -20°C for 10-30 minutes. The tube was centrifuged for 30 minutes at 4°C in a microfuge, and the pellet was washed with 70% ethanol, air dried and dissolved in 15 µl of TE pH8.0. The amount of DNA was estimated by running an aliquot on a gel along with DNA standard.

III.B.8. Ligation Reaction

The following reaction was set up with vector to insert ratio of 1:2. One control was set up without the insert DNA, and the other without vector DNA. The reaction was set up in total volume of 10 µl and incubated overnight in 12°C water bath.

100 ng insert DNA
50 ng vector DNA
1 µl 10x TM
1 µl ATP (2 mM)
1 µl 0.1 M DTT
1 µl T4 DNA Ligase
1 µl T4 RNA Ligase

III.B.9. End Filling of DNA Fragments by Klenow Enzyme

The reaction contained the following in a total volume of 25 µl adjusted with DW, and was incubated at room temperature for 30 minutes:

1-2 µg DNA with recessed 3' termini
1 µl 2mM dATP
1 µl 2mM dCTP
1 µl 2mM dGTP
1 µl 2mM dTTP
2.5 µl 10x NT salts
1 µl Klenow enzyme (large fragment of DNA polymerase I)

III.B.10. Radio-labelling of DNA Fragments [Feinberg and Vogelstein, 1983]

The radio-labelling of DNA fragments was done by using the random oligo-primed method in a final volume of 25 µl:

5 µl DTM (100 µM each dATP, dGTP, dTTP in 250 mM Tris HCl pH8.0, 25 mM $MgCl_2$, 50 mM 2-MCE)
5 µl 1 M HEPES pH7.6
1.4 µl OL/2 (45 units/ml pd N6 in 1 mM Tris HCl pH7.5, 1mM EDTA)
1 µl BSA (10 mg/ml)
50 ng of DNA fragment (boiled for 2 minutes and quenched on ice before being added to the mixture)

1 µl (10 µCi/µl) α ^{32}P-dCTP (1000 Ci/mmole, Amersham)
1 µl Klenow enzyme

As the contents were incubating at room temperature for 40 minutes, a sephadex G-50 spin column was prepared. The bottom of a sterile plastic syringe was plugged with a small amount of glass wool and sephadex G-50 equilibrated in STE buffer was added to the syringe. The syringe was transferred to a 15 ml glass tube and centrifuged for 4 minutes. More resin was added to the syringe and recentrifuged until the volume of packed column was 0.9 ml. The column was washed twice with 100 µl of STE buffer and centrifuged as above.

The radio-labelled DNA was applied to the column in a final volume of 100 µl (volume adjusted with STE buffer) and then recovered by centrifugation. Two samples of 1:10 dilution were spotted onto separate Whatman GFC filters. One filter was air dried whereas the other was treated with trichloroacetic acid (TCA) by washing the filter three times with ice cold TCA/0.1 M tetra-sodium pyrophosphate. This was follwed by washing the filter once with methanol and acetone. Both the filters were put in scintillation vials containing toluene-based scintillation fluid and counted in a Geiger counter to estimate the percentage incorporation of ^{32}P-dCTP.

III.B.11. Radio-labelling of HindIII Digested Lambda Marker DNA

One µg of HindIII digested lambda marker DNA was mixed with 1 µl of 10x NT salts, 1 µl of each of 2 mM dATP, dGTP and TTP, 3 µl of α-^{32}P dCTP and 1 unit of Klenow enzyme in a final volume of 25 µl. This was incubated at room temperature for 30 minutes and then two samples of 2 µl (1:10 dilution) were spotted onto Whatman GFC filters and treated as described above.

III.B.12. Southern Transfer and Hybridization [Southern, 1975]

After photography, the gel was washed briefly in DW and soaked in depurination solution (0.2 N HCl) for 10 minutes with shaking. The gel was left shaking in denaturation solution (1.5 M NaCl, 0.5 M NaOH), for 60 minutes, and the solution was replaced with fresh solution after 30 minutes. The gel was then neutralized (0.5 M Tris HCl pH7.2, 1.5 M NaCl, 0.001 M EDTA) for 45 to 60 minutes. Meanwhile, a nylon membrane (Hybond N) was cut to the same size as the gel and soaked in 20xSSC (3 M NaCl, 0.3 M sodium citrate) for 5 to 10 minutes. The gel was arranged on a tray containing several layers of whatman paper soaked in 20xSSC, and the membrane was placed on top of the gel and trapped air bubbles were released. Both were sealed on all sides with parafilm, and stacks of absorbent paper were placed on top for capillary action. After overnight transfer, the gel was checked on UV transilluminator to ensure that DNA transfer was complete. The membrane was exposed to UV light for 2-3 minutes to covalently attach nucleic acids to the membrane, and then stored at 4°C, or prehybridized.

The membrane was prehybridized in a sealed plastic bag at 65°C with shaking for 12-18 hours, using 250 µl/cm^2 of the following solution:

SSC	5x
Denhardts solution	5x
SDS	0.5%
Salmon sperm DNA	100 µg/ml (boiled for 2 minutes, and quenched on ice)

After prehybridization, the denatured probe (1x10^7 cpm) was added to the plastic bag and further incubated at 65°C for 12-18 hours. The membrane was sequentially washed at 65°C as follows:

2xSSC	15 minutes (twice)
2xSSC, 0.1% SDS	30 minutes
0.1xSSC	20 minutes

The membrane was air dried after the last wash and exposed to Kodak XAR film at -70°C with intensifying screens.

III.B.13. Colony Hybridization [Grunstein and Hogness, 1975]

Screening of small numbers of colonies (40-60) was done by plating these in duplicate onto agar plates containing the appropriate antibiotic, and incubating at 37°C overnight. Colony lifts were performed by using a circular Hybond N membrane that was arranged on the agar surface. The membrane was removed after 1 minute and placed colony side up, on a pad of Whatman 3MM paper soaked in denaturation solution for 7 minutes. It was then transferred to a similar pad soaked in neutralization solution for 3 minutes and then same incubation was repeated with a fresh pad of same solution. The membrane was rinsed in 2xSSC, air dried and fixed with UV light for 2-3 minutes. This was prehybridized as described for Southern transfer, hybridized with $1x10^6$ cpm of denatured probe and washed before exposure to an X-ray film.

Where large numbers of colonies (60+) were to be screened, these were first grown overnight in a 96 well plate (Nunclon) in LB medium containing appropriate antibiotic. A drop of glycerol was added to each of the wells and mixed by vortexing. A sterile template apparatus was used to transfer the culture from the wells, in sets of 47, to a nylon membrane placed on an agar plate. This was incubated for 4-5 hours at 37°C, the membrane was removed and treated as described above.

III.B.14. Polymerase Chain Reaction (PCR)

III.B.14.a. Elution of Primers from Columns

Oligonucleotide primers were synthesized on a Cyclone DNA Synthesizer and were provided still linked to the columns, and eluted as described in the instruction manual.

One ml of ammonium hydroxide solution was drawn into a 5 ml syringe. The needle was discarded and the syringe was inserted into one end of the column. Another, empty syringe was similarly inserted in the other end of the column and the ammonia solution was drawn through the column 3 to 4 times. The column/syringe assembly was left at room temperature for 45 minutes, before cycling the solution back and forth and incubating again. After 1½ hour, all the solution was drawn into one syringe, emptied into a screw capped freezing vial and incubated at 50°C overnight. The ammonia was removed by chromatography on a PD10 column (Pharmacia), which was equilibrated with 25 ml of 10 mM Tris HCl pH7.5. The primer ammonia solution was added to the column and allowed to flow through. The same buffer was added and 12 fractions, of 10 drops each were collected in tubes, and OD_{260} was read for all fractions.

III.B.14.b. PCR Protocol

PCR amplification was performed as described by Saiki et al., [1988]. The following primers were used:

CMV1 Primer [29 bp] 5' GCGAAGCTTCAACGAAATCCTGTTGGGCA 3'
HindIII

CMV2 Primer [30 bp] 5' GCGGATCCCTAGGTGGAGATACTGCTGAGG 3'
BamHI

CMV3 Primer [28 bp] 5' GCGAATTCCAACGAAATCCTGTTGGGCA 3'
EcoRI

CMV4 Primer [29 bp] 5' GCGGATCCACGGTGGAGATACTGCTGAGG 3'
BamHI

The reaction mixture was set up in 0.5 ml eppendorf tubes in a final volume of 100 µl adjusted with DW, and contained the following:

1 pg of linearized plasmid DNA
25 mM Tris HCl pH8.9
17 mM ammonium sulphate
10 mM 2-MCE
3 mM $MgCl_2$
0.002% gelatin
1 mM of each of the deoxynucleotides [dATP, dCTP, dGTP, and dTTP]
1 µM of each primer
1 unit of Taq polymerase

A separate negative control, without the plasmid DNA was set up for each reaction. The mixture was overlaid with 100 µl of mineral oil and the samples were denatured at 95°C for 6 minutes, followed by amplification by 35 PCR cycles. Each cycle involved denaturation at 94°C for 90 seconds, primer annealing at 42°C for 90 seconds and, finally, extension at 72°C for 120 seconds. After the last cycle, the samples were further incubated at 72°C for 10 minutes. Following amplification, the solutions were extracted twice with phenol-chloroform, once with phenol-chloroform-isoamylalcohol (25:24:1) and precipitated with two volumes of ethanol in presence of 0.2 M NaCl, and stored at -20° C for 2 to 3 hours. The DNA was pelleted, resuspended in 30 µl of TE pH8.0, and 0.5 µl of PCR product from samples and control tubes was loaded in duplicate on a 2% agarose gel.

III.B.15. DNA Sequencing by Chain Termination Method [Sanger et al., 1977]

III.B.15.a. Preparation of Insert and Vector DNA for Ligation

The DNA fragment to be sequenced, was double-digested with appropriate enzymes, and the fragment of interest was gel purified.

The CsCl-EtBr gradient purified, M13mp18 vector DNA (2 µg) was digested with a pair of appropriate restriction enzymes that cleaved within the multiple cloning site. After analyzing an aliquot on agarose gel, the DNA was extracted, precipitated with ethanol and pelleted.

A ligation reaction was set up between 100 ng of digested M13mp18, and 200 ng of insert DNA along with a control containing vector or insert DNA alone.

III.B.15.b. Transformation and Recombinant Selection

Competent E.coli were prepared and transformed with the ligation products as described above [III.B.2 and III.B.3]. Several colourless plaques were picked up and used for making templates as described below.

III.B.15.c. Preparation of Single Stranded DNA Templates

An overnight culture of TG2 cells was diluted 1:100 in YT broth and 1.5 ml was transferred to bijoux bottles. The culture was inoculated with recombinant M13 plaques, and incubated at 37°C for 5½ hours. The culture was transferred to an eppendorf tube and centrifuged for 5 minutes in a microfuge at room temperature. The supernatant was collected in a fresh tube, and vortexed with 150 µl of 20% PEG 6000 in 2.5 M NaCl, and left at room temperature for 30 minutes. The contents were centrifuged for 5 minutes, and the supernatant was discarded. The pellet was resuspended in 100 µl of TE pH8.0, phenol-chloroform extracted once, and ethanol precipitated in presence of 0.1 volume of 3 M sodium acetate. After storage at -20°C, the DNA was pelleted in a microfuge for 10 minutes, rinsed in 70% ethanol, air dried and dissolved in 30 µl of TE pH8.0 and stored at -20°C.

III.B.15.d. Sequencing Reaction (Sequenase Version 2.0 kit; United States Biochemical Corporation)

(1) Annealing Template and Primer

The following annealing reaction was set up for each template:

7 µl template DNA
2 µl 5x sequencing buffer (200 mM Tris HCl pH7.5, 100 mM $MgCl_2$, 250 mM NaCl)
1 µl primer (5' GTTTTCCCAGTCACGAC)

The tube was heated at 65°C for 2 minutes and then allowed to cool slowly to room temperature.

(2) Labelling Reaction

To the annealed primer template above, following were added:

0.4 µl 5x Labelling mix (7.5 µM of each dGTP, dCTP and dTTP)
1 µl 0.1 M DTT
0.5 µl (10 µCi/µl) α-^{35}S dATP (1000 Ci/mmol; Amersham)
1.6 µl DW
2 µl diluted Sequenase version 2.0 (0.25 µl bacteriophage T7 DNA polymerase + 1.75 µl of enzyme dilution buffer {10 Mm Tris HCl pH7.5, 5 mM DTT, 0.5 mg/ml BSA})

The contents were mixed and incubated for 2½ minutes at room temperature.

(3) Termination Reactions

To a set of four eppendorf tubes labelled as A, C, G, and T, 2.5 µl of each termination mix containing the appropriate ddNTP was added (e.g ddGTP termination mix contained 80 µM of each dATP, dCTP, dGTP and dTTP, 8 µM ddGTP, 50 mM NaCl).

When the incubation for labelling reaction above was complete, 3.5 µl was added to each of A, C, G, and T tubes. The contents were mixed, centrifuged briefly and incubated at 37°C for 3 to 5 minutes. Then 4 µl of stop solution (95% Formamide, 20 mM EDTA, 0.05% Bromophenol blue, 0.05% Xylene cyanol FF) was added to each tube and samples were stored at -20°C, until loaded on sequencing gel.

III.B.16. Direct Sequencing of Double Stranded Plasmid DNA [Winship, 1989 (modification of the protocol by V.Emery)]

The reaction was set up in a total volume of 10 µl and contained:

100 ng linearized plasmid DNA

140 ng primer DNA

(HBc primer: 5' GTCCACGTACTCGTAGGCCG)

(BEVS primer: 5' TAAGTATTTTACTGTTT)

1 µl of DMSO

The solution was boiled in a water bath for 3 minutes and placed on dry ice for 20 seconds, after which following were added to the template (buffers from Sequenase version 2.0 kit):

2 µl 5x sequencing buffer

2 µl Labelling mix dilution (1 µl 5x mix + 4 µl DW)

1 µl 0.1 M DTT

0.5 µl DMSO

0.5 µl (10µCi/µl) α- ^{35}S dATP (1000 Ci/mmol; Amersham)

2 µl Sequenase version 2.0 dilution (0.5 µl enzyme + 4 µl of ice cold 1 M TE pH7.4)

The reaction was incubated at room temperature for 10 minutes. Meanwhile, 2.5 µl of appropriate termination mix, and 0.6 µl of DMSO was added to tubes labelled A, C, G, and T. After incubation, 3.5 µl of reaction mixture was added to A, C, G, and T tubes and kept at 37°C for 5 minutes. Then 4 µl of stop solution was added, and the samples were stored at

-20°C. Before loading on polyacrylamide gel, the samples were heated at 90°C for 5 minutes.

III.B.17. Denaturing Polyacrylamide Sequencing gel

The following were mixed in 20-30 ml of DW:

29.4 gm urea

10.5 ml 40% acrylamide solution

7 ml 10xTBE

The volume was adjusted to 70 ml, and 420 µl of ammonium persulphate and 70 µl of TEMED were added. Immediately after this the solution was poured into a gel mould, and allowed to polymerize for 45 minutes and pre-run for 60 minutes in 1xTBE at 60 watts. The samples were heated at 90°C for 5 minutes and run on the gel in duplicate, the first set allowed to run longer than the second set in order to read more nucleotides. The gel was dried for 45 minutes at 80°C, and exposed to Kodak XAR film at room temperature overnight.

III.B.18. Induction and Purification of HBcAg fusion proteins [Stahl et al., 1982; Stahl and Murray, 1989]

III.B.18.a. Several hybridization positive HBcAg recombinant clones were grown in 20, 100 or 500 ml cultures, and the tac promoter was induced by IPTG. At the same time, control cultures were also induced. The protocol for induction of a 20 ml culture is given below.

A single colony of each recombinant clone that had been grown from glycerol stocks, was used to inoculate 2 ml of LB-amp medium and incubated overnight at 37°C with shaking. The culture was diluted 1:10 with LB medium without ampicillin and at the same time 60 µg/ml of IPTG (40 µl of 30 mg/ml stock solution) was added. The culture was incubated for 6 hours at 37°C in a shaker and the bacterial cells were pelleted by

centrifugation at 8000 rpm for 15 minutes at room temperature. The pellet was either stored at -20°C for overnight or processed further by resuspending in 60 µl of solution 1 (25% sucrose, 50 mM Tris HCl pH8.0) and 10 µl of solution 2 (5 mg/ml lysozyme in 250 mM Tris HCl pH8.0). The tube was kept on ice for 5 minutes and then 25 µl of solution 3 (250 mM EDTA pH8.0) was added. The contents were stored on ice for 5 minutes, followed by addition of 100 µl of solution 4 (1% Triton x-100, 0.4% sodium deoxycholate, 50 mM Tris HCl pH8.0, 62.5 mM EDTA pH8.0). The lysate was again transferred to ice for 10 minutes and 10 µl of 1 M $MgCl_2$ and 2 µl of 10 mg/ml pancreatic deoxyribonuclease were added. The contents were incubated at 37°C for 1 hour and centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was stored at -20°C for analysis by electron microscopy or immunodot analysis.

III.B.18.b. In the second method, cultures of E.coli strain RB791 harbouring control and recombinant plasmids directing the expression of HBcAg and HBcAg fusion proteins, were grown overnight at 37°C with shaking in LB-amp medium (containing 100 µg/ml of ampicillin). The following day when high cell density was obtained, medium was diluted with fresh LB (or LB-amp) (1:50). When culture reached OD_{600} of 1.0, protein expression was induced with IPTG to 0.5mM (using a stock of 1M IPTG in dw). Before adding IPTG, some culture medium was stored at -20°C as a control. After 16-18 hours of shaking at 37°C, bacteria were harvested by centrifugation (12,000x g) for 10 minutes at 4°C. The cell pellet was resuspended in lysis buffer (50 mM Tris-HCl pH8.0; 1% Triton X-100 v/v) in 1/100 volume of the culture medium (10 ml for 1 litre culture). Cells were lysed by sonicating for 8-10 times, 30 seconds a burst, in Soniprobe (Dawe Instrument type 7530A). The cellular debris in the lysate was sedimented by centrifugation (12,000x g) for 10 minutes at 4°C and the resulting supernatant, the crude extract, was recovered.

Proteins in the crude extract were precipitated by adding ammonium sulphate to 30% saturation. The salt was added to the crude extract over a period of 30 minutes with stirring at 4°C, followed by further stirring for 30 minutes at the same temperature. The precipitate was centrifuged (27,000x g) for 10-15 minutes at 4°C and the pellet resuspended in the same volume of Tris Triton lysis buffer as above. The ammonium sulphate fraction was dialysed against three changes of 50 mM Tris HCl pH8.0 at 4°C overnight or longer and centrifuged (12,000x g) for 10 minutes at 4°C to remove debris. The supernatant was stored at -20°C and the amount of protein estimated by Lowry method. An aliquot was subjected to SDS-PAGE analysis to confirm the presence of proteins of appropriate molecular weight. The supernatant was then chromatographed on sepharose 4B-CL in 0.1M $NaHCO_3$ pH7.0 in a cold room. The fractions containing the fusion proteins were identified again by SDS-PAGE and pooled. The fusion protein particles were collected by ultracentrifugation (100,000x g) for 60 minutes at 4°C (TH641 rotor, Combi, Sorvall). The resulting pellet was resuspended by rocking overnight or longer in 0.5 ml PBS at 4°C.

III.B.19. Protein Methods

III.B.19.a. Protein Estimation by Lowry Method

Lowry Reagent:

- A 2% Na_2CO_3 (anhydrous) in 0.1 N NaOH
- B 0.5% $CuSO_4.5H_2O$ in 1% sodium citrate
- C 50 ml A (freshly prepared) + 1 ml B
- D Folin-Ciocalteau reagent (Sigma) diluted 1:1 with water

Protein sample (1, 2 and 5 µl in final volume of 0.4 ml distilled water) was added to 2 ml of reagent C, mixed immediately and left at room temperature for 10 minutes. Freshly prepared reagent D (0.2 ml) was added, mixed well

and stood for 30 minutes at room temperature. OD_{550nm} was measured for each sample. The assay was calibrated using BSA at a concentration of 10-300 µg/ml.

III.B.19.b. Protein Estimation by Bradford Method (Coomassie blue binding)

Method 1:

100 mg Coomassie brilliant blue G-250 is dissolved with vigorous agitation in 50 ml of 95% ethanol, then mixed with 100 ml 85% phosphoric acid. The mixture is diluted to 1 litre with distilled water, and filtered to remove undissolved dye. The solution is stable for 1-2 weeks if kept in the cold.

To 0.1 ml sample containing up to 50 µg protein, add 2.5 ml of Coomassie blue reagent and read absorbance at 595 nm after 2-30 minutes

Method 2:

600 mg Coomassie brilliant blue G-250 is dissolved in 1 litre 2% perchloric acid, and filtered to remove undissolved material. This solution is stable indefinitely.

To 1.5 ml sample containing up to 50 µg protein, add 1.5 ml Coomassie blue reagent, and read absorbance at 595 nm after 2-30 minutes. For dealing with concentrated protein solutions ($\geq$ 5 mg/ml^{-1}), it is convenient to take 1.5 ml water or dilute sodium chloride, add the sample from a microlitre syringe, mix well, then add the dye reagent. Concentrated protein solution will precipitate on contact with the dye reagent. Reagent 1 gives a higher absorbance reading than reagent 2, but is less stable.

III.B.20. Electrophoresis of Proteins in SDS-polyacrylamide Gels (SDS-PAGE)

Acrylamide stock solution (30%):

Acrylamide	29.2% (w/v)
N,N'-bismethylene acrylamide	0.8% (w/v)

in water, filter and store in dark bottles at 4°C.

Resolving gel: (volumes shown in ml)

Gel	30% Acrylamide	1.5M TrisHCl pH8.8	Water
5%	1.66	4.18	4
7.5	2.5	3.34	4
10	3.34	2.5	4
12.5	4.16	1.68	4
15	5	0.84	4

Add SDS (10% w/v), ammonium persulphate (10% w/v) and TEMED.

Stacking gel:

Tris-HCl (0.6M pH6.8)
Acrylamide stock 30%
DW
SDS 10%
Ammonium persulphate 10%
TEMED

Runner buffer (10x):

Tris-HCl pH8.3	0.25 M
Glycine	1.92 M
SDS	1%

2x Protein dissociation buffer (10 ml):

Tris HCl pH6.8 (0.6M)	2 ml
Glycerol	2 ml
SDS 10% (or 0.4 gm powder)	4 ml
Bromophenol blue in water	2 ml

Proteins were separated by SDS-PAGE as described by Laemmli (1970). Resolving gel solution was prepared and poured between two sealed glass plates (Biorad equipment), overlaid with water and allowed to polymerise at 60°C for 20-30 minutes. The water was discarded and plates dried with Whatmann filter paper. The stacking gel solution was prepared and poured onto resolving gel and allowed to polymerise as above. The gel assembly was transferred to an electrophoresis tank in 1x runner buffer. Samples were prepared by heating with an equal volume of 2x protein dissociation buffer at 100°C for 5 minutes. Low molecular weight markers (Pharmacia), including phosphorylase b (94 kD), albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20 kD) and lactalbumin (14.4 kD) were also loaded. Rainbow coloured protein molecular weight markers were included in the gels processed for immunoblot analysis. Electrophoresis was performed at 25-30 mA.

III.B.21. Coomassie Staining of Proteins following SDS-PAGE

After electrophoresis of the proteins, gels were stained with a solution containing Coomassie brilliant blue (0.2% w/v) in methanol (40% v/v) and acetic acid (10% v/v), with gentle shaking at room temperature. Staining was done for 20-30 minutes depending on the thickness of the gel and polyacrylamide concentration. This was followed by destaining in methanol (40% v/v) and acetic acid (10% v/v) for 40-60 minutes or longer with shaking at room temperature. Glycerol (5% v/v) was added to the destaining solution and the gels were dried after transferring and sealing the gels between two layers of a plastic film (Biorad) and left overnight or longer.

III.B.22. Immunoblotting (Western Blotting)

Immunoblotting involves separation of proteins by electrophoresis, transfer and immobilisation of the proteins onto nitrocellulose membranes, and detection of specific proteins using antibodies as probes. The blotting was carried out as described by Towbin et al (1979) with some modifications.

Transfer Buffer:

Tris HCl pH8.3	25 mM
Glycine	192 mM
Methanol	20% (v/v)

Tris Saline (10x):

Tris HCl pH8.1	100 mM
NaCl	1500 mM

Blocking Solution:

Nonfat dry milk powder (Marvel)	5% (w/v)
[or BSA (IgG free, fraction V, BDH)	3% (w/v)]
Sodium azide	0.02% (w/v)
in 1x Tris Saline	

Alkaline Phosphatase Buffer:

Tris HCl pH9.5	100 mM
NaCl	100 mM
$MgCl_2$	50 mM

III.B.22.a. Transfer of Proteins from Gels to Nitrocellulose Membranes

After separation of proteins by SDS-PAGE, the stacking gel was discarded and the separation gel equilibrated in transfer buffer before blotting. The electroblotting was done using the Trans-Blot Cell (Biorad). The gel was overlaid with

a nitrocellulose membrane (Bioblot-NC, Costar, Cambridge, Massachussets, USA) and was sandwiched between 3MM filter paper and foam sponge. The components were soaked in transfer buffer before assembly. The electroblot apparatus was assembled and filled with transfer buffer, with the nitrocellulose membrane facing the anode. The proteins were transferred to nitrocellulose at 60V (350 mA) for 1 hour.

After transferring the proteins but prior to antibody probing of the blots, the proteins can be reversibly stained using Ponceau S (0.5% Ponceau S (Sigma) in 3% TCA) for 30-60 seconds at room temperature with agitation and destained with distilled water. In some gels, Rainbow coloured molecular weight proteins markers (Pharmacia) were run to serve as internal markers for transfer and molecular weight calibrations.

III.B.22.b. Immunostaining of Proteins

Prior to antigen detection, the blot was incubated in blocking solution for at least 1 hour at room temperature, with shaking, to prevent nonspecific adsorption of the immunological reagents. Following blocking, the membrane was incubated with appropriate dilution of primary antibody in blocking solution for 2 hours or longer at room temperature with shaking. The membrane was washed five times with 1x Tris Saline for 5 minutes each. Depending on the animal source of the primary antibody, the secondary antibody was either sheep anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (Sigma) or goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma). The blot was incubated with a 1:7500 dilution of the secondary antibody in blocking solution for 2 hours at room temperature with agitation. The blot was washed with 5 changes of 1x Tris Saline for 5 minutes each. Before developing the blot, fresh substrate solution was prepared each time by adding nitroblue tetrazolium (NBT, 5% in 70% dimethylformamide; 66 µl) and 5-bromo 4-chloro 3-indolyl

phosphate (BCIP, 50 mg/ml in water or 5% in 100% dimethylformamide; 33 µl) to alkaline phosphatase buffer (10 ml). Substrate solution was added to the blot with shaking, to develop the colour reaction within 10-20 minutes. The reaction was stopped by washing the blot in several changes of distilled water.

III.B.23. Electron Microscopy (EM)

The IPTG-induced sample, obtained above was used for making EM grids. A drop was placed on a glass slide, and an EM grid held with forceps was placed on the drop with its matt side towards the sample. After being in contact with the sample for 1 minute, the grid was lifted and blotted dry by touching its edge with a piece of Whatman 3MM paper. The grid was then placed on a drop of 10% phosphotungstic acid solution pH 5.3, for 30 to 40 seconds and blotted dry again. It was kept in a labelled petri dish, and examined by transmission Electron Microscopy.

III.B.24. Immune Electron Microscopy

Some EM grids were prepared with samples that had been incubated with antibody. In these preparations, 10 µl of sample was mixed with same volume of undiluted antibody and incubated at 4°C overnight. The contents were centrifuged for 30 minutes in a microfuge, and the pellet was resuspended in 10 µl of DW and used for making an EM grid.

III.B.25. Immunodot Assay for Expression of Proteins

The modified protocol outlined below, was used for performing the assays [Young, 1989].

A nitrocellulose membrane (Schleicher and Schull, S&S

membrane filter) and two Whatman 3MM sheets cut to the same size as the manifold apparatus were prewetted in TBS buffer (20 mM Tris HCl pH7.5, 150 mM NaCl) for 30 minutes and then arranged in the manifold. The sample and the control dilutions were prepared using PBS in a final volume of 100 µl, added to the wells of the manifold and spotted on the membrane by suction with a vacuum pump. The membrane was allowed to air dry and cut into strips of 0.5 cm. Each strip was separately blocked with 2% non-fat milk powder (Marvel, Cadbury's) for 2 hours at room temperature with shaking, and then washed for 10 minutes, in PBS containing 0.05% Tween 20 (PBS-T). The primary antibody dilution was prepared in PBS containing 0.05% Tween 20 and 2% milk powder (PBS-T-MP) in 2 ml and the strip was incubated for 2 hours at room temperature with shaking, with appropriate antibodies, depending on the type of assay.

The strips were washed three times for 10 minutes in PBS-T, and incubated with 1:1000 dilution of secondary antibody (goat anti-rabbit, or goat anti-mouse antibody conjugated to HRP, Jackson Immunoresearch Labs), in PBS-T-MP for 1 hour. The strips were washed with PBS-T twice for 10 minutes, and in TBS buffer for 10 to 15 minutes. During the last wash, HRP substrate mixture was prepared by dissolving 30 mg of chloronaphthol in 10 ml cold methanol. This solution was added to 50 ml of TBS buffer, to which 30 µl of 30% hydrogen peroxide was added just prior to immersion of strips. The colour development was allowed for 10 to 15 minutes, and the strips were washed under stream of tap water for 5 minutes, fixed with 10 mM sulphuric acid for 5 seconds. After a final rinse in tap water for 5 minutes, the strips were air dried and stored in plastic bags in dark.

Some strips were prepared using an IPTG-induced sample that had been incubated with the same volume of undiluted antibody. The solution was either kept at 37°C for 2 hours or left at 4°C overnight, after which it was centrifuged for 30 minutes. The supernatant was carefully removed and used in the immunodot assay.

III.B.26. Conversion of HBcAg to HBeAg [Mackay et al., 1981]

To 50 µl of sample, 2.5 µl of 20 mg/ml pronase, and 2-MCE (final concentration 0.1%) were added and incubated at 37°C for 2 hours. This was spotted on a nitrocellulose membrane in a manifold apparatus and the assay was performed as described above with the exception that the primary antibody was anti-HBe mouse monoclonal A2C12.

III.B.27. Immunological Methods

III.B.27.a. Preparation of Immunogen for Immunization

The HBcAg fusion protein (30 µg) was diluted in 0.5 ml PBS and 0.5 ml of complete Freund's adjuvant (CFA) for immunisation or same volume of incomplete Freund's adjuvant (IFA) for booster injections. The two phases were mixed by using a tissue homogeniser (Omni-mixer; Sorvall, Newtown, Conn., USA) or by passing the mixtures from a syringe into a glass universal bottle and back vigorously, and repeated until a thick emulsion developed.

III.B.27.b. Immunisation and Bleeding of Animals

Outbred dutch male rabbits (6 months of age; 1.5 kg) were immunised intramuscularly with 30 µg of immunogen emulsified in CFA on day 0 and subsequently boosted twice with the same antigen emulsified in IFA on days 30 and 60. Blood was collected from rabbits on days 0 (preimmune; PI), 10 (first test bleed; ITB), 40 (second test bleed; 2TB) and 70 (third test bleed; 3TB).

The injection and bleeding of animals was carried out by the staff of the Medical Microbiology Transgenic Unit, University of Edinburgh.

III.B.27.c. Separation of Sera from Blood

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

III.B.28. Solid-phase Radioimmunoassays (RIA)

III.B.28.a. Preparation of Antibody-coated Solid Phase

Polystyrene beads (polystyrene balls, 6.4 mm diameter, specular finish, NBL) were placed in 50 ml centrifuge tube and coated with rabbit anti-HBc antibody in coating buffer (0.2 M $NaHCO_3$, 0.02% Sodium azide) for overnight at room temperature with shaking (for mouse ascites fluid use 1:150 or 1:200 dilution in coating buffer, for polyclonal anti-HBc IgG use 60 µg/ml in coating buffer). The antibody-coated beads were washed three times with PBS, blotted dry on a tissue paper and stored at 4°C in a universal.

III.B.28.b. Assay of HBc Antigenicity by Sandwich RIA

HBc antigenicity was determined by a modification of the sandwich RIA described previously by Stahl et al, 1982 (see fig 36).

Polystyrene beads coated with rabbit anti-HBc antibody (section III.B.28.a.) were used for capturing HBcAg and HBcAg-fusion proteins. The proteins were diluted with blocking buffer (PBS containing 10% sheep serum and 0.02% sodium azide) in a final volume of 200 µl and added to wells containing beads. Each sample was assayed in duplicate.

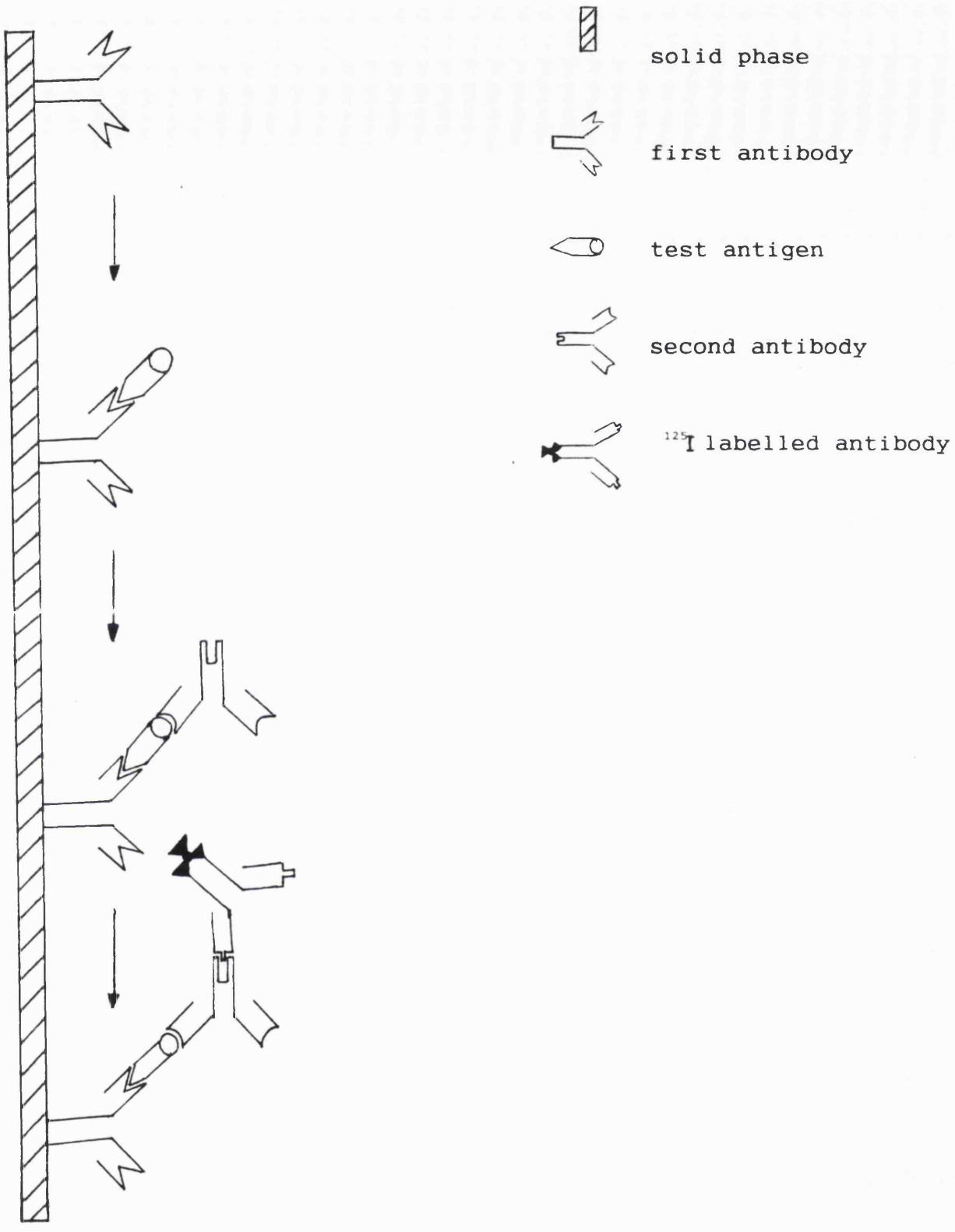


FIG 36: Antigen capture Sandwich RIA

After incubation at 37°C for 2 hours, the samples were aspirated and the beads washed with washing buffer (PBS containing 0.05% Tween 20) for 5-6 times. A suitable dilution (1:250 or 1:500) of mouse polyclonal anti-HBc antibody (B10.S Group 1 Day 43 anti-HBc serum and Balb/c Group 21 Day 73 anti-HBcS serum from A.L.Shiau [IgG fraction was purified and used in the experiments]) in a final volume of 200 µl blocking buffer was added to each well, and incubated at 37°C for 1 hour. The beads were washed as above and ^{125}I labelled sheep anti-mouse Immunoglobulin (200 µl of 250,000 cpm/ml in blocking buffer [50,000 cpm per bead] Amersham) was added to each well. After a further hour at 37°C, the beads were washed for 8-10 times, transferred to counting tubes and counting done in a Gamma counter (1275 Minigamma, LKB).

III.B.28.c. Assay of HCMV Antigenicity by Sandwich RIA

The assay used to determine binding of HBcAg-fusion protein containing HCMV epitope to anti-HCMV monoclonal (7-17) and anti-HCMV polyclonal antibody (human) was performed as detailed in section III.B.28.b. with some exceptions. In the first experiment, instead of using mouse anti-HBc polyclonal antibody, anti-HCMV monoclonal 7-17 was used (1:250 dilution). In the second experiment, anti-HCMV polyclonal antibody (human; 1:80-1:100 dilution) replaced anti-HBc antibody, followed by incubation with ^{125}I labelled sheep anti-human Immunoglobulin (250,000 cpm/ml; Amersham).

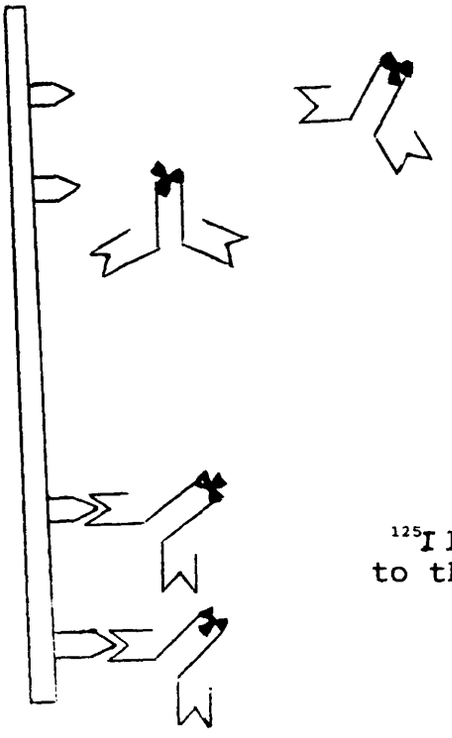
III.B.28.d. Preparation of HBcAg-coated Solid Phase

Polystyrene beads (6.4 mm diameter, specular finish, NBL) were placed in a 50 ml Falcon tube and incubated with reference HBcAg (0.54 or 0.72 µg/ml; 1:5000 dilution; 1 µl/5 ml) in coating buffer (Na_2CO_3 pH9.2, 0.05M) for 6-7 hours or overnight at room temperature, with rocking. The beads were

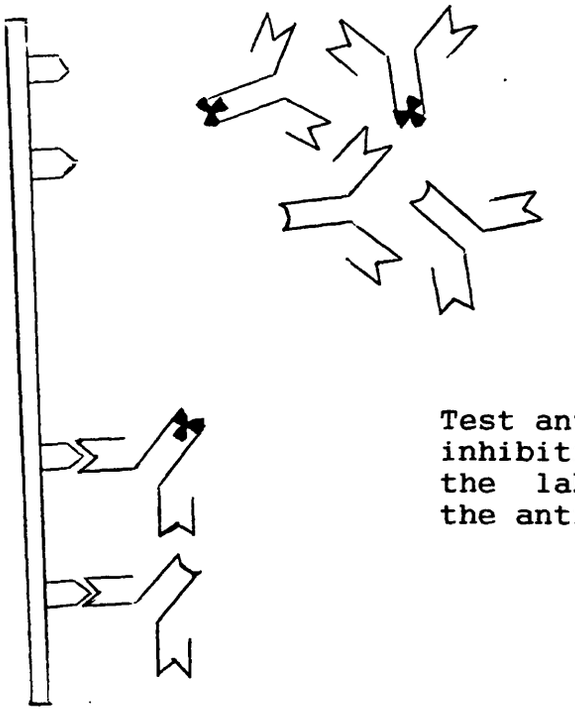
washed with PBS and blotted dry on a tissue paper before storing at 4°C in a Universal bottle.

III.B.28.e. Detection of anti-HBc Antibody by Competitive RIA

This competitive RIA was performed to detect anti-HBc in test sera, as described by Murray et al., 1984 (see fig 37). Test serum dilutions (1:10, 1:30, 1:100) along with anti-HBc positive control serum (rabbit 86) and negative control (PBS) were prepared using PBS and 100 µl of each was added to the wells of the tray followed by 100 µl of ^{125}I human anti-HBc IgG (480,000 cpm/ml in PBS; 60,000 cpm/bead) provided by G.Leadbetter of Department of Medical Microbiology, University of Edinburgh. A bead coated with HBcAg was added to each well and the tray incubated overnight at room temperature. After incubation, the beads were washed 8-10 times with PBS or DW, transferred to counting tubes and radioactivity counted by Gamma counter (1275 Minigamma, LKB).



^{125}I labelled antibody binding to the antigen on the solid phase



Test antibody (unlabelled) inhibiting the binding of the labelled antibody to the antigen

FIG 37: Antibody capture Competitive RIA

III.B.29. Bacterial Strains

Name	Genotype	Reference
TG1	supE, hsdΔ5, thi, Δ(lac-proAB), F'(traD36, proAB$^+$, lacI^q, lacZΔM15)	Gibson (1984)
RB791	ara, Δ(lac-pro), straA, thi, lacZΔM15	Brent & Ptashne (1981)

III.B.30. Antibodies

Antibodies	Source
Polyclonal anti-HBc (rabbit) Polyclonal anti-HBc (mouse)	C.R.Howard, A.L.Shiau A.L.Shiau
Monoclonal anti-HBc; RFc17	J.A.Waters
Monoclonal anti-HBc; 35H2, 18CE11B12, 18CH5G1, A2	E.Korec
Monoclonal anti-HBe; A2C12	E.Korec
Monoclonal anti-HCMV; 7-17 Polyclonal anti-HCMV	V.C.Emery V.C.Emery, J.Peutherer
Human anti-HBs	A.Kitchen

III.C. RESULTS

III.C.1. Construction of an Expression Plasmid Containing the HBcAg Amino Terminal Fusion with HCMV-gp58: pv404CMV

Plasmids p73HCMV and pPV404 were used to construct an HBcAg amino terminal fusion with HCMV-gp58. Plasmid pPV404 had EcoRI and BamHI restriction enzyme sites in the precore region to facilitate ligation to foreign sequences.

III.C.1.a. PCR Amplification of p73HCMV using Primers CMV3 and CMV4

One pg of EcoRI digested p73HCMV DNA was PCR amplified using the CMV3 and CMV4 primer pair, followed by visualization of 0.5 µl of extracted and precipitated product on a 2% agarose gel. A fragment of 150 bp was present in both of the lanes containing the PCR product [fig 38A, lanes 2 and 4], although not in the negative control lanes [lanes 3 and 5]. After Southern transfer and hybridization with $1x10^7$ cpm of radioactively labelled HCMV probe, a fragment of 150 bp was seen in both of the lanes containing the product [fig 38B].

III.C.1.b. Digestion of PCR Product and pPV404

The CMV3 and CMV4 primers had EcoRI and BamHI restriction enzyme sites incorporated at their respective 5' ends, to help ligation to pPV404. After visualization on the gel, the remaining 29 µl of PCR product was double digested with EcoRI/BamHI and, after extraction and precipitation, was

FIG 38: Electrophoresis of products of PCR amplification of plasmid p73HCMV with CMV3 and CMV4 primers, on a 2% agarose gel (A), and Southern blot hybridization with radioactively labelled HCMV probe (B).

(A)

LANES:

1) 1 µg of HindIII digested lambda marker DNA (fragment size shown in kb)

2 & 4) 0.5 µl of PCR product

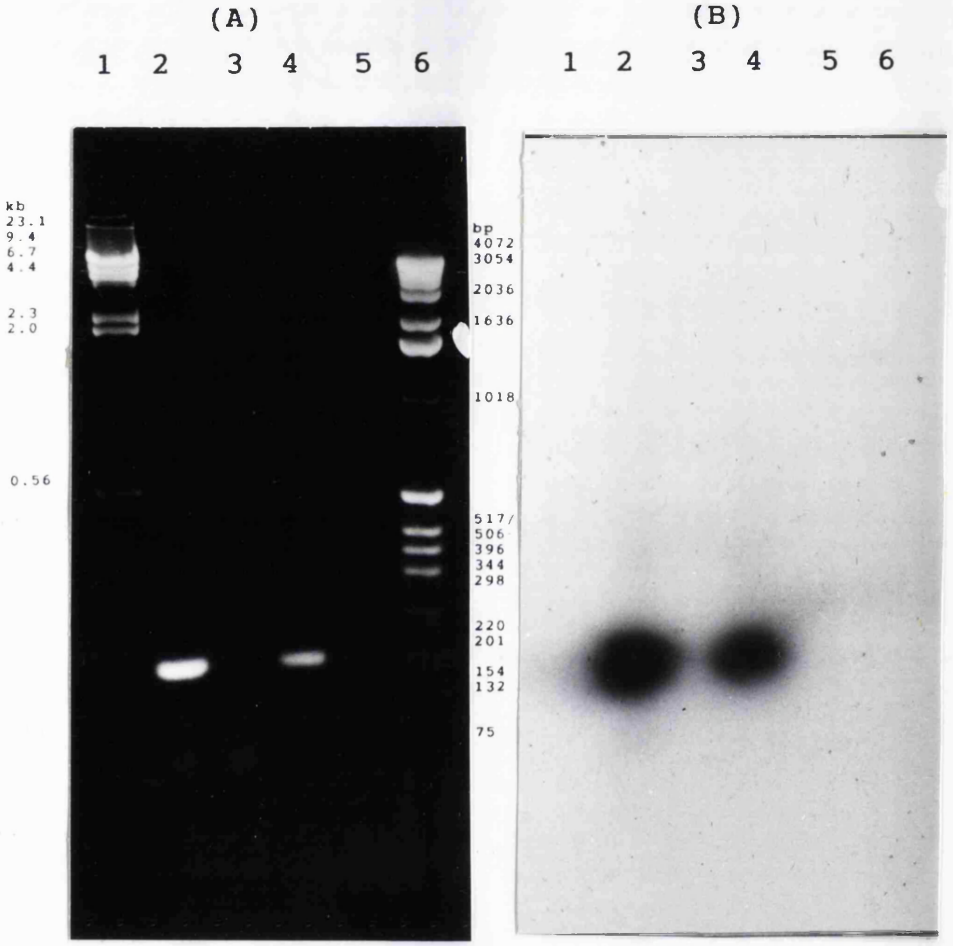
3 & 5) 0.5 µl of PCR products from negative control reactions without plasmid DNA

6) 1 µg of 1 kb ladder marker DNA (fragment size shown in bp)

(B)

Southern hybridization of (A) with ^{32}P labelled HCMV probe.

Lanes 2 & 4) 150 bp fragment


(A)
1 2 3 4 5 6
kb
23.1
9.4
6.7
4.4
2.3
2.0
0.56
bp
4072
3054
2036
1636
1018
517/
506
396
344
298
220
201
154
132
75
(B)
1 2 3 4 5 6

dissolved in 6 µl of DW. An aliquot of 0.5 µl was run on an agarose gel to estimate the amount of DNA present.

Eight µg of pPV404 was double digested with EcoRI/BamHI, and visualized on a 1% LMP agarose gel. Two fragments of 4000 bp and 30 bp were produced. The latter contained the sequences encoding one of the neutralizing epitopes of the polio virus whereas the 4000 bp fragment containing the plasmid was excised, purified and used for ligation of the PCR product.

III.C.1.c. Ligation

The ligation reaction was set up with 100 ng of EcoRI/BamHI digested PCR product and 50 ng of EcoRI/BamHI digested pPV404 DNA, and used to transform competent E.coli TG2 strain. A control containing EcoRI/BamHI double digested plasmid DNA was also set up. Several colonies were present on the plates containing the ligation product, and a few on the negative control plate. Eleven recombinant colonies and one control pPV404 colony were grown for analysis.

III.C.1.d. Analysis of pv404CMV Clones by Restriction Enzyme Digestion of Mini-prep DNA

Plasmid DNA obtained by the mini-prep method was analyzed by double digesting DNA from 5 pv404CMV clones with EcoRI/BamHI and running it on an agarose gel. In addition, EcoRI/BamHI digested pPV404 DNA from control clone and p73HCMV DNA (as a positive control size marker) were run on the same gel. The lanes containing DNA from the pv404CMV clones showed one fragment of 4000 bp in each lane, corresponding to the digested vector [fig 39A, lane 2-6], but no band corresponding to the expected HCMV fragment of 150 bp could be seen on the gel. The lane containing the control pPV404 clone showed numerous fragments [fig 39A, lane 7], whereas the lane with

FIG 39: Electrophoresis of EcoRI/BamHI digested DNA obtained from pv404CMV clones, on a 1% agarose gel (A), and Southern blot hybridization with a radioactively labelled HCMV probe (B).

(A)

LANES:

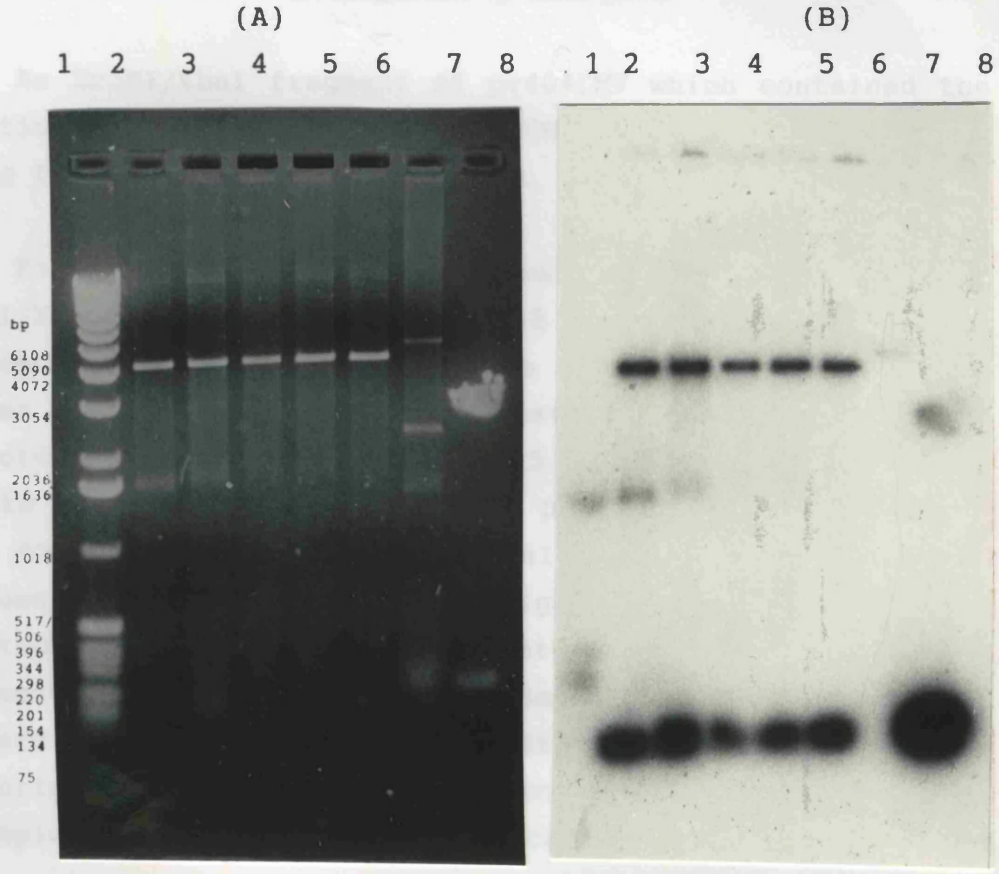
1) 1 µg of 1 kb ladder marker DNA (fragment size shown in bp)
2) EcoRI/BamHI digested pv404CMV clone 1
3) EcoRI/BamHI digested pv404CMV clone 2
4) EcoRI/BamHI digested pv404CMV clone 3
5) EcoRI/BamHI digested pv404CMV clone 4
6) EcoRI/BamHI digested pv404CMV clone 5
7) EcoRI/BamHI digested pPV404 DNA (negative control)
8) EcoRI/BamHI digested p73HCMV DNA (positive control)

(B)

Southern hybridization of (A) with ^{32}P labelled HCMV probe.

Lanes 2-6) diagnostic fragments of 150 bp

Lane 8) control 150 bp fragment as a size marker


(A)
(B)
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
bp
6108
5090
4072
3054
2036
1636
1018
517
506
396
344
298
220
201
154
134
75

p73HCMV DNA had two fragments of 3039 bp and 150 bp [lane 8]. The DNA was transferred and after hybridization with a HCMV probe showed a strong band of 150 bp in all five lanes [fig 39B, lanes 2-6] which were of the same size as the positive control fragment [lane 8]. No band was seen in the lane containing the negative control pPV404 [lane 7].

III.C.1.e. Nucleotide Sequencing Analysis

An EcoRI/XbaI fragment of pv404CMV which contained the junction between the HBV core and HCMV sequences was sequenced using the chain termination method.

Five µg of pv404CMV DNA was double digested with EcoRI/XbaI, and analyzed on a 1% LMP agarose gel. Two fragments of 3849 and 300 bp were seen on the gel and the latter was excised. The DNA was extracted, precipitated and dissolved in 10 µl TE pH8.0 and 0.5 µl was run on an agarose gel to estimate the amount of DNA present. MI3mp18 DNA was also double digested with EcoRI/XbaI and 100 ng was run on an agarose gel to make sure that digestion was complete. A ligation reaction was set up between 200 ng of ptacCMV fragment and 100 ng of digested M13mp18 DNA. The product was transformed into competent E.coli strain (TG2), plated and 12 colourless plaques were grown for analysis. The samples from 4 templates were analyzed in duplicate.

The nucleotide sequence obtained for pv404CMV [Fig 40] was exactly as predicted [fig 34A]. The EcoRI site [5'] present in pPV404 was utilized for ligation, by incorporating the same site in the CMV3 primer. This site also contained the precore ATG, although the nucleotide 'A' of this codon was lost during cloning of EcoRI/XbaI fragment of pv404CMV in M13mp18. Therefore, it was not possible to confirm the presence of 'A' from this sequencing analysis, and thus to establish that the HCMV sequence was in-frame with the

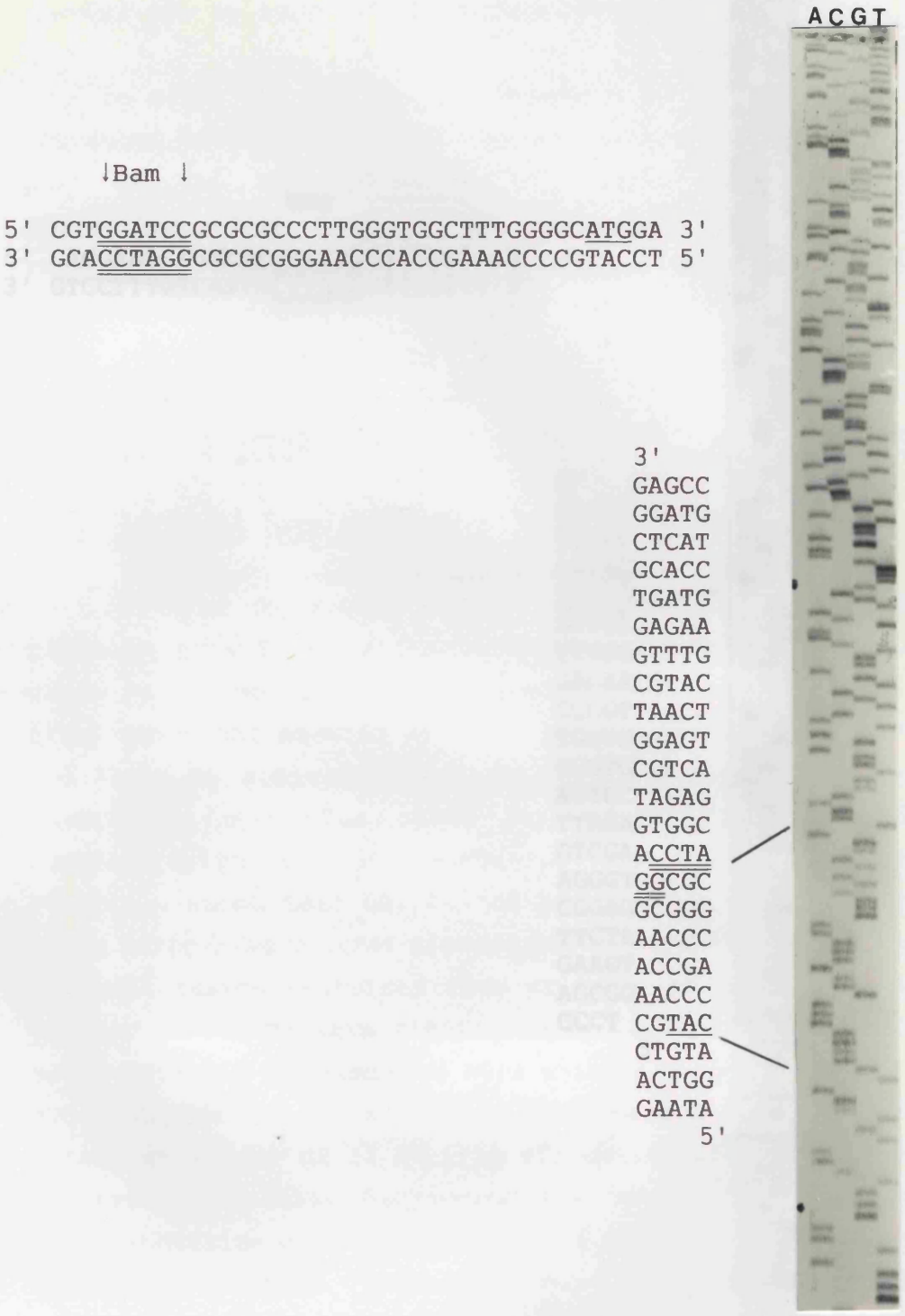


FIG 40: Nucleotide sequencing of a part of pv404CMV, as determined by dideoxy chain termination method.

The regenerated BamHI site and Core initiation codon have been indicated. Compare with predicted nucleotide sequence in fig 34A.

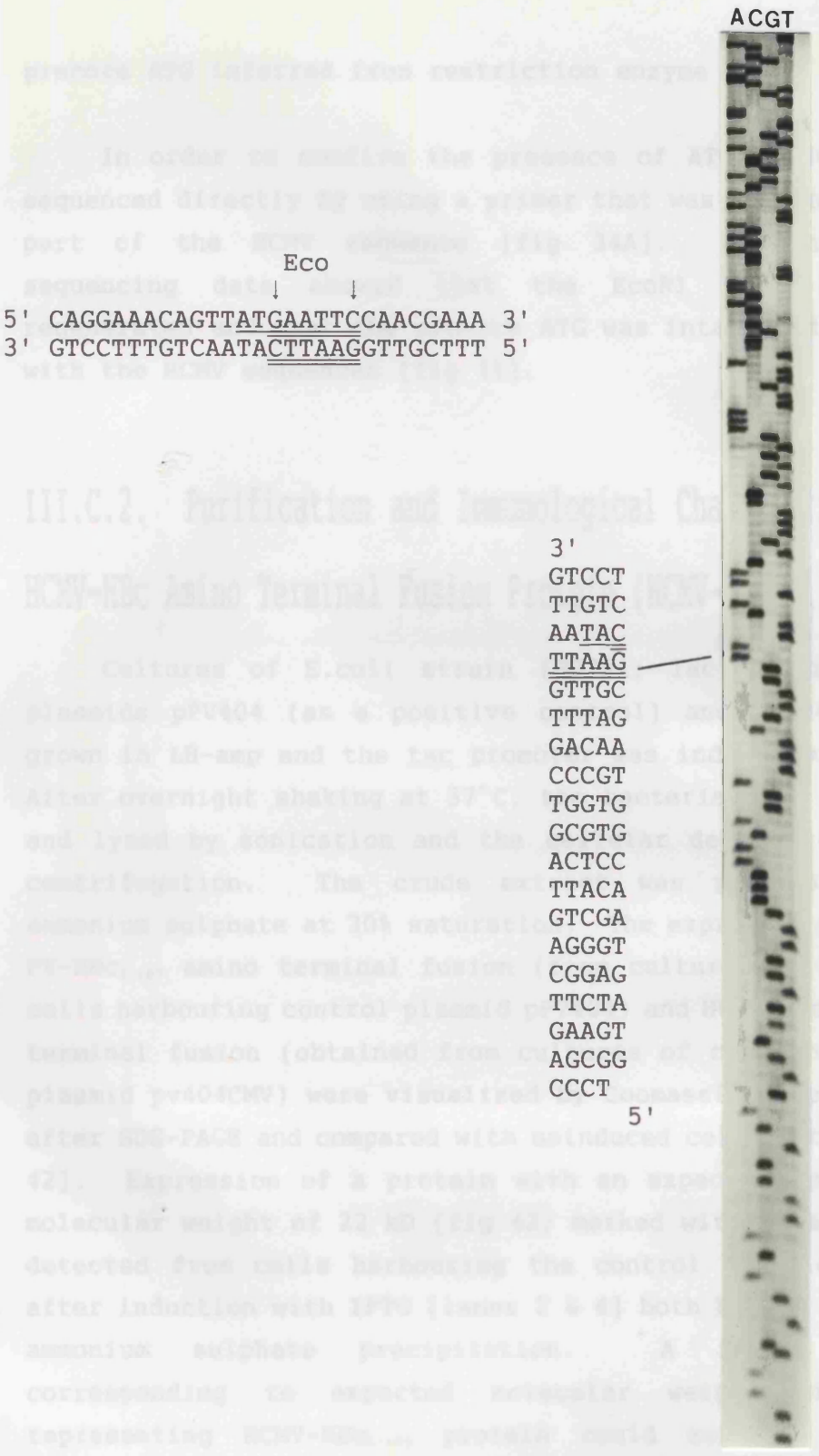


FIG 41: Direct sequencing of a part of pv404CMV.

The regenerated EcoRI site and preC initiation codon have been indicated.

precore ATG inferred from restriction enzyme site.

In order to confirm the presence of ATG, pv404CMV was sequenced directly by using a primer that was homologous to a part of the HCMV sequence [fig 34A]. The nucleotide sequencing data showed that the EcoRI site had been regenerated and that the precore ATG was intact and in-frame with the HCMV sequences [fig 41].

III.C.2. Purification and Immunological Characteristics of HCMV-HBc Amino Terminal Fusion Protein (HCMV-HBc_{1-183})

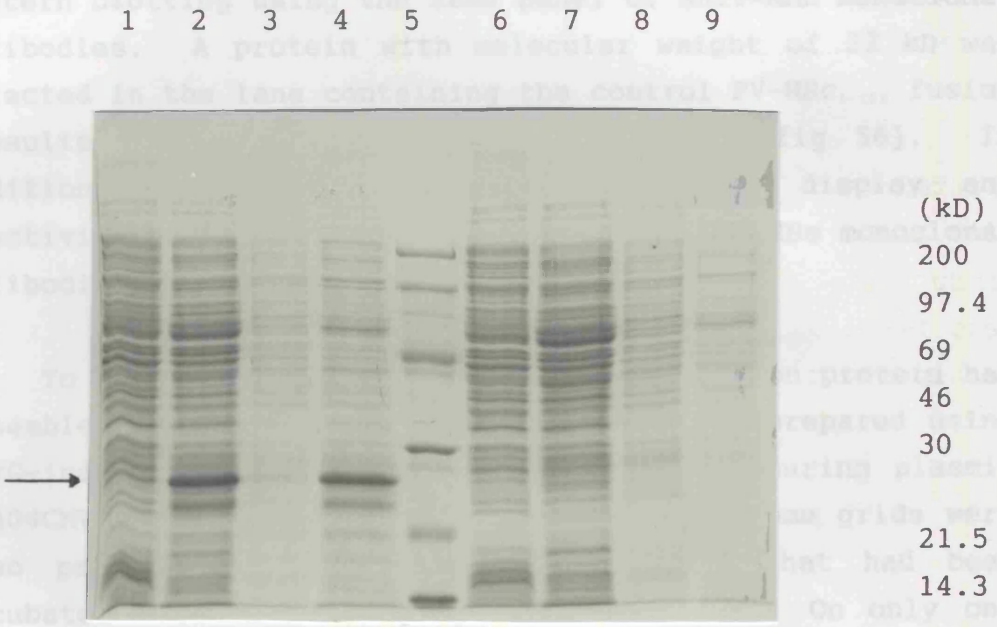
Cultures of E.coli strain (RB791; lac I^q) harbouring plasmids pPV404 (as a positive control) and pv404CMV were grown in LB-amp and the tac promoter was induced with IPTG. After overnight shaking at 37°C, the bacteria were harvested and lysed by sonication and the cellular debris removed by centrifugation. The crude extract was precipitated by ammonium sulphate at 30% saturation. The expressed proteins, PV-HBc_{1-183} amino terminal fusion (from cultures of bacterial cells harbouring control plasmid pPV404) and HCMV-HBc_{1-183} amino terminal fusion (obtained from cultures of cells harbouring plasmid pv404CMV) were visualized by Coomassie blue staining after SDS-PAGE and compared with uninduced cell extracts [fig 42]. Expression of a protein with an expected approximate molecular weight of 22 kD [fig 42; marked with an arrow] was detected from cells harbouring the control plasmid pPV404, after induction with IPTG [lanes 2 & 4] both before and after ammonium sulphate precipitation. A fusion protein corresponding to expected molecular weight of 24 kD representing HCMV-HBc_{1-183} protein could not be detected. Although further purification was not carried out for HCMV-HBc_{1-183} protein, cultures were examined immunologically and by electron microscopy.

FIG 42: SDS-PAGE and Coomassie blue staining of proteins expressed after IPTG-induction of cultures harbouring plasmids pPV404 (polio virus-HBc_{1-183} amino terminal fusion) and pv404CMV (HCMV-HBc_{1-183} amino terminal fusion).

Protein samples from soluble fractions of crude extracts after 30% ammonium sulphate precipitation from uninduced and IPTG-induced cultures harbouring plasmids pPV404 (positive control) and pv404CMV, were fractionated on 12.5% acrylamide gel and stained with Coomassie brilliant blue.

LANES:

1) PV-HBc_{1-183}; pre-IPTG, before $(NH_4)_2SO_4$ precipitation
2) PV-HBc_{1-183}; post-IPTG, before $(NH_4)_2SO_4$ precipitation
3) PV-HBc_{1-183}; pre-IPTG, after $(NH_4)_2SO_4$ precipitation
4) PV-HBc_{1-183}; post-IPTG, after $(NH_4)_2SO_4$ precipitation
5) protein molecular weight markers
6) HCMV-HBc_{1-183}; pre-IPTG, before $(NH_4)_2SO_4$ precipitation
7) HCMV-HBc_{1-183}; post-IPTG, before $(NH_4)_2SO_4$ precipitation
8) HCMV-HBc_{1-183}; pre-IPTG, after $(NH_4)_2SO_4$ precipitation
9) HCMV-HBc_{1-183}; post-IPTG, after $(NH_4)_2SO_4$ precipitation


1 2 3 4 5 6 7 8 9
(kD)
200
97.4
69
46
30
21.5
14.3

To determine the HBc antigenicity of the HCMV-HBc_{1-183} fusion protein, dot blot and Western blot immunoassays were performed. In dot blot assays using a panel of anti-HBc monoclonal antibodies, no HBc reactivity could be demonstrated for HCMV-HBc_{1-183}. However, the control PV-HBc_{1-183} fusion reacted with all anti-HBc monoclonal antibodies tested and always produced a strong colour reaction compared to truncated $HBcAg_{3-144}$, which was used as a second positive control (protein expressed from IPTG-induced bacterial cultures harbouring plasmid ptacHpaII-R2 [figs 52, 53 & 54]. Furthermore, no HBc reactivity could be detected for the HCMV-HBc_{1-183} fusion by Western blotting using the same panel of anti-HBc monoclonal antibodies. A protein with molecular weight of 22 kD was detected in the lane containing the control PV-HBc_{1-183} fusion (results only shown for one Western blot) [fig 56]. In addition, the HCMV-HBc_{1-183} fusion did not display any reactivity with anti-HCMV (MAb 7-17) and anti-HBe monoclonal antibodies in dot blot or Western blot assays.

To determine whether the HCMV-HBc_{1-183} fusion protein had assembled into core particles, EM grids were prepared using IPTG-induced cultures of bacterial cells harbouring plasmid pv404CMV, along with appropriate controls. Some grids were also prepared using IPTG-induced cultures that had been incubated with anti-HBc polyclonal antibody. On only one occasion, core-like particles were visualized for HCMV-HBc_{1-183} fusion [fig 43A]. These hybrid core particles were similar to HBcAg particles produced in E.coli but appeared somewhat larger than the core particles visualized for the PV-HBc_{1-183} fusion [fig 43B]. This difference could be explained by the fact that HCMV epitope was 50 amino acids compared to 20 amino acid epitope that had been derived from poliovirus and consequently produced slightly larger core particles.

Electron microscopy of the cultures that had been induced with IPTG for a variable length of time did not improve the chance of visualizing more core particles in the recombinant cultures. On the other hand, core particles were present in

abundance in the control cultures proving that IPTG-induction was not defective and therefore was not responsible for reduced expression of the fusion protein. Another strategy of treating IPTG-induced cultures with anti-HBc polyclonal antibody was also not fruitful, although the control cultures showed more core particles compared to the cultures that were not treated with antibody.

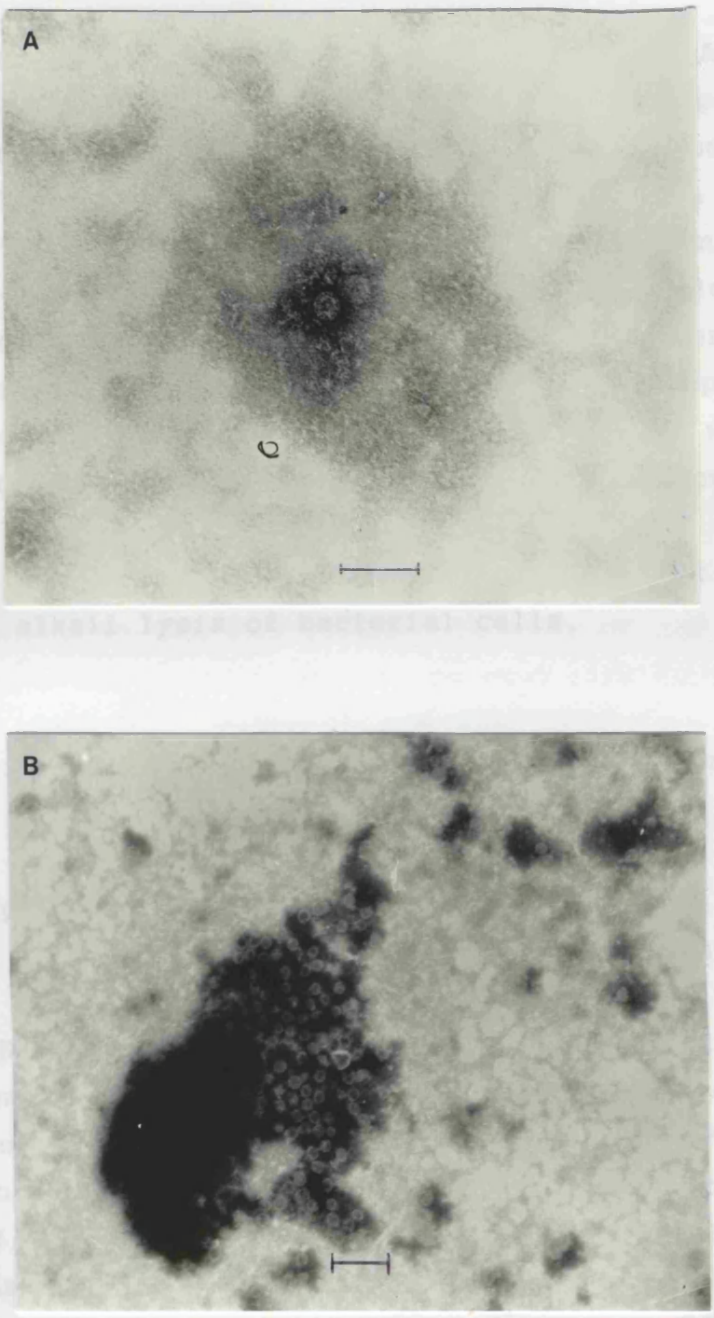


FIG 43: Electron micrographs showing core-like particles in IPTG-induced cultures of cells harbouring plasmids pv404CMV (HCMV-HBc_{1-183}) [A] and pPV404 (PV-HBc_{1-183}) [B].
Size bar = 100 nm

III.C.3. Construction of an Expression Plasmid Containing the HBcAg Carboxyl Terminal Fusion with HCMV-gp58: ptacCMV

Two plasmids were used to construct an HBcAg carboxyl terminal fusion with HCMV-gp58. Plasmid ptacHpaII-R2 was derived from ptacHpaII [Stahl and Murray, 1989] and contained only one EcoRI site instead of the two present in the parent plasmid [Professor K.Murray, personal communication]. Plasmid ptacHpaII-R2 contained a HindIII site at nucleotide 431 (equivalent to amino acid 144 of HBcAg {adw}), for insertion of foreign sequences. The PCR amplified 149 bp HCMV-gp58 coding sequences from a clinical isolate of HCMV were cloned in pT7T3 to form plasmid p73HCMV, as described above [section III.B.1.b].

Both plasmids were purified on CsCl/EtBr gradients following alkali lysis of bacterial cells.

III.C.3.a. PCR Amplification of p73HCMV using Primers CMV1 and CMV2

The primers were designed with HindIII at 5' (upstream) and BamHI restriction enzyme sites at the 3' (downstream) ends (section III.B.1.b) in order to amplify part of plasmid p73HCMV DNA (containing the 149 bp HCMV-gp58 encoding region).

Three µg of p73HCMV DNA was digested with EcoRI and 0.2 µg was run on an agarose gel, along with undigested DNA, to ensure that digestion was complete. The digested DNA was extracted, precipitated, dissolved in 10 µl of TE pH8.0 and 0.5 µl was run on a gel to estimate the amount of DNA present. The DNA was diluted in TE pH8.0, and 1 pg was PCR amplified using the CMV1 and CMV2 primer pair. In addition, a negative control was set up containing primers only. The PCR products, after extraction and precipitation, were dissolved in 30 µl of TE pH8.0, and 0.5 µl was analyzed on a 2% agarose gel containing EtBr, in duplicate.

A fragment of 150 bp was visualized on the gel in both the lanes containing the PCR products [fig 44A, lanes 2 and 4], whereas no fragment was present in the lanes containing the negative controls [lanes 3 and 5]. BamHI/EcoRI digested p73HCMV was also run along with the PCR products, this comprised two fragments of 3039 bp and 150 bp [fig 44A, lane 7]. The latter fragment which contained the HCMV insert, was of the same size as the PCR products, thus serving as a size marker.

Analysis of the PCR products was extended by Southern transfer of the DNA and hybridization with an HCMV probe. The probe was prepared by digesting 10 µg of p73HCMV DNA with BamHI/EcoRI and purifying the 150 bp fragment containing the HCMV insert by electrophoresis on an LMP agarose gel. Fifty nanogram of the fragment was radioactively labelled and $1x10^7$ cpm was used for hybridization. The blot showed a band corresponding to the PCR product in each of the lanes [fig 44B, lanes 2 and 4] which was of the same size as control HCMV fragment [lane 7]. Although the HCMV probe overlapped the primer region present in the PCR product, the intensity of the two bands obtained from the PCR product was greater than the 150 bp size marker control band [lane 7]. No band was present in the negative control lanes [fig 44B, lanes 3 and 5].

III.C.3.b. Digestion of PCR Product and ptacHpaII-R2

The CMV1 and CMV2 primers had, respectively, HindIII and BamHI restriction enzyme sites at their 5' ends, to help ligation to ptacHpaII-R2. Therefore, the remaining 29 µl of the PCR product was double digested with HindIII/BamHI, extracted once with phenol chloroform and the organic phase was back extracted with TE pH8.0. The DNA was precipitated, the pellet dissolved in 4 µl of DW, and 0.5 µl was run on an agarose gel to estimate the amount of DNA present.

FIG 44: Electrophoresis of products of PCR amplification of plasmid p73HCMV with primers CMV1 and CMV2, on a 2% agarose gel (A), and Southern blot hybridization with a radioactively labelled HCMV probe (B).

(A)

LANES:

1) 1 µg of HindIII digested lambda marker DNA (fragment size shown in kb)

2&4) 0.5 µl of PCR product obtained from reaction containing the p73HCMV plasmid DNA

3&5) 0.5 µl of PCR product obtained from reaction containing no plasmid DNA (negative control)

7) BamHI/EcoRI digested p73HCMV DNA as a positive control size marker

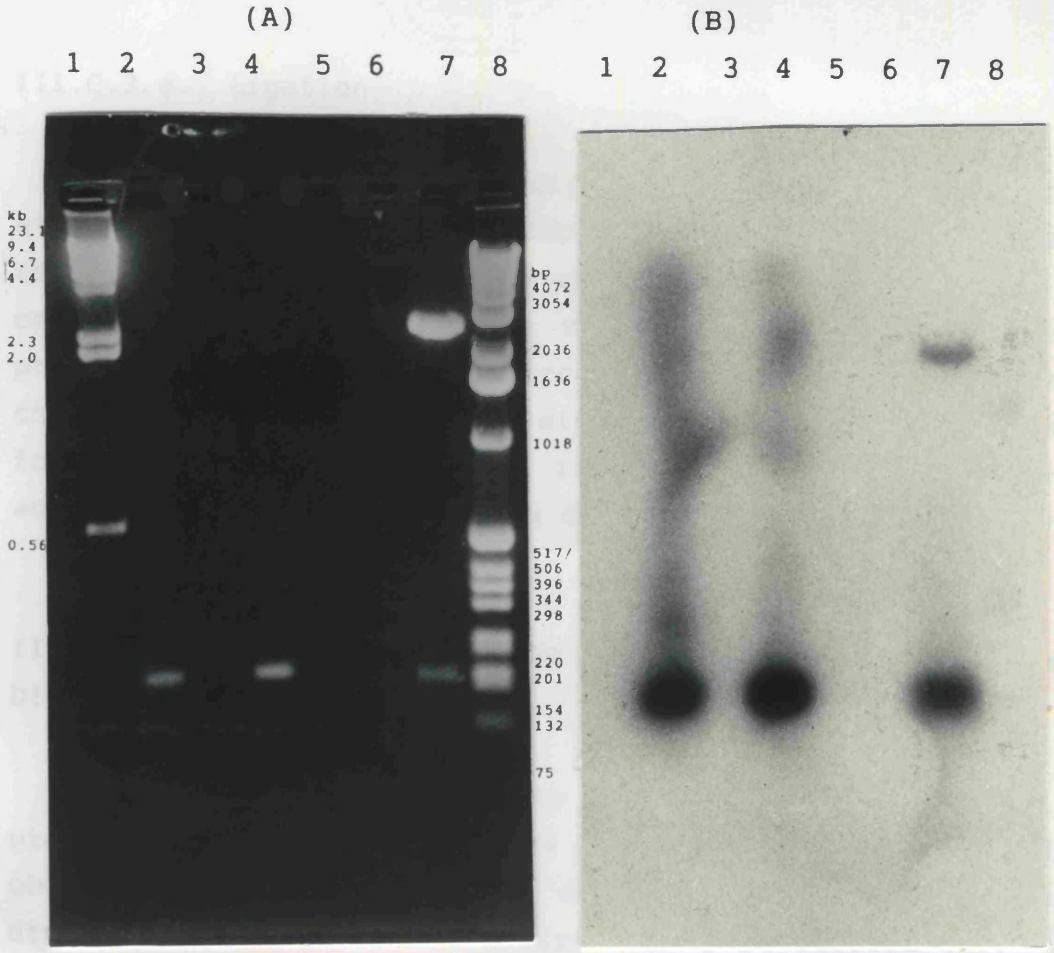
8) 1 µg of 1 kb ladder marker DNA (fragment size shown in bp)

(B)

Southern blot hybridization of PCR products in (A), with ^{32}P labelled HCMV probe.

Lanes 2 & 4) hybridized PCR products of 150 bp

Lane 7) positive control size marker of p73HCMV.


(A)
1 2 3 4 5 6 7 8
kb
23.1
9.4
6.7
4.4
2.3
2.0
0.56
bp
4072
3054
2036
1636
1018
517/
506
396
344
298
220
201
154
132
75
(B)
1 2 3 4 5 6 7 8

Ten µg of ptacHpaII-R2 was double digested with HindIII/BamHI and run on a 1% agarose gel. The BamHI site was located 346 bp downstream of the HindIII site and digestion produced two fragments of 4806 and 346 bp [fig 45]. The 4806 bp fragment was excised from the gel, electrophoresed into a dialysis bag and purified on a DEAE sephacel column. The DNA was precipitated, dissolved in 10 µl of DW and 0.5 µl was run on an agarose gel to estimate the amount of DNA recovered.

III.C.3.c. Ligation

200 ng of HindIII/BamHI digested PCR product and 100 ng of HindIII/BamHI digested ptacHpaII-R2 were ligated, and used to transform competent E.coli strain [TG2] as described. A control ligation was set up using HindIII/BamHI digested ptacHpaII-R2 alone. On two occasions, 20-30 well separated colonies were obtained and a single colony was also present for the control ptacHpaII-R2 ligation. The colonies were analyzed by restriction enzyme digestion as described below.

III.C.3.d. Analysis of ptacCMV Clones by Restriction Enzyme Digestion of Mini-prep DNA

Plasmid DNA was purified by the mini-prep method from 11 ptacCMV colonies and one control ptacHpaII-R2 colony. The DNA obtained from 6 recombinant and the control colony was digested with HindIII and analyzed on a 1% agarose gel. A fragment of 4955 bp, the predicted size of the recombinant plasmid, was present for all the candidate ptacCMV clones tested. In addition, one fragment of similar size was also present in the lane containing the control ptacHpaII-R2 digested DNA (results not shown).

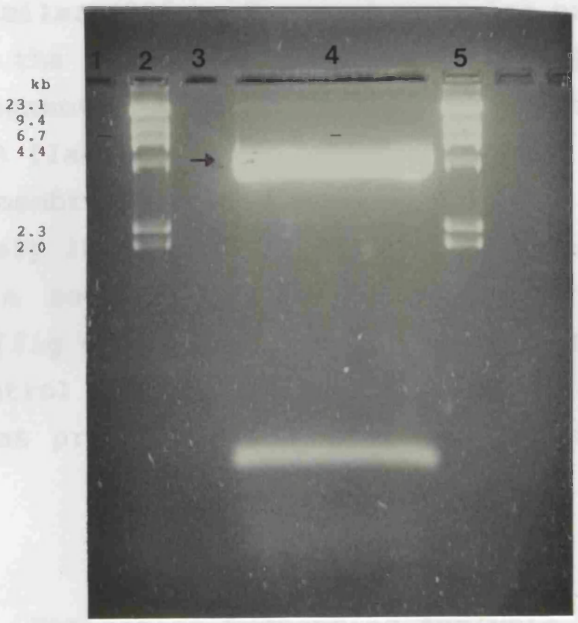


FIG 45: Separation of fragments obtained by HindIII/BamHI digestion of plasmid ptacHpaII-R2, by electrophoresis on a 1% agarose gel.

The fragment marked with an arrow was cut out from the gel and purified for ligation (Lanes 2 and 5 contain 1 µg of HindIII digested lambda marker DNA; fragment size shown in kb).

Following this, DNA from two ptacCMV clones and the control ptacHpaII-R2 clone was digested with BamHI/HindIII and run on a 1% agarose gel. Plasmid p73HCMV digested with BamHI/EcoRI was also run on the same gel as a positive control size marker. A fragment of 4806 bp, and a very faint band of 150 bp (which was only visible on 1 second exposure), were present in the lanes containing ptacCMV clones [fig 46A, lanes 2-3]. A similar 4806 bp fragment was also present in the lane containing the control ptacHpaII-R2 clone [fig 46A, lane 4] and two fragments of 3039 bp and 150 bp in the lane containing p73HCMV DNA [lane 5]. The DNA was Southern transferred onto a nylon membrane and hybridized with $1x10^7$ cpm of a radioactively labelled HCMV probe. The presence of a 150 bp fragment in both the lanes containing ptacCMV clones was confirmed [fig 46B, lanes 2 and 3] which was of the same size as the control HCMV insert size marker [lane 5]. No such fragment was present in ptacHpaII-R2 control lane [fig 46B, lane 4].

III.C.3.e. Nucleotide Sequencing Analysis

Nucleotide sequencing was done to ensure that no insertion or deletion of nucleotides had occurred during construction of ptacCMV, thus disrupting the reading frame. Therefore a part of the plasmid ptacCMV (EcoRI/BamHI fragment) containing the junction between HBV core gene and HCMV insert was sequenced by the chain termination method.

III.C.3.e.(1). Digestion of ptacCMV and M13mp18 DNA

Five µg of ptacCMV DNA was double digested with EcoRI/BamHI and run on a 1% LMP agarose gel. Two fragments of 4368 bp and 586 bp were visualized on the gel and the latter fragment was excised. The DNA was extracted, precipitated, and dissolved in 10 µl TE pH8.0 and 0.5 µl was run on an

FIG 46: Electrophoresis of HindIII/BamHI digested ptacCMV DNA, on a 1% agarose gel (A), and Southern blot hybridization with a radioactively labelled HCMV probe (B).

(A)

LANES:

1) 1 µg of HindIII digested lambda marker DNA (fragment size shown in kb)
2) HindIII/BamHI digested ptacCMV DNA from clone 5
3) HindIII/BamHI digested ptacCMV DNA from clone 6
4) HindIII/BamHI digested DNA obtained from ptacHpaII-R2 (negative control)
5) EcoRI/BamHI digested p73HCMV DNA (positive control)
6) 1 µg of 1 kb ladder marker DNA (fragment size shown in bp)

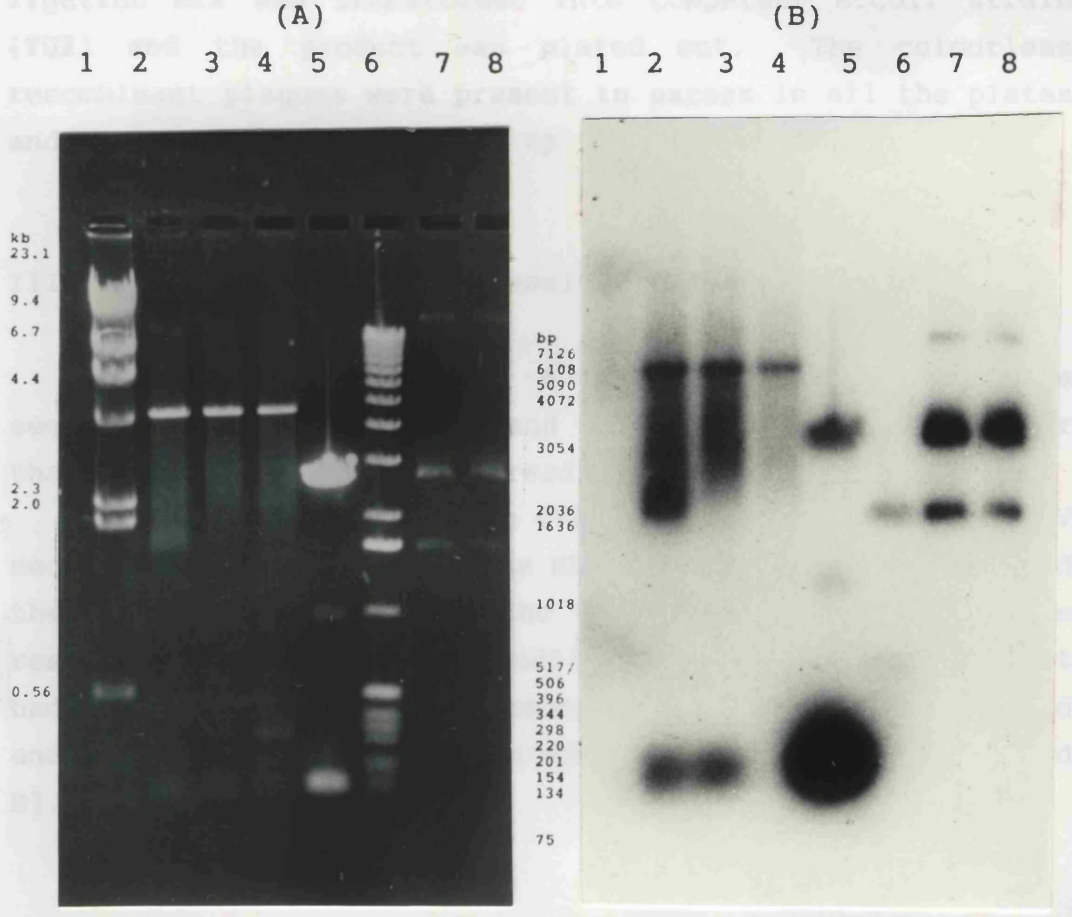
7&8) undigested ptacCMV DNA from clones 5 and 6.

(B)

Southern hybridization of (A) with ^{32}P labelled HCMV probe.

Lanes 2 & 3) 149 bp diagnostic fragments

Lane 5) positive control size marker of p73HCMV.


(A)
1 2 3 4 5 6 7 8
kb
23.1
9.4
6.7
4.4
2.3
2.0
0.56
(B)
1 2 3 4 5 6 7 8
bp
7126
6108
5090
4072
3054
2036
1636
1018
517/
506
396
344
298
220
201
154
134
75

agarose gel to estimate the amount of DNA present. Three µg of M13mp18 DNA was double digested with EcoRI/BamHI and 100 ng was run on an agarose gel to ensure complete digestion. The DNA was extracted, precipitated and dissolved in 10 µl TE pH8.0 and 0.5 µl was run on an agarose gel.

The ligation reaction was set up between 200 ng of ptacCMV fragment and 100 ng of digested M13mp18 DNA. The ligation mix was transformed into competent E.coli strain (TG2) and the product was plated out. The colourless recombinant plaques were present in excess in all the plates and 12 were picked and grown up to make DNA templates.

III.C.3.e.(2). Sequencing Results

Samples prepared from 6 templates were run on a sequencing gel in duplicate and the first set was run longer than the second in order to read more sequence.

The sequencing data [fig 47A and B] proved that the HCMV sequence was in-frame with the HBV core gene, as this matched the predicted sequence of the gene fusion [fig 35]. The restriction enzyme sites {HindIII (5') and BamHI (3')} that had been utilized for this construction had been regenerated and the presence of stop codon was confirmed also [fig 47A and B].

III.C.4. Purification of ptacCMV

Cultures of E.coli strain (RB791; lac I^q) harbouring plasmids ptacHpaII-R2 (as a positive control) and ptacCMV were grown in LB-amp and the tac promoter was induced with IPTG. After induction, the bacteria were lysed by sonication and the crude extract was precipitated by ammonium sulphate at 30% saturation. The expressed proteins, truncated $HBcAg_{3-144}$ (from plasmid ptacHpaII-R2) and HBc_{3-144}-HCMV (from plasmid ptacCMV) were visualized by Coomassie blue staining after SDS-PAGE.

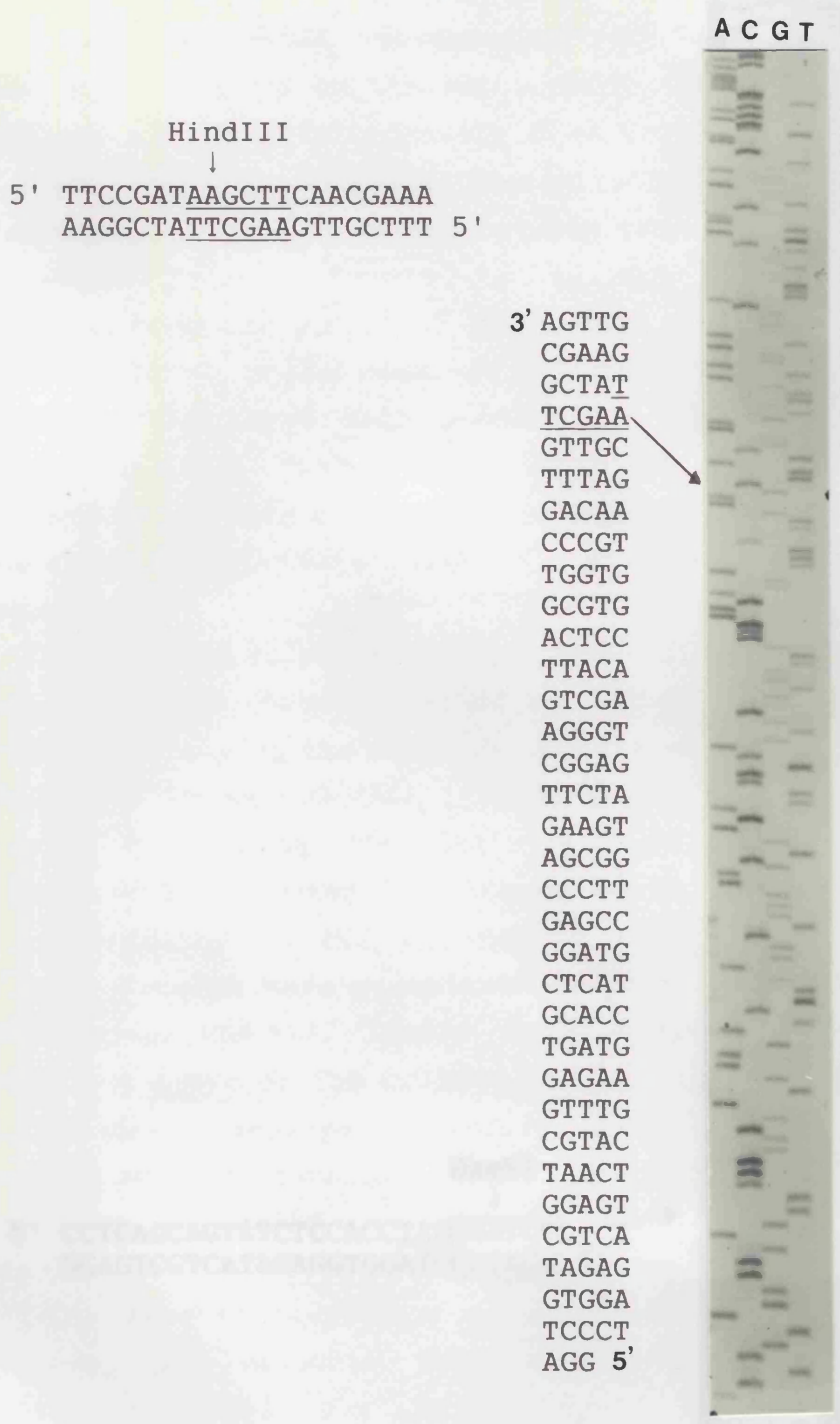


FIG 47: (A) Nucleotide sequencing of ptacCMV, as determined using the dideoxy chain termination method.

The sequence around junction region of HBcAg-HCMV gp58 encoding gene fusion, has been shown along with regenerated HindIII site (underlined). Compare with predicted nucleotide sequence in fig 35A.

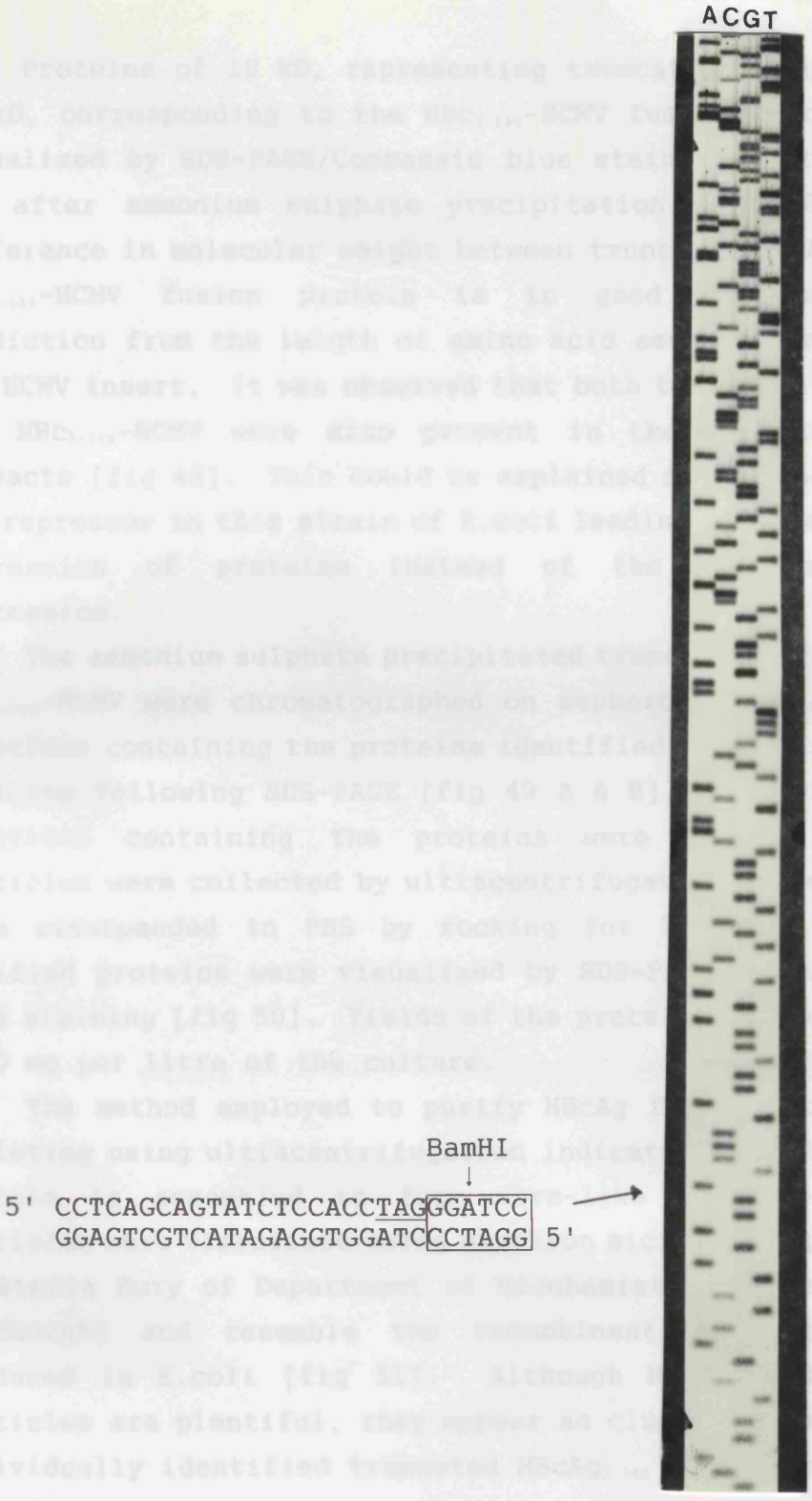


FIG 47: (B) Nucleotide sequence of ptacCMV (continued).

The Core termination codon has been underlined and the BamHI site is shown as a boxed area. Compare with predicted nucleotide sequence in fig 35A.

Proteins of 18 kD, representing truncated $HBcAg_{3-144}$, and 22 kD, corresponding to the HBc_{3-144}-HCMV fusion protein were visualized by SDS-PAGE/Coomassie blue staining, both before and after ammonium sulphate precipitation [fig 48]. The difference in molecular weight between truncated $HBcAg_{3-144}$ and HBc_{3-144}-HCMV fusion protein is in good agreement with prediction from the length of amino acid sequence encoded by the HCMV insert. It was observed that both truncated $HBcAg_{3-144}$ and HBc_{3-144}-HCMV were also present in the uninduced cell extracts [fig 48]. This could be explained due to the lack of lac repressor in this strain of E.coli leading to constitutive expression of proteins instead of the IPTG regulated expression.

The ammonium sulphate precipitated truncated $HBcAg_{3-144}$ and HBc_{3-144}-HCMV were chromatographed on sepharose 4B-CL and the fractions containing the proteins identified by Coomassie blue staining following SDS-PAGE [fig 49 A & B]. The identified fractions containing the proteins were pooled and the particles were collected by ultracentrifugation. The pellets were resuspended in PBS by rocking for 2-3 days and the purified proteins were visualized by SDS-PAGE and Coomassie blue staining [fig 50]. Yields of the proteins ranged between 2-10 mg per litre of the culture.

The method employed to purify HBcAg fusion proteins by pelleting using ultracentrifugation indicates that the fusion protein is assembled to form core-like particles. The particles were visualized using electron microscopy (performed by Stella Bury of Department of Biochemistry, University of Edinburgh) and resemble the recombinant HBcAg particles produced in E.coli [fig 51]. Although HBc_{3-144}-HCMV fusion particles are plentiful, they appear as clumps as compared to individually identified truncated $HBcAg_{3-144}$ control particles [fig 51].

FIG 48: SDS-PAGE and Coomassie blue staining of proteins expressed after IPTG-induction of cultures harbouring plasmids ptacHpaII-R2 (truncated $HBcAg_{3-144}$) and ptacCMV (HBc_{3-144}-HCMV).

Protein samples from soluble fractions of crude extracts after 30% ammonium sulphate precipitation both from uninduced and IPTG-induced cultures harbouring plasmids ptacHpaII-R2 (positive control) and ptacCMV, were fractionated on 12.5% acrylamide gel and stained with Coomassie brilliant blue.

LANES:

1) protein molecular weight markers in kD
2) truncated $HBcAg_{3-144}$; pre-IPTG, before $(NH_4)_2SO_4$ precipitation
3) truncated $HBcAg_{3-144}$; post-IPTG, before $(NH_4)_2SO_4$ precipitation
4) truncated $HBcAg_{3-144}$; pre-IPTG, after $(NH_4)_2SO_4$ precipitation
5) truncated $HBcAg_{3-144}$; post-IPTG, after $(NH_4)_2SO_4$ precipitation
6) empty
7) HBc_{3-144}-HCMV; pre-IPTG, before $(NH_4)_2SO_4$ precipitation
8) HBc_{3-144}-HCMV; post-IPTG, before $(NH_4)_2SO_4$ precipitation
9) HBc_{3-144}-HCMV; pre-IPTG, after $(NH_4)_2SO_4$ precipitation
10) HBc_{3-144}-HCMV; post-IPTG, after $(NH_4)_2SO_4$ precipitation

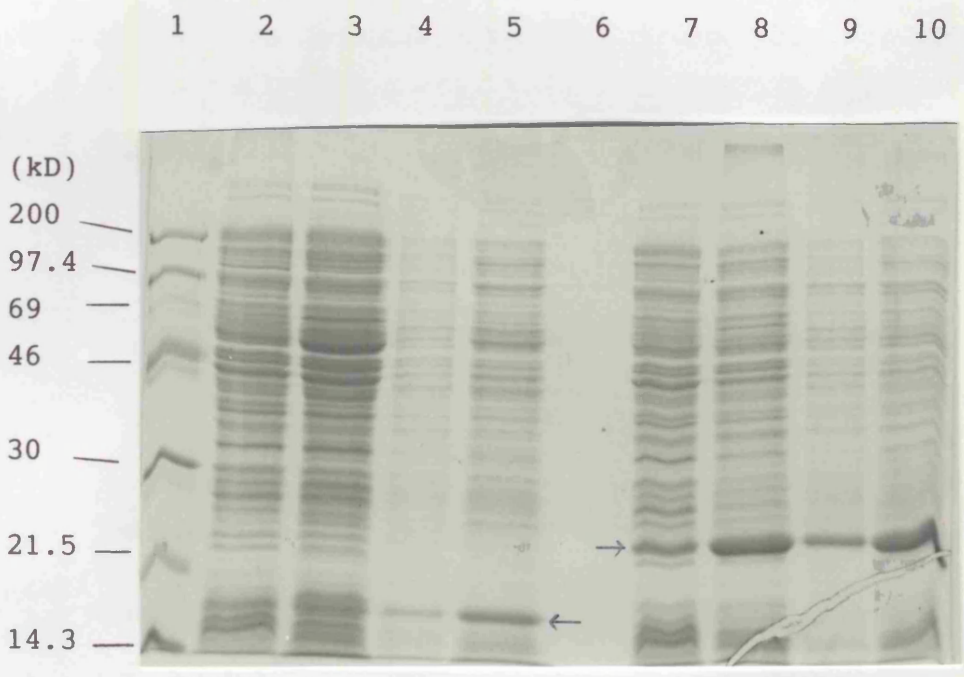

1 2 3 4 5 6 7 8 9 10
(kD)
200
97.4
69
46
30
21.5
14.3

FIG 49: Identification of fractions containing truncated $HBcAg_{3-144}$ and HBc_{3-144}-HCMV obtained by chromatography on sepharose 4B-CL, by SDS-PAGE and Coomassie blue staining.

(A) truncated $HBcAg_{3-144}$ fractions

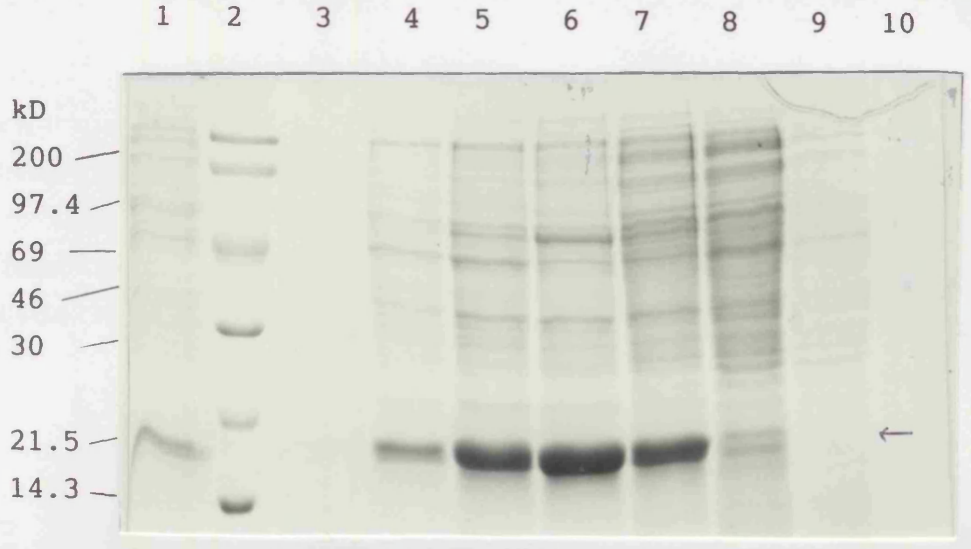
LANES:

1) Reference protein preparation of truncated $HBcAg_{3-144}$
2) Protein molecular weight markers
3) Fraction No: 1
4) Fraction No: 2
5) Fraction No: 3
6) Fraction No: 5
7) Fraction No: 7
8) Fraction No: 9
9) Fraction No: 11
10) Fraction No: 13

(B) HBc_{3-144}-HCMV fractions

LANES:

1) Protein molecular weight markers
2) Reference protein preparation of HBc_{3-144}-HCMV
3) Fraction No: 1
4) Fraction No: 3
5) Fraction No: 5
6) Fraction No: 7
7) Fraction No: 9
8) Fraction No: 11
9) Fraction No: 13
10) Fraction No: 15

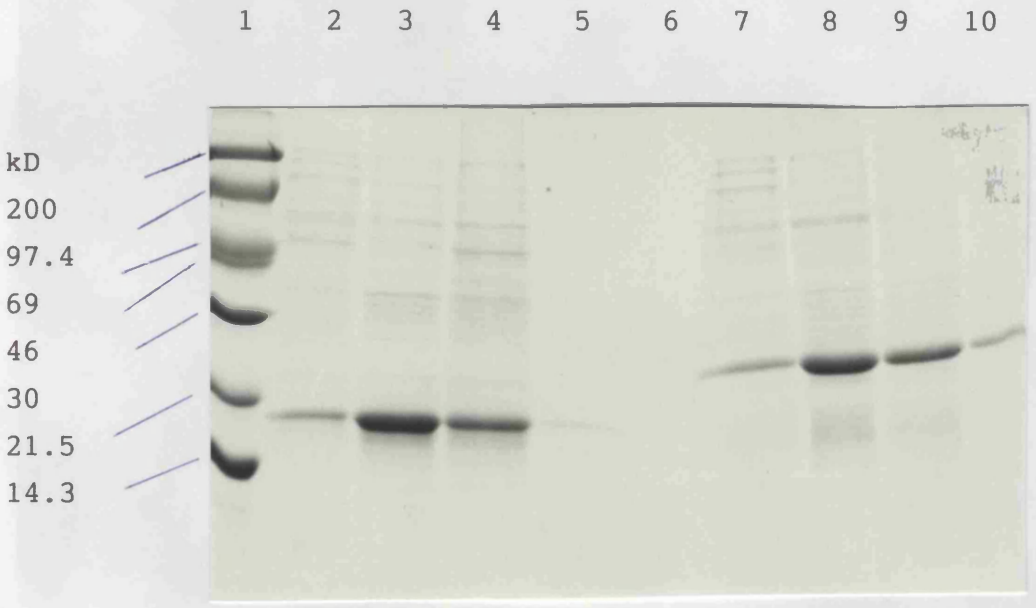

1 2 3 4 5 6 7 8 9 10
kD
200
97.4
69
46
30
21.5
14.3


1 2 3 4 5 6 7 8 9 10
kD
200
97.4
69
46
30
21.5
14.3

FIG 50: SDS-PAGE and Coomassie blue staining of purified truncated $HBcAg_{3-144}$ and HBc_{3-144}-HCMV fusion protein obtained after ultracentrifugation.

LANES:

1) Protein molecular weight markers
2) Reference protein preparation of truncated $HBcAg_{3-144}$
3) First batch of purified truncated $HBcAg_{3-144}$ recovered after 48 hours of shaking of the protein pellet (obtained after ultracentrifugation) in PBS
4) Second batch of truncated $HBcAg_{3-144}$ recovered after further shaking of the remaining protein pellet in a fresh batch of PBS for 48 hours
5) Third batch of truncated $HBcAg_{3-144}$ recovered from the same pellet
6) empty
7) Reference protein preparation of HBc_{3-144}-HCMV
8) First batch of purified HBc_{3-144}-HCMV fusion protein recovered from the pellet after 48 hours in PBS
9) Second batch of HBc_{3-144}-HCMV recovered after further shaking of the pellet for 48 hours in fresh PBS
10) Third batch of HBc_{3-144}-HCMV recovered from the remaining pellet


1 2 3 4 5 6 7 8 9 10
kD
200
97.4
69
46
30
21.5
14.3

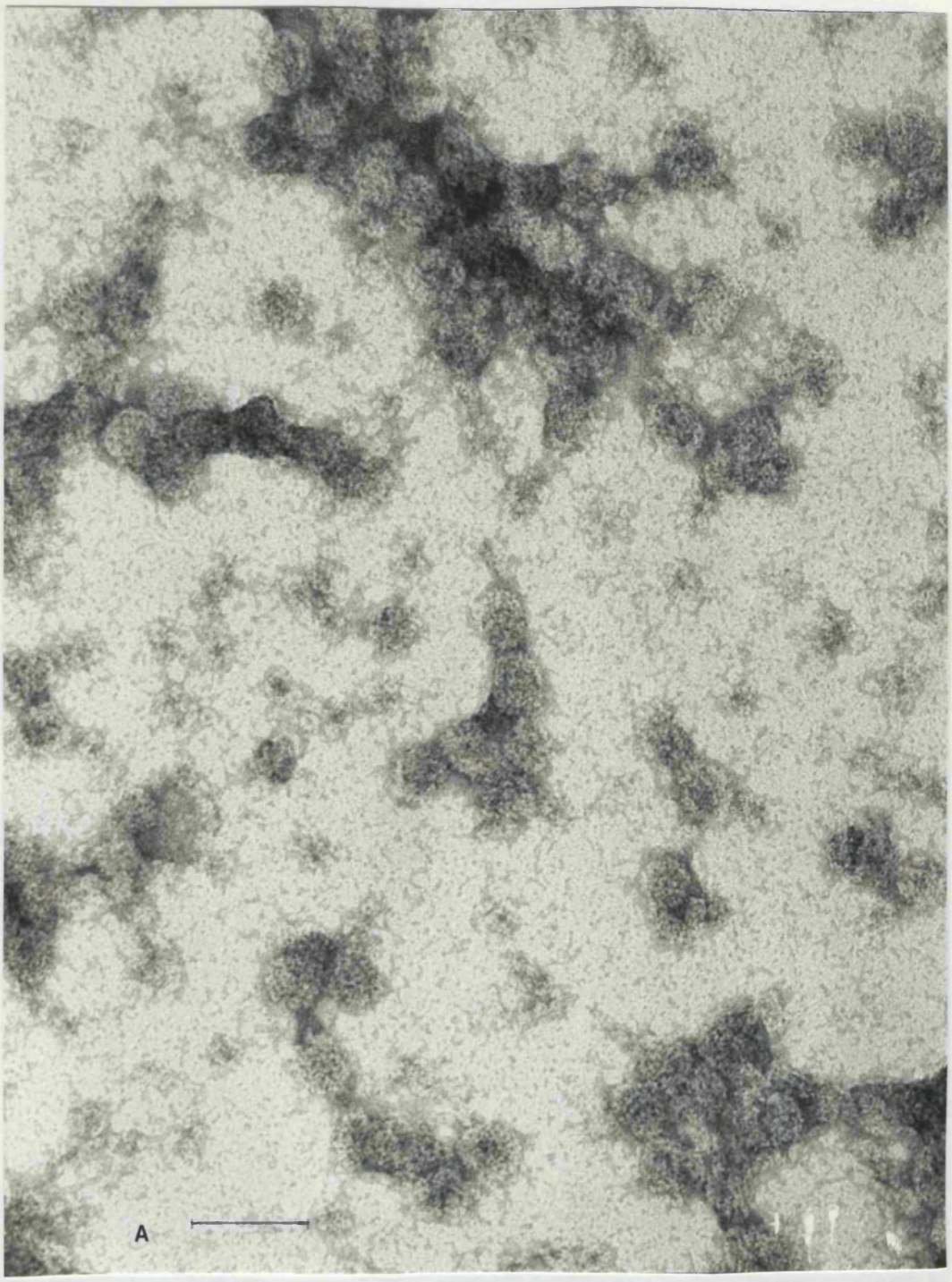
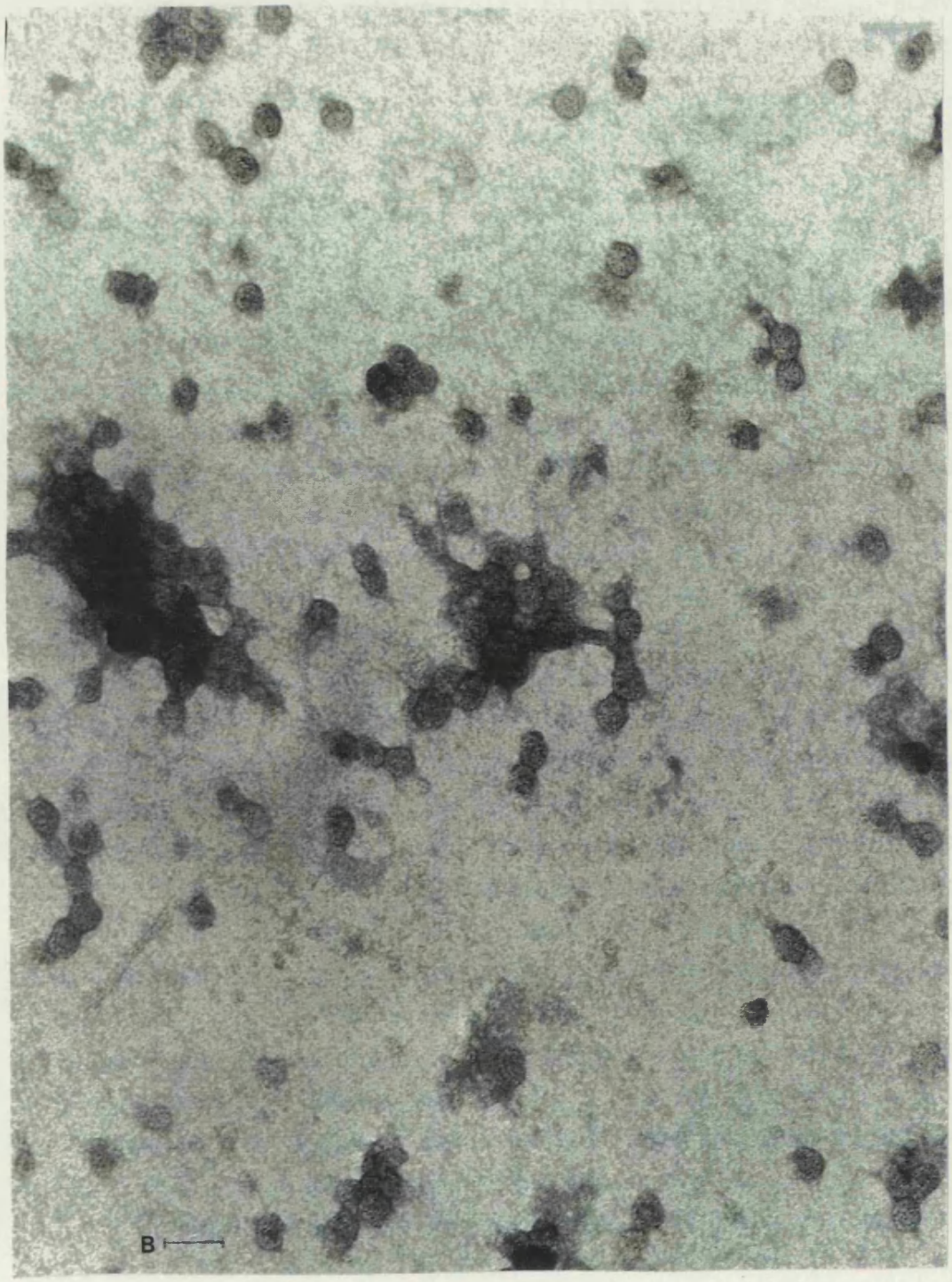


FIG 51: Electron micrographs showing core-like particles in IPTG-induced cultures of cells harbouring plasmids (A) ptacCMV (HBc_{3-144}-HCMV fusion) and (B) positive control ptacHpaII-R2 (truncated $HBcAg_{3-144}$) [see next page]. Size bar = 50 nm


B

III.C.5. Immunological Characteristics of HBc-HCMV Carboxyl Terminal Fusion Protein (HBc_{3-144}-HCMV)

The HBc and HCMV antigenicity of the purified HBc_{3-144}-HCMV fusion protein was demonstrated using dot blot, western blot and radioimmunoassays.

III.C.5.a. HBc Antigenicity

The results of several dot blot assays are shown in figures 52, 53 and 54. In the first set of experiments, dot blots were performed using a polyclonal rabbit anti-HBc antibody. Bacterial cultures harbouring plasmids ptacHpaII-R2 and ptacCMV (several transformed bacterial clones were tested) were induced with IPTG for 5-6 hours. Results from several experiments indicated that an antibody dilution of 1:200 produced a maximal colour reaction using a horseradish peroxidase detection system. However, it was not possible to draw any conclusions from these assays, as the negative control (IPTG-induced TG2 cells) consistently reacted with anti-HBc polyclonal antibody (results not shown). As the source of the anti-HBc polyclonal antibody were rabbits immunized with HBcAg prepared in E.coli, this may account for non-specific results. To alleviate this problem, another experiment was set up in which the IPTG-induced culture was preincubated with an equal volume of anti-HBc polyclonal antibody for 12-18 hours at 4°C and then centrifuged for 30 minutes. The supernatant was collected and then used in the dot blot assay as described above. The results were still inconclusive, as a faint reaction was observed with negative control TG2 cells (results not shown).

Following this, another assay was performed using anti-HBc monoclonal antibody (18CE11B12) instead of the polyclonal antibody. Only two ptacCMV clones (4 & 6) out of six tested, produced enough HBc_{3-144}-HCMV fusion protein that could be

detected with the horseradish peroxidase system [fig 52]. This suggests that the level of expression of the fusion protein differs between the clones and that several clones should be tested to determine optimum expression. No reaction was observed in any of these assays for TG2 cells [fig 52A, well 11; fig 52B, wells 4-5] or any of the IPTG-induced pv404CMV clones [fig 52A, wells 7-9; fig 52B, wells 7-8]. HBc reactivity of HBc_{3-144}-HCMV fusion protein was further analyzed using a panel of four monoclonal antibodies (35H2, 18CH5G1, A2 and RFc17) and a separate assay was performed for each antibody. HBc_{3-144}-HCMV fusion protein reacted with all monoclonal antibodies [fig 53 A-D, well 5] except monoclonal A2 [fig 53E, well 5]. These results indicate that fusion of a heterologous sequence to the truncated carboxyl terminus of HBcAg has preserved the antibody binding sites of HBcAg recognized by a panel of anti-HBc monoclonal antibodies.

As induction of the tac promoter with IPTG would be an important factor in determining optimum expression of the fusion protein, IPTG-induction time was varied (1-2 hours & 12-18 hours) [fig 54]. A faint reaction obtained for cultures that had been induced with 1-2 and 12-18 hours compared to the cultures that had been induced for 5-6 hours suggests that IPTG-induction for 5-6 hours allows optimum expression of the fusion protein. The results are only shown for 12-18 hours IPTG-induced cultures [fig 54 A-E, wells 1-6]. For comparison, 5-6 hour IPTG-induced cultures were analyzed simultaneously [fig 54 A-E, wells 9-12].

These results indicate that the epitopes recognized by at least four anti-HBc monoclonal antibodies (I8CE11B12, 35H2, 18CH5G1, and RFc17) are accessible and perhaps exposed on the surface of hybrid core particles.

The IPTG-induced cultures were treated with pronase and 2-MCE, to convert HBcAg reactivity into HBeAg reactivity. In the dot blot assay that had been performed with anti-HBe monoclonal A2C12, no reactivity was observed suggesting that HBe antigenicity was not displayed by truncated $HBcAg_{3-144}$ or HBc_{3-144}-HCMV fusion protein.

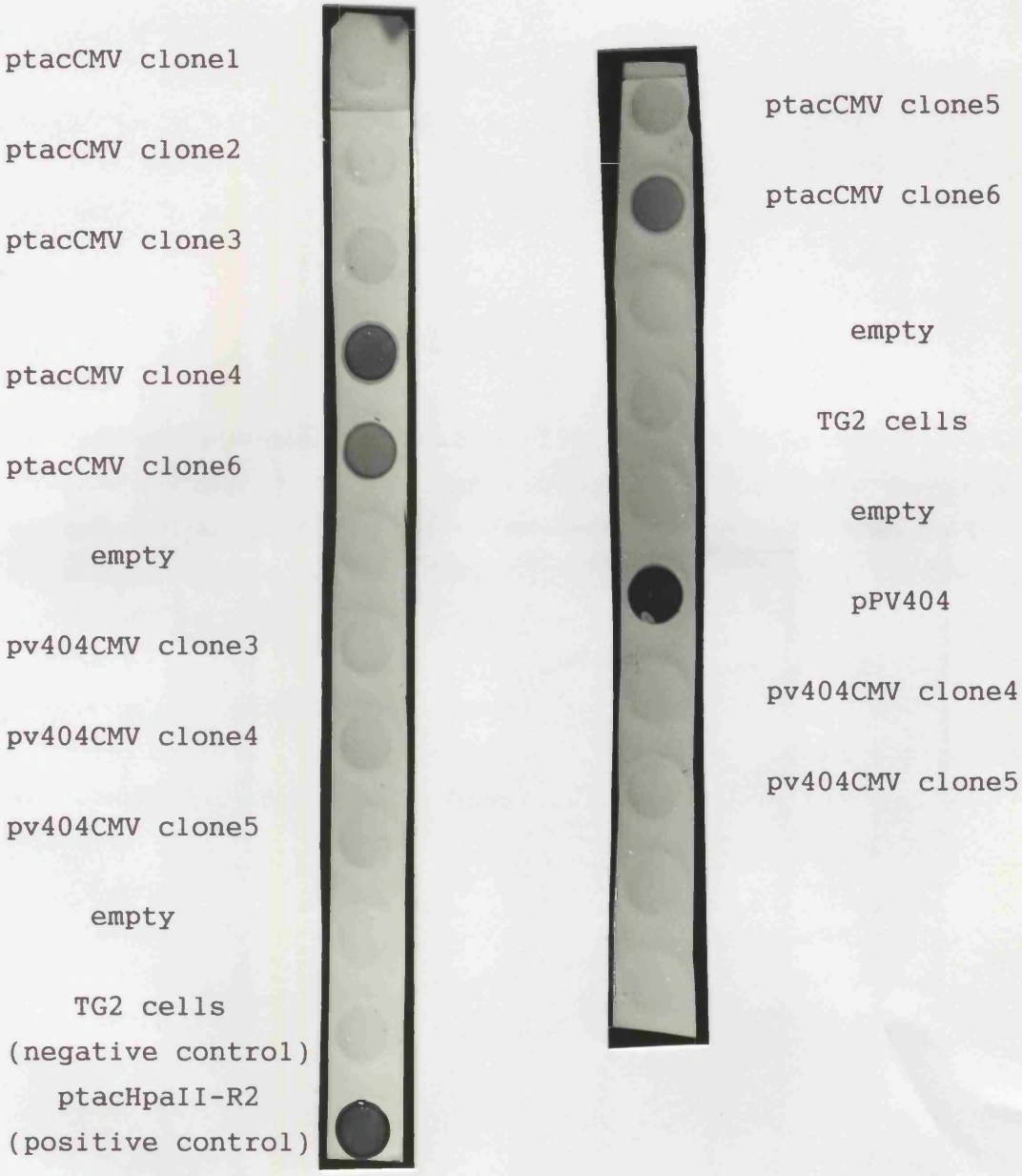


FIG 52: Dot blot assay performed on IPTG-induced bacterial cultures harbouring plasmids ptacCMV (HBc_{3-144}-HCMV fusion protein) and pv404CMV (HCMV-HBc_{1-183} fusion protein), using anti-HBc monoclonal antibody 18CE11B12.

FIG 53: Dot blot assays performed on IPTG-induced bacterial cultures (5-6 hour induction) harbouring plasmids ptacCMV (HBc_{3-144}-HCMV fusion protein) and pv404CMV (HCMV-HBc_{1-183} fusion protein), using a panel of anti-HBc monoclonal antibodies.

WELLS:

1) truncated $HBcAg_{3-144}$ (positive control)
2) PV-HBc_{1-183} (positive control)
3) TG2 cells, IPTG-induced for 5-6 hours (negative control)
4) HBc_{3-144}-HCMV
5) HBc_{3-144}-HCMV
7, 8, 10, 11 & 12) HCMV-HBc_{1-183}

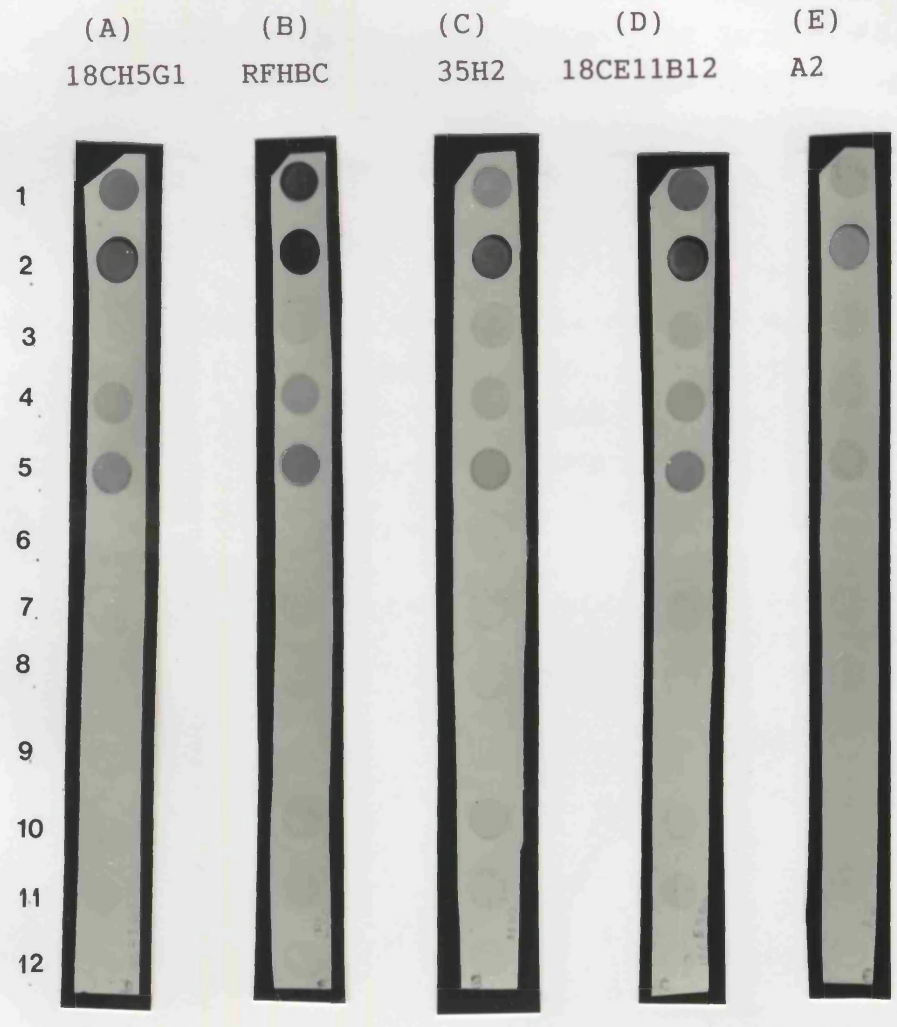
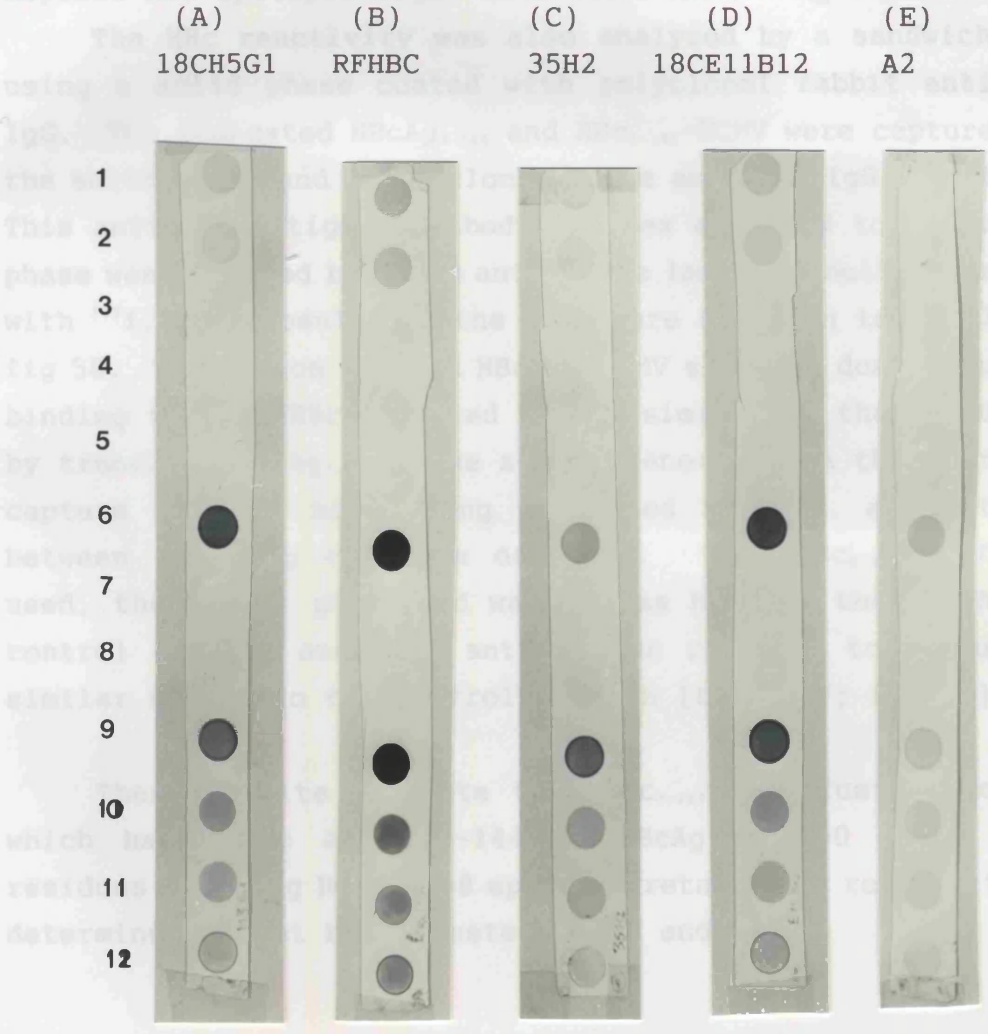

(A)
18CH5G1
(B)
RFHBC
(C)
35H2
(D)
18CE11B12
(E)
A2
1
2
3
4
5
6
7
8
9
10
11
12

FIG 54: Dot blot assays performed on IPTG-induced cultures (12-18 hours) of ptacCMV (HBc_{3-144}-HCMV fusion protein) and pv404CMV (HCMV-HBc_{1-183} fusion protein), using a panel of anti-HBc monoclonal antibodies.

WELLS:
{wells 1-6 have 12-18 hours IPTG-induced cultures}
1,2&3) HBc_{3-144}-HCMV fusion
4&5) HCMV-HBc_{1-183}
6) truncated $HBcAg_{3-144}$
7) empty

{Wells 8-12 contain 5-6 hours IPTG-induced cultures}
8) TG2 cells
9) PV-HBc_{1-183}
10,11&12) HBc_{3-144}-HCMV


(A)
18CH5G1
(B)
RFHBC
(C)
35H2
(D)
18CE11B12
(E)
A2
1
2
3
4
5
6
7
8
9
10
11
12

The HBc reactivity of HBc_{3-144}-HCMV fusion protein was further analyzed by Western blotting [figs 55 & 56]. The polyclonal (rabbit) anti-HBc serum recognized the truncated $HBcAg_{3-144}$ and HBc_{3-144}-HCMV fusion protein in monomeric, dimeric and high molecular weight forms [fig 55]. Only one anti-HBc monoclonal antibody, RFc17 [fig 56], detected HBc reactivity in a western blot, whereas monoclonal antibodies 18CE11B12, 35H2 and H5G1 failed to react with the truncated $HBcAg_{3-144}$ or HBc_{3-144}-HCMV. As the latter three antibodies did detect HBc reactivity in a dot blot assay previously [figs 52, 53 & 54], it is suggested that a conformational change of several exposed HBc epitopes might have resulted during SDS-PAGE.

The HBc reactivity was also analyzed by a sandwich RIA using a solid phase coated with polyclonal rabbit anti-HBc IgG. The truncated $HBcAg_{3-144}$ and HBc_{3-144}-HCMV were captured by the solid phase and a polyclonal mouse anti-HBc IgG was added. This antibody-antigen-antibody complex attached to the solid phase was detected by sheep anti-mouse immunoglobulin labelled with ^{125}I. The results of the assay are shown in table 57 and fig 58. The fusion protein HBc_{3-144}-HCMV showed a dose-response binding to anti-HBc labelled beads, similar to that produced by truncated $HBcAg_{3-144}$. The signal generated in this antigen capture RIA was high using truncated $HBcAg_{3-144}$ and antigen between 1-100 ng could be detected. When HBc_{3-144}-HCMV was used, the signal generated was not as high as that with the control antigen and more antigen was required to produce a similar signal to the control antigen [table 57; fig 58].

These results indicate that HBc_{3-144}-HCMV fusion protein which has amino acids 3-144 of HBcAg and 50 amino acid residues encoding HCMV-gp58 epitope, retains HBc reactivity as determined by dot blot, Western blot and RIA.

FIG 55: Western blot analysis of HBc_{3-144}-HCMV fusion protein using polyclonal anti-HBc serum.

Purified protein samples were electrophoresed through a 12.5% SDS-PAGE and transferred to nitrocellulose membrane. HBc reactivity was detected by using polyclonal rabbit anti-HBc serum with the alkaline phosphatase conjugate detection system.

LANES:

1) Protein molecular weight markers
2) Empty
3) HBc_{3-144}-HCMV (2 µg)
4) HBc_{3-144}-HCMV (1 µg)
5) truncated $HBcAg_{3-144}$ (2 µg)
6) truncated $HBcAg_{3-144}$ (1 µg)
7) HBc_{3-144}-HCMV (1.5 µg)
8) HBc_{3-144}-HCMV (0.5 µg)
9) truncated HBc_{3-144} (1.5 µg)
10) truncated HBc_{3-144} (1 µg)

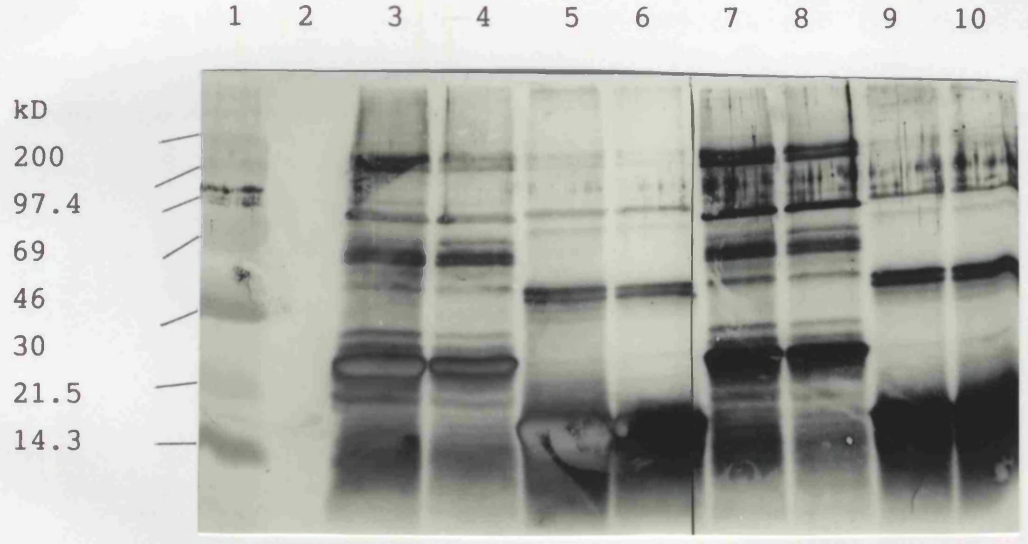
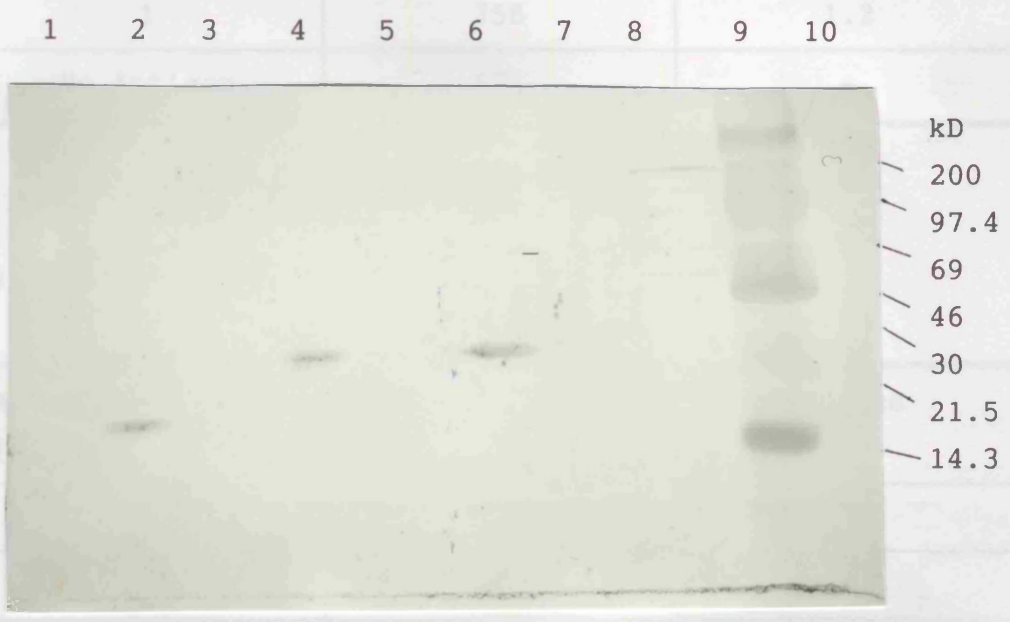

1 2 3 4 5 6 7 8 9 10
kD
200
97.4
69
46
30
21.5
14.3

FIG 56: Western blot analysis of HBc_{3-144}-HCMV using anti-HBc monoclonal antibody RFc17.

Purified protein samples were electrophoresed through a 12.5% SDS-PAGE and transferred to nitrocellulose membrane. HBc reactivity was detected by using polyclonal rabbit anti-HBc serum with the alkaline phosphatase conjugate detection system.

LANES:

1) truncated $HBcAg_{3-144}$ (0.1 µg)
2) truncated $HBcAg_{3-144}$ (1 µg)
3) HBc_{3-144}-HCMV (0.1 µg)
4) HBc_{3-144}-HCMV (1 µg)
5) PV-HBc_{1-183} (0.1 µg)
6) PV-HBc_{1-183} (1 µg)
7) HCMV-HBc_{1-183} (0.1 µg)
8) HCMV-HBc_{1-183} (1 µg)
9) Protein molecular weight markers
10) Empty


1
2
3
4
5
6
7
8
9
10
kD
200
97.4
69
46
30
21.5
14.3

(A) HBc_{3-144}-HCMV

Antigen (ng)	Counts per minute (cpm)	Signal/Noise Ratio (S/N)
100	3250	5.2
50	2196	3.5
30	1961	3.1
10	1236	1.9
3	897	1.4
1	758	1.2
No Antigen	621	-

(B) truncated $HBcAg_{3-144}$

Antigen (ng)	Counts per minute (cpm)	Signal/Noise Ratio (S/N)
100	7738	12.4
30	5879	9.4
10	6063	9.7
3	4262	6.8
1	1096	1.7
No Antigen	621	-

Table 57: Results of the Sandwich RIA used to determine HBc Antigenicity of the fusion protein HBc_{3-144}-HCMV and the control truncated $HBcAg_{3-144}$.

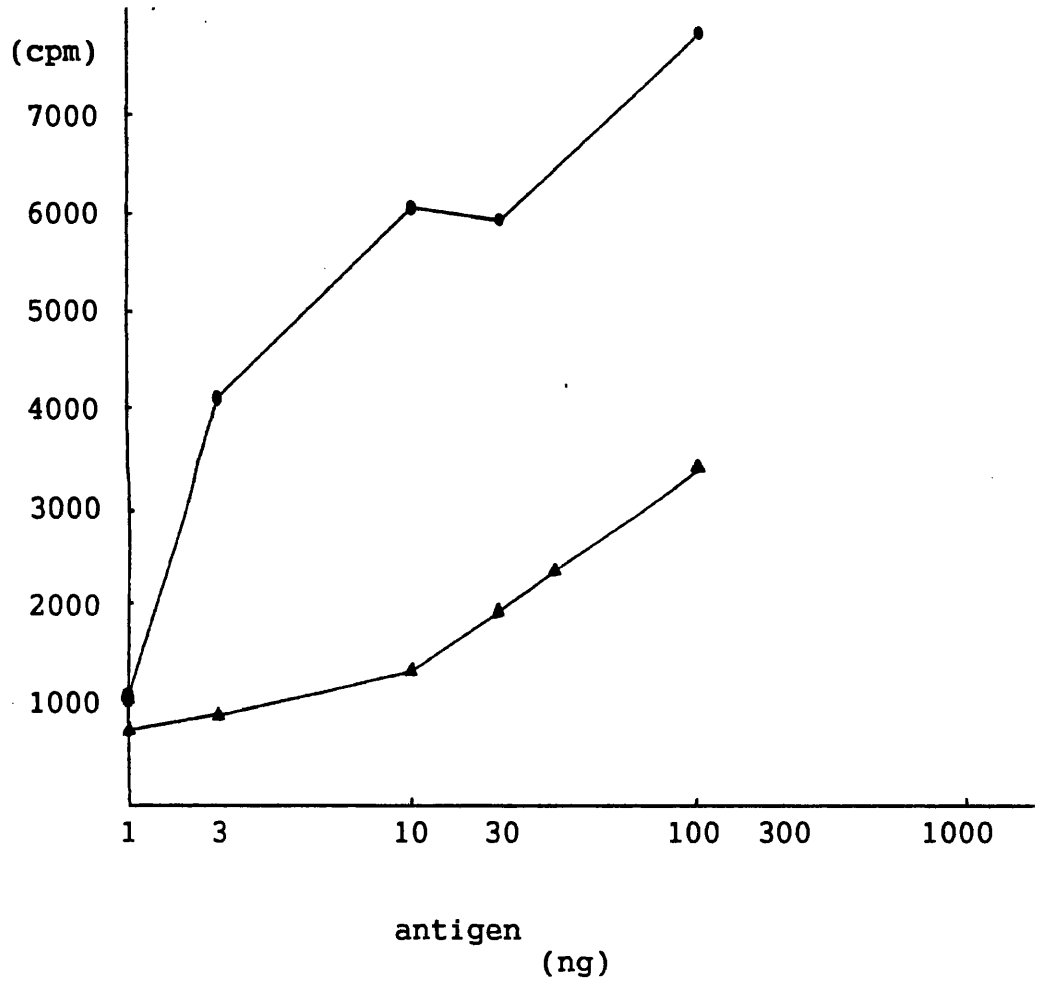


FIG 58: Graphic representation of the results (as shown in table 57) of the Sandwich RIA used to determine HBc reactivity of HBc_{3-144}-HCMV (▲) and truncated $HBcAg_{3-144}$ (●).

III.C.5.b. HCMV Antigenicity

The HCMV antigenicity of HBc_{3-144}-HCMV fusion protein was analyzed in dot blot immunoassays, western blot and by RIA. Anti-HCMV monoclonal 7-17 and anti-HCMV human polyclonal antibodies were utilised in both dot blot and western blotting experiments. The HCMV antigenicity of HBc_{3-144}-HCMV could not be demonstrated using these antibodies in any of the assays. Furthermore, negative results were obtained using RIA in which the capturing solid phase was same as the one used for HBc antigenicity, that is polystyrene beads coated with rabbit anti-HBc antibody but with the exception that the second antibody was either a monoclonal or a polyclonal anti-HCMV antibody [section III.B.28.c; page 205]. The failure to detect HCMV antigenicity suggests that the HCMV epitope might not be exposed on the surface of the hybrid core particles.

III.C.6. Immunization of rabbits with HBc_{3-144}-HCMV

Three rabbits (R730, R731 and R732) were immunized by intramuscular injection of 30 µg of HBc_{3-144}-HCMV fusion protein in CFA on day 0. Booster injections were given three times at four weekly intervals on days 30, 60 and 90 using the same amount of antigen in IFA. Blood samples were collected on days 0 (preimmune serum), 10 (first test bleed; ITB), 40 (second test bleed; 2TB) 70 (third test bleed; 3TB) and 100 (fourth test bleed; 4TB). Sera from the three rabbits were analyzed for the presence of anti-HBc and anti-HCMV antibodies.

III.C.6.a. Anti-HBc Response

Rabbit serum dilutions (1 in 10, 1 in 30 and 1 in 100) were assayed for the presence of anti-HBc antibody by a competitive RIA using ^{125}I human anti-HBc IgG as a competitor

and HBcAg coated polystyrene beads as the capturing solid phase. Serum from a control rabbit (R86) that had been immunized with HBcAg using the same regimen was used as a positive control and was assayed in parallel with the test sera. Tables 59-61 and figure 62 summarise the results of the assay. All three rabbits mounted an efficient immune response against the fusion protein HBc_{3-144}-HCMV by producing anti-HBc antibodies, which was detected 10 days after commencing immunization. A maximum anti-HBc response was achieved at day 40, ten days after the first booster injection and this antibody level was maintained or rose only slightly after the second booster injection on day 60. The fourth test bleed obtained on day 100 was not analyzed for anti-HBc response. Although all three rabbits produced a similar response, the anti-HBc levels in rabbit 732 were higher than the other two rabbits. As one of the rabbits (R730) died because of a chest infection on day 65, it was not possible to analyse the third test bleed from this rabbit.

In conclusion, the results obtained demonstrated that the fusion protein HBc_{3-144}-HCMV elicited a high anti-HBc response in the three immunized rabbits.

III.C.6.b. Anti-HCMV Response

The presence of anti-HCMV antibodies in rabbit sera was analyzed using Immunofluorescense technique and was performed by Dr Vince Emery. Insect cells (Sf21) infected with a recombinant baculovirus expressing glycoprotein B (gB) of HCMV were fixed to the glass slides. The cells were incubated with preimmune or immune rabbit sera at doubling dilutions of 1 in 10, 1 in 20, 1 in 40 , 1 in 60, 1 in 80 and 1 in 100. Uninfected Sf21 cells were used as a control. Only serum obtained from the third and the fourth test bleeds was tested against HCMVgB infected and uninfected Sf21 cells. Any reactivity between insect cell expressed gB and anti-HCMV

specific antibody in rabbit sera was detected by using fluorosciene isothiocyanate (FITC) conjugated anti-rabbit IgG.

Although HCMV antigenicity had not been detected so far, immunofluorescence studies confirmed the presence of anti-HCMVgB specific antibodies in the three rabbit sera. Results have only been shown for R731 and R732 [fig 63 A-I]. Strong nuclear immunofluorescence was detected when immune rabbit serum from the third [fig 63 B & C] and the fourth [fig 63 E & F] test bleeds was incubated with gB infected insect cells. Only weak immunofluorescence that was diffusely spread in the insect cell was seen when the preimmune serum was used as a control [fig 63 H]. Similar weak fluorescence was noted when immune rabbit serum was tested against uninfected insect cells [fig 63 I]. These results provide the evidence that HCMV-gp58 epitope is immunogenic when presented to the immune system with HBcAg.

Rabbit 730 (days)	Serum Dilution	Counts per minute (cpm)	Signal/Noise Ratio
0 (PI)	1 in 10	5814	-
	1 in 30	6654	-
	1 in 100	7283	-
10 (1TB)	1 in 10	1579	3.6
	1 in 30	2078	3.2
	1 in 100	5095	3.5
40 (2TB)	1 in 10	721	8.0
	1 in 30	884	7.5
	1 in 100	1633	4.4
70 (3TB)	-	-	-
	-	-	-
	-	-	-
No serum		12817	
R86 (+ C)	1 in 10	508	11.7
	1 in 30	829	8.2
	1 in 100	1581	4.6

Table 59: Results of the Competitive RIA used to determine induction of anti-HBc antibody in Rabbit 730.

PI; preimmune serum

ITB; first test bleed

2TB; second test bleed

3TB; third test bleed

+ C; positive control serum from rabbit 86 immunized with HBcAg

No serum; negative control containing ^{125}I anti-HBc and PBS.

Rabbit 731 (days)	Serum dilution	Counts per minute (cpm)	Signal/Noise Ratio
0 (PI)	1 in 10	6400	-
	1 in 30	6976	-
	1 in 100	7096	-
10 (1TB)	1 in 10	2457	2.6
	1 in 30	3866	1.8
	1 in 100	7325	0.9
40 (2TB)	1 in 10	531	12.0
	1 in 30	1223	5.7
	1 in 100	2527	2.8
70 (3TB)	1 in 10	407	15.7
	1 in 30	898	7.7
	1 in 100	2435	2.9
No serum	-	12817	-
R86 (+ C)	1 in 10	508	11.7
	1 in 30	829	8.2
	1 in 100	1581	4.6

Table 60: Results of the Competitive RIA used to determine induction of anti-HBc antibody in Rabbit 731.

PI; preimmune serum

ITB; first test bleed

2TB; second test bleed

3TB; third test bleed

+ C; positive control serum from rabbit 86 immunized with HBcAg

No serum; negative control containing ^{125}I anti-HBc and PBS.

Rabbit 732 (days)	Serum dilution	Counts per minute (cpm)	Signal/Noise Ratio
0 (PI)	1 in 10	5620	-
	1 in 30	6886	-
	1 in 100	7693	-
10 (1TB)	1 in 10	1349	4.1
	1 in 30	2565	2.6
	1 in 100	5093	1.5
40 (2TB)	1 in 10	612	9.1
	1 in 30	965	7.1
	1 in 100	3223	2.3
70 (3TB)	1 in 10	511	10.9
	1 in 30	1063	6.4
	1 in 100	2046	3.7
No serum	-	12817	-
R86 (+ C)	1 in 10	508	11.7
	1 in 30	829	8.2
	1 in 100	1581	4.6

Table 61: Results of the Competitive RIA used to determine induction of anti-HBc antibody in Rabbit 732.

PI; preimmune serum

ITB; first test bleed

2TB; second test bleed

3TB; third test bleed

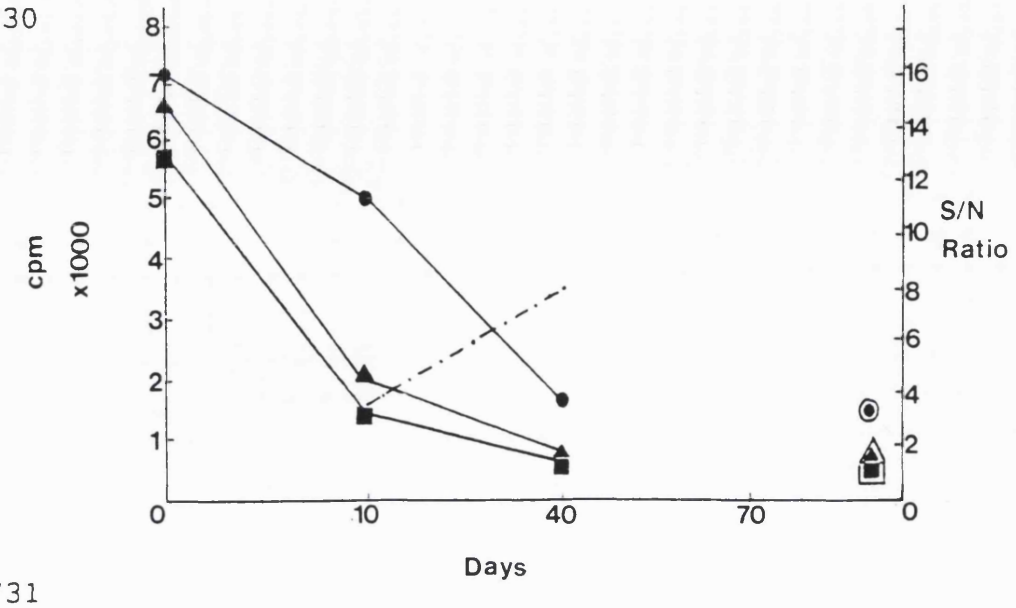
+ C; positive control serum from rabbit 86 immunized with HBcAg

No serum; negative control containing ^{125}I anti-HBc and PBS.

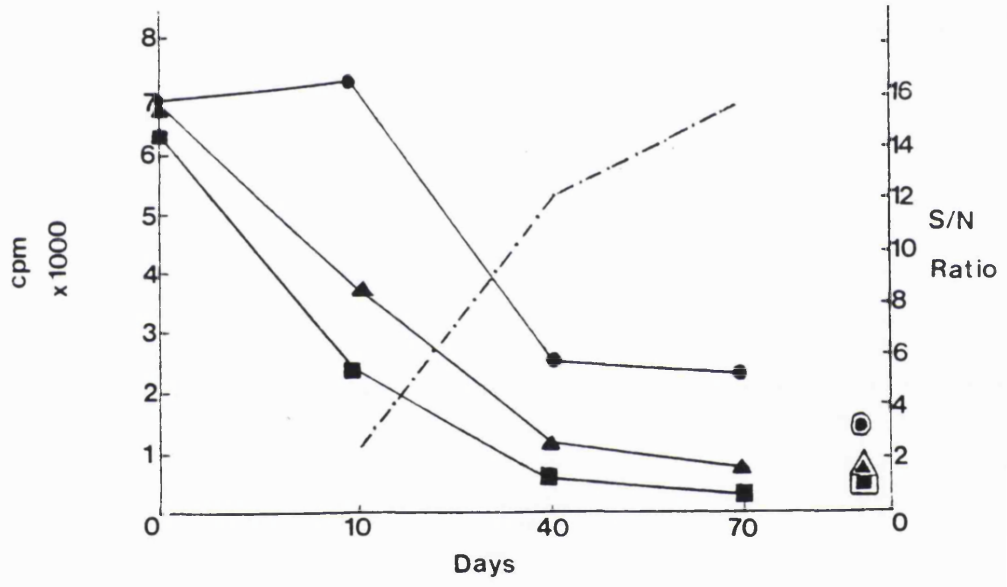
FIG 62: Induction of anti-HBc antibody in three rabbits immunized with HBc_{3-144}-HCMV, determined using a competitive RIA.

R730, R731 and R732 were immunized with HBc_{3-144}-HCMV on days 0, 30 (first booster injection) 60 (second booster injection) and 90 (third booster injection). Serum samples were obtained on day 0 before immunization and days 10 (1TB), 40 (2TB) 70 (3TB) and 100 (4TB). Sera from first, second and third test bleeds were diluted 1 in 10 (■), 1 in 30 (▲) and 1 in 100 (●) and tested in duplicate for the presence of anti-HBc antibody by competitive RIA. A high titre positive control serum obtained from R86 immunized with HBcAg was also diluted 1 in 10 (▣), 1 in 30 (◬) and 1 in 100 (◉) and assayed concomitantly. Signal/Noise (S/N) Ratio is indicated as a broken line.

A: RABBIT 730



B: RABBIT 731



C: RABBIT 732

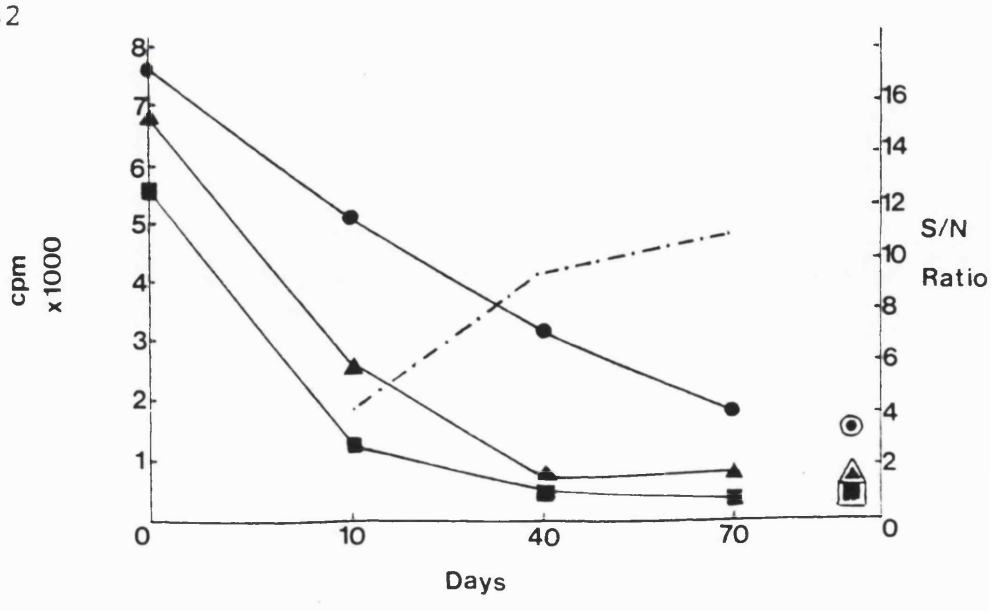


FIG 63: Analysis of anti-HCMV specific antibody response in rabbit sera by Immunofluorescence.

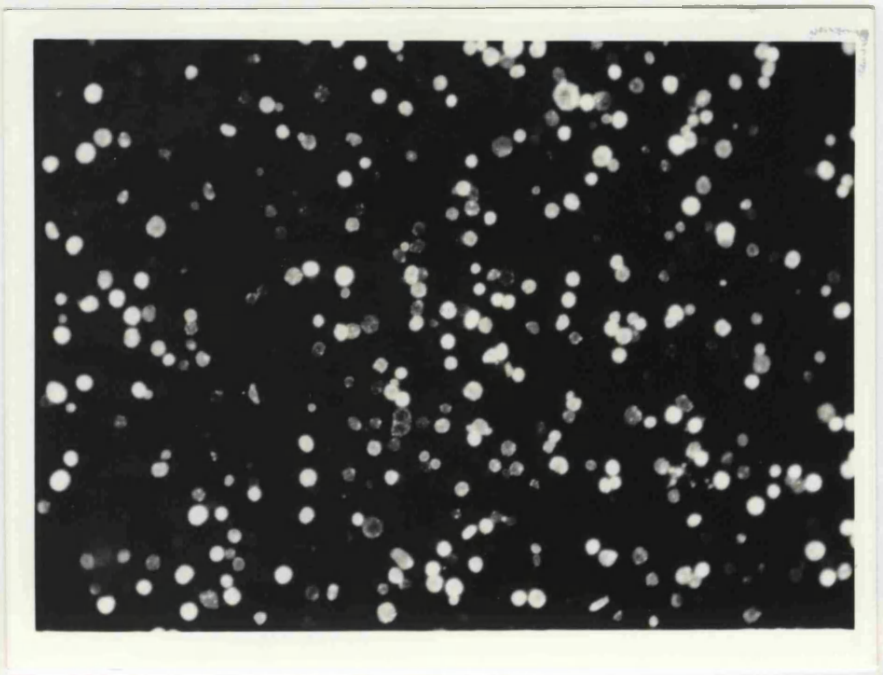
Sf21 cells infected with a recombinant baculovirus expressing HCMVgB were fixed to the glass slide and incubated with immune or preimmune rabbit sera. FITC conjugated anti-rabbit antibody was added to detect any reactivity between HCMVgB and rabbit sera.

A: positive control anti-HCMVgB specific antibody (C23) against HCMVgB expressing Sf21 cells (magnification 100x for A, B & C).

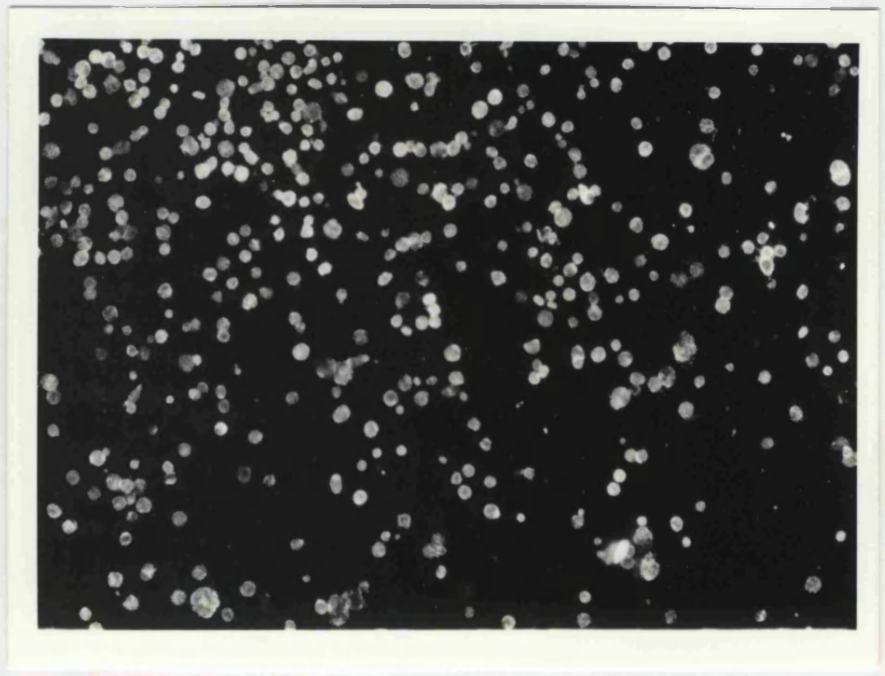
B: serum from R731 (third test bleed) against HCMVgB expressing Sf21 cells.

C: serum from R732 (third test bleed) against HCMVgB expressing Sf21 cells.

A



B



C

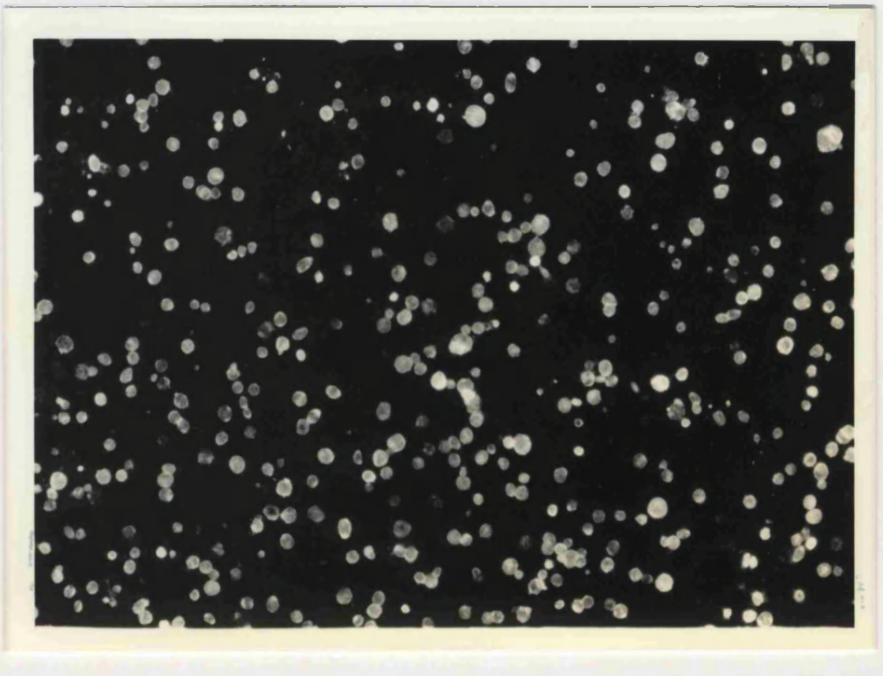


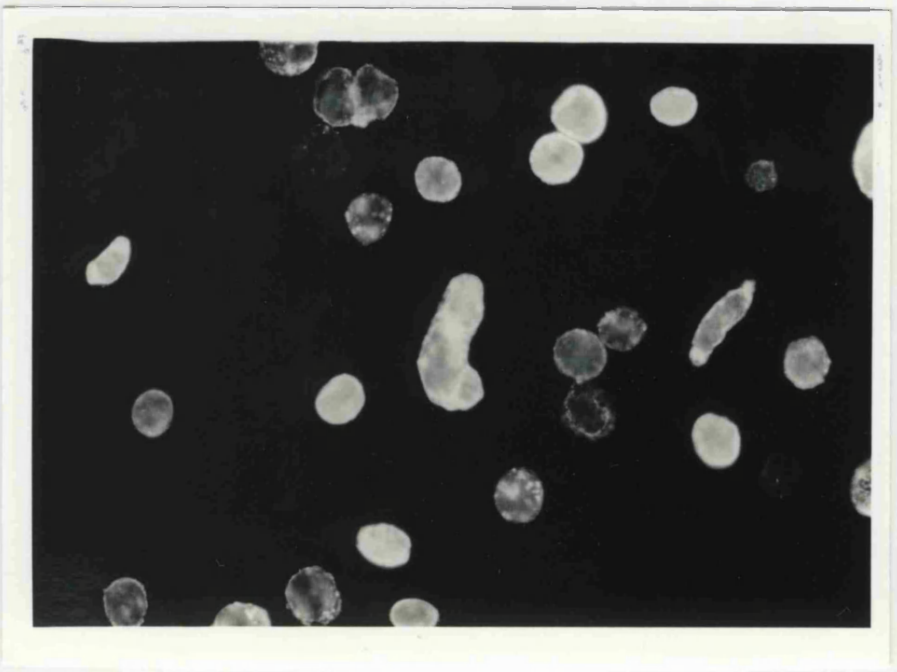
FIG 63: (continued)

D: positive control anti-HCMVgB specific antibody (C23) against HCMVgB expressing Sf21 cells (magnification 400x for D, E & F).

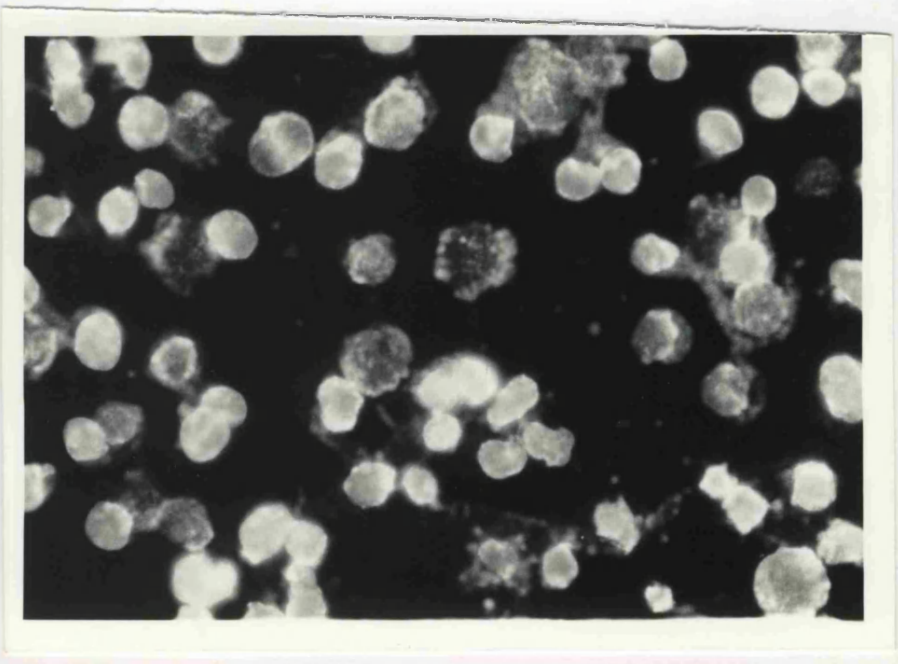
E: serum from R731 (fourth test bleed) against HCMVgB expressing Sf21 cells.

F: serum from R732 (fourth test bleed) against HCMVgB expressing Sf21 cells.

D



E



F

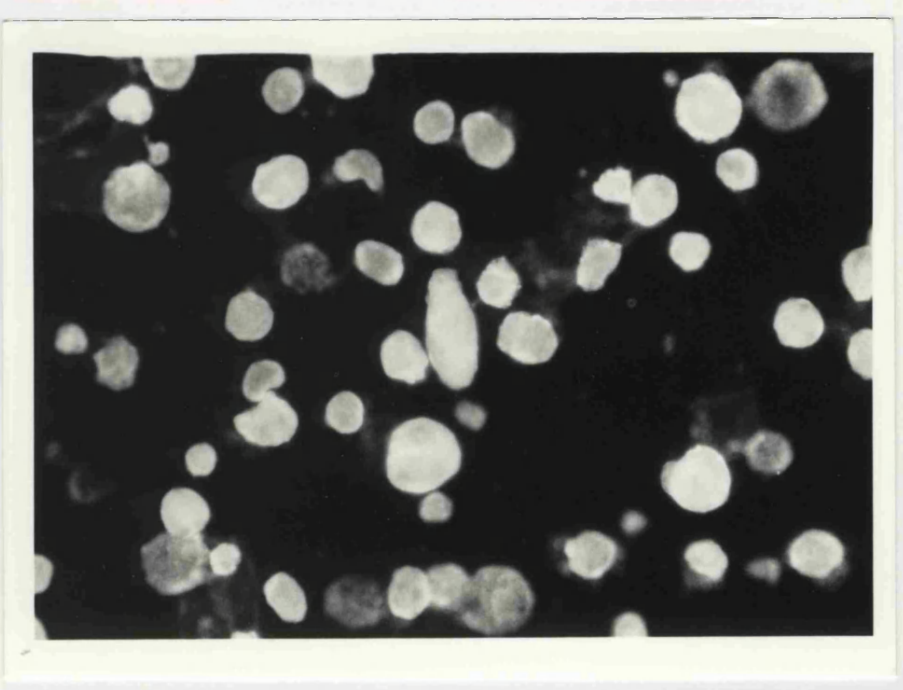


FIG 63: (continued)

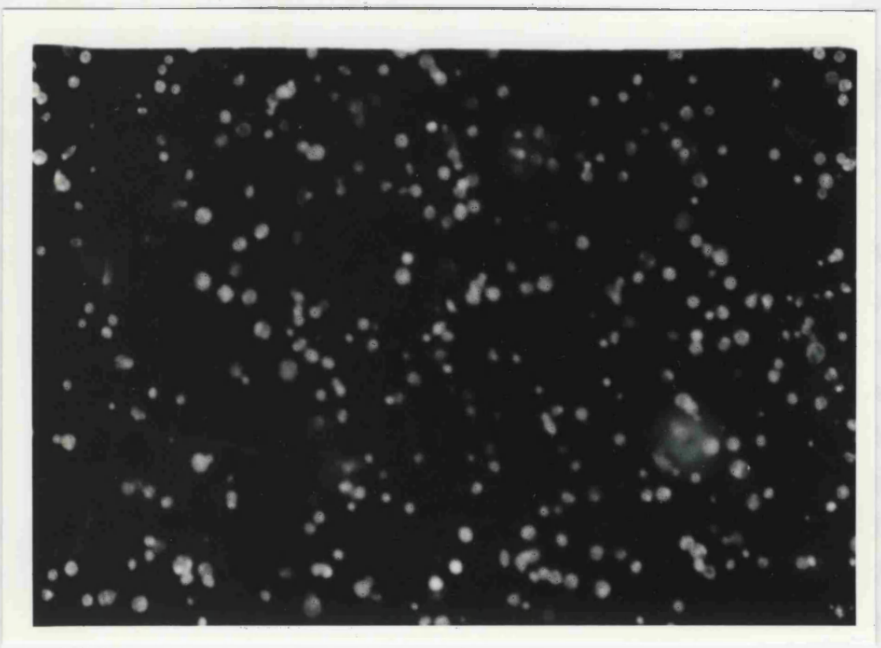
G: negative control in which primary antibody (rabbit serum or antibody C23) was not added to HCMVgB infected Sf21 cells (magnification 100x for G, H & I).

H: preimmune serum from R731 against HCMVgB infected Sf21 cells (same results for R732).

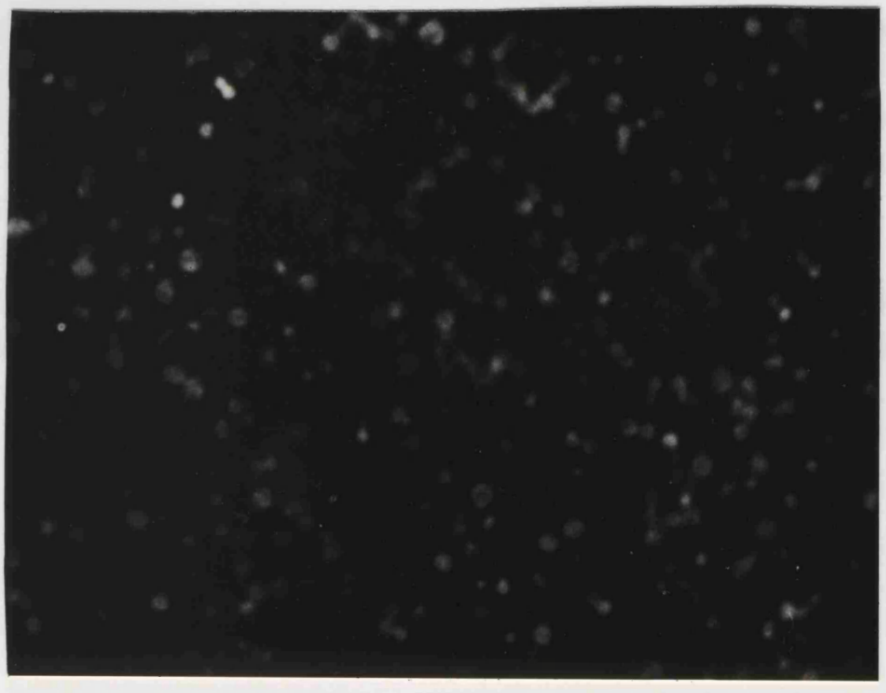
I: serum from R731 against uninfected Sf21 cells (same results for R732).

G

H



I



III.D. DISCUSSION

Several immunogenic epitopes ranging in length from 13 to 35 amino acids have been fused to the amino terminus of HBcAg, without affecting native HBc antigenicity [Clarke et al., 1987; Francis and Clarke, 1989; Francis et al., 1990; Schödel et al., 1992] (section III.A.1). On the other hand, up to 65 amino acids have been accommodated at the truncated carboxyl terminus of HBcAg and this has similarly preserved HBc antigenicity [Stahl and Murray, 1989] (section III.A.2).

III.D.1. HBcAg Amino Terminal Fusion with HCMV-gp58 Epitope: HCMV-HBc_{1-183}

In this study, the strategy for construction of an HBcAg amino terminal fusion with a 50 amino acid HCMV-gp58 epitope was similar to the one used by other groups [Francis and Clarke, 1989; Clarke et al., 1990; Francis et al., 1990] as described in sections III.A.1 and III.B.1.a. The HCMV-gp58 epitope was joined to the precore region through linker sequences and followed by the full length HBcAg sequence. In the reports mentioned above, immunogenic epitopes from FMDV, poliovirus type 1 and human rhinovirus type 2 have been joined in a similar fashion to HBcAg sequence via the precore sequence and have been found to be immunogenic in experimental animals. Recently Schödel et al [1992] have shown that a preS1 epitope (aa 12-47) of HBV fused to the amino terminus through a precore linker sequence into precore amino acid position 26, was surface accessible and highly immunogenic.

The HBcAg fusion proteins have been successfully expressed in E.coli and purified on the basis of assembly of the fusion proteins into core-like particles [Stahl et al.,

1982; Stahl and Murray, 1989]. Although the same method was employed here for protein purification, results obtained in this study suggest that the fusion protein HCMV-HBc_{1-183} was either not expressed at all or was only expressed in very low quantities as the protein could not be detected by Coomassie blue staining, dot blot immunoassays or western blotting. In another study by Shiau [1993], it was noted that HBcAg fusion proteins (using sequences encoding S, preS1 and preS2 regions of HBV) carrying inserts at both amino and carboxyl termini of truncated HBcAg displayed different physical and/or chemical characteristics from those carrying only carboxyl terminal inserts. They were insoluble when pelleted by ultracentrifugation after ammonium sulphate precipitation of the soluble fractions from crude extracts. Therefore, HBcAg fusion proteins with inserts at both termini were not purified. It was suggested that the aggregation of these fusion proteins might be a due to a peculiarity of the sequences used for making the HBcAg fusions. In the present study, a similar problem might have occurred due to some inherent properties of gp58 epitope, making it an unfavourable candidate for fusion, especially at the amino terminus of HBcAg. Utz et al., [1989] have reported that a stretch of amino acids in HCMV-gp58 that bind to the HCMV neutralizing antibody 7-17 are hydrophobic and indeed this characteristic has been displayed in hydrophobicity profiles, generated for both HBcAg amino and carboxyl terminal recombinants using the Staden Plus programme (see fig 64) [Kyte and Doolittle, 1982]. This hydrophobicity could be responsible for poor solubility of the recombinant polypeptide as has been noticed while preparing synthetic peptides derived from HCMV-gp58 [Utz et al., 1989].

This problem could also be explained in the light of findings reported by Zhou et al., [1992]. Core particle assembly has been noted to be a highly cooperative process that requires a threshold concentration of p21.5 dimers and proceeds in the absence of easily detectable intermediates. It has been observed that, if the level of p21.5 synthesis is

sufficiently high, all p21.5 assembles into core particles but if the concentration of cellular p21.5 is reduced fivefold, the conversion of free p21.5 into core particles becomes almost negligible [Zhou et al., 1992]. In the present study, reduced expression and/or concentration of HCMV-HBc_{1-183} fusion protein might have resulted in lower concentration of fusion protein that was not detected immunologically and was below the threshold limit that is essential for initiation of the core particle assembly process. Although HCMV-HBc_{1-183} could not be purified for immunological study, the presence of core fusion particles in one of the electron micrographs suggested that the fusion protein was capable of assembling into core particles and this finding supports the view given above by Zhou et al [1992].

In summary, the investigations emphasize that addition of bulky foreign sequences, especially those having majority of hydrophobic residues, can have unpredictable effects on protein structure and consequently on hybrid core particle assembly. Thus, the problems associated with such situations can be minimized to an extent by using small foreign epitopes with less hydrophobic sequences, that would less radically disrupt protein structure. This in not only applicable to particulate HBcAg but would also apply to other particulate structures that have been utilized as carriers of heterologous sequences such as HBsAg.

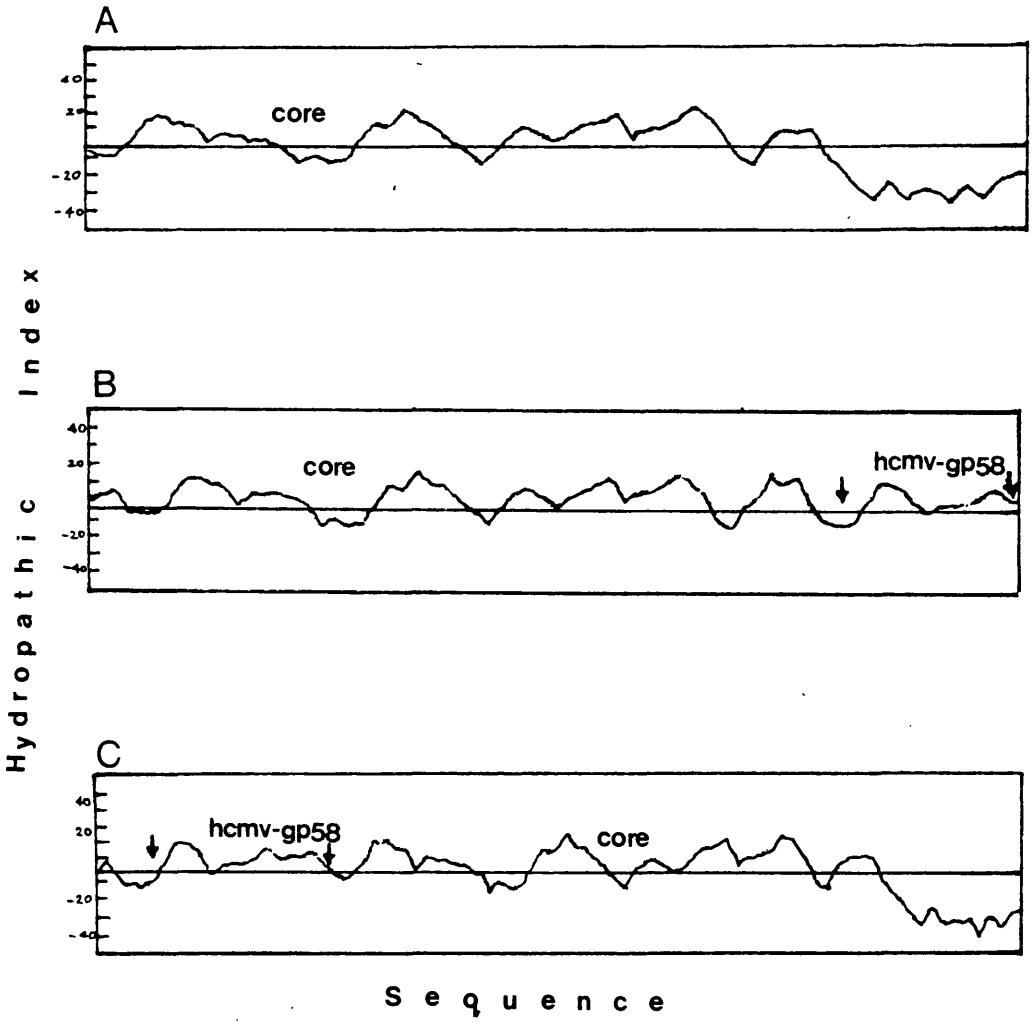


FIG 64: Hydrophobicity profiles of:

(A) HBcAg

(B) HBc_{3-144}-HCMV

(C) HCMV-HBc_{1-183}

III.D.2. HBcAg Carboxyl Terminal Fusion with HCMV-gp58 Epitope: HBc_{3-144}-HCMV

The fusion to the carboxyl terminus of HBcAg was made at amino acid 144 via a short linker sequence as described in sections III.A.2 and III.B.1.c. [Stahl and Murray, 1989]. The fusion protein HBc_{3-144}-HCMV was easily purified on the basis of its particulate nature and was evaluated for both HBc and HCMV antigenicity and immunogenicity.

III.D.2.a. HBc_{3-144}-HCMV retains HBc Antigenicity

HBc antigenicity of the fusion protein HBc_{3-144}-HCMV was analyzed using dot blot immunoassays, western blotting and radioimmunoassays. Both HBc_{3-144}-HCMV and the control truncated HBcAg ($HBcAg_{3-144}$) display a similar HBc antigenicity in these assays and indicate that fusion of foreign sequences to the carboxyl terminus of HBcAg preserves HBc antigenicity. However, it has been noticed that insertion of a foreign sequence between residues 75 and 83 of HBcAg results in loss of HBc antigenicity when detected by several anti-HBc monoclonal and human anti-HBc antibodies, emphasizing the importance of this region in antibody recognition of HBcAg [Schodel et al., 1992].

In dot blot immunoassays, a panel of five anti-HBc monoclonal antibodies was used for detecting HBc antigenicity and all but one (monoclonal antibody A2) reacted with the purified HBc_{3-144}-HCMV. Interestingly, in this blot no HBc antigenicity was detected for the positive control $HBcAg_{3-144}$ [fig 53E, page 248]. However, PV-HBc_{1-183} (polio virus amino terminal fusion) that was used as the other positive control did react with monoclonal antibody A2 indicating that the epitope reactive with this monoclonal was probably located in the carboxyl terminal region of the core antigen beyond amino

the carboxyl terminal region of the core antigen beyond amino acid 144, that had been deleted during construction of the plasmid ptacHpaII-R2 (section III.A.2).

In western blotting, when the same panel of anti-HBc monoclonal antibodies was used, only one monoclonal (RFc17) reacted with HBc_{3-144}-HCMV and the control proteins [fig 56, page 256]. Both in western blotting and in dot blot immunoassays, the same solid support was used for binding the fusion protein. In western blotting, however, the protein would probably not be in the native form, since it was denatured by treatment with ionic detergent and boiling under reducing conditions. Although electrophoretic transfer of the protein to the nitrocellulose membrane could remove the detergent and the reducing agent, the covalent forces which result in binding of the protein to the membrane would prevent complete renaturation of the polypeptide. Therefore, it is likely that western blotting would favour linear determinants [Shiau, 1993]. This suggests that monoclonal RFc17 recognized a linear HBc determinant whereas the other monoclonal antibodies probably recognized a conformational HBc determinant/s present only on the native protein.

In the antigen capture RIA using rabbit polyclonal anti-HBc antibody, HBc_{3-144}-HCMV displayed a similar antigenicity to the control truncated $HBcAg_{3-144}$. Although the signal generated was weaker with HBc_{3-144}-HCMV compared to the control, these results are in accordance with the previous findings that HBc antigenicity is retained in carboxyl terminal fusions [Borisova et al., 1989; Stahl and Murray, 1989; Schodel et al., 1992].

III.D.2.b. HBc_{3-144}-HCMV loses HCMV Antigenicity

The HCMV-gp58 epitope was found not to be antigenic in either dot blot immunoassays or SDS-PAGE immunoblotting using anti-HCMV monoclonal and (human) polyclonal antibodies. The absence of reactivity with anti-HCMV antibodies raises the

possibility that the HCMV epitope might not have been expressed at all. However, the results obtained by sequencing both fusion constructs indicate that there was no stop codon or disruption of the reading frame that could have prevented the expression of the fusion proteins.

Instability or misfolding of a hybrid polypeptide has been the main impediment to the development of generally applicable strategies for chimeric particle formation [Bruss and Ganem, 1991a]. In this project, a similar problem might have arisen due to presence of hydrophobic domains in the gp58 epitope as discussed above [section III.D.1.]. This suggestion is strengthened by the finding in this study that gp58 antigenicity was not preserved and could have resulted from misfolding of the hybrid polypeptide in such a way that HBc B-cell epitopes were exposed on the surface of the particle but gp58 epitopes were masked, being sequestered inside the particle. Because loss of gp58 antigenicity was observed for both fusion proteins, this further supports the notion that conformational change is the result of hydrophobic properties of gp58. Thus, it is possible that hydrophobic amino acids have altered the topology of the already overstretched molecule in a way that makes its assembly into a hybrid core particle an increasingly difficult task.

A recent study [Schödel et al., 1992] has shown that the HBV preS1 epitope (comprising aa 27-53) inserted between the core methionine and the second core amino acid, was not accessible on the surface of core particle or immunogenic. When another preS1 epitope (aa 12-47) was fused to the amino terminus through a precore linker sequence into precore amino acid position 26, it was surface accessible and highly immunogenic. Similarly, a preS2 epitope fused to carboxyl terminus of HBcAg proved to be surface accessible but weakly immunogenic [Schödel et al., 1992]. In view of the findings described above, the HCMV epitope used in this study should have been highly antigenic at least for HBcAg amino terminal

fusion because the epitope was inserted in the precore region. Therefore, one explanation for the loss of antigenicity of the gp58 epitope could be some peculiarity of the HCMV epitope (as discussed above), that might have influenced the external positioning of the epitope. Another interesting point that emerged from the work mentioned above [Schödel et al., 1992] was that the fused preS1 and preS2 epitopes were surface accessible under nondenaturing conditions, when the particles were bound to ELISA plates. Thus, it is possible that denaturing conditions used both in dot blot immunoassays and SDS-PAGE/Immunoblotting could have been responsible for loss of antigenicity of the gp58 epitope. This suggestion has been reinforced by a recent study in which the humoral immune response to the gp58 epitope has been studied by establishing cell lines producing specific human monoclonal antibodies. The cell lines were generated from peripheral blood lymphocytes obtained from a healthy carrier [Ohlin et al., 1993]. Even though five of the six gp58-specific antibodies recognized the antigen after SDS-PAGE and western blotting, it was observed that several or all of these antibodies recognized discontinuous epitopes. The individual epitope integrity was however more or less sensitive to denaturing and reducing conditions [Ohlin et al., 1993].

In a study by Borisova et al [1989], epitopes derived from transmembrane protein gp41 of human immonodeficiency virus (HIV-1), preS region of HBV and envelope protein gp51 of bovine leukaemia virus were fused to the truncated carboxyl terminus of HBcAg at amino acid 144 or inserted at an internal site between amino acid 144 and 145 in the full length HBcAg. The external positioning of these antigenic determinants was confirmed by antigen capture sandwich ELISA and immunogold microscopy. However, in another study [Yon et al., 1992] the inserted epitope derived from simian immunodeficiency virus fused to the carboxyl terminus of the full length HBcAg could not be detected by antigen capture sandwich ELISA. It was suggested that arginine rich carboxyl terminus of HBcAg

prevented the foreign epitope from being presented externally on the surface of hybrid HBcAg particle. Although in the studies described here truncated HBcAg, instead of the full length HBcAg, was used to present the HCMV gp58 epitope on the surface of the hybrid core particle, no evidence was obtained to suggest the surface localization of the HCMV epitope. The explanation for this could be that the epitope was either presented in a different conformation on the surface of the hybrid core particle unrecognized by the antibodies used in this study or was sequestered inside the particle and surface inaccessible.

III.D.3. Immunization Studies

The HBc and HCMV immunogenicity of HBcAg fusion protein HBc_{3-144}-HCMV was determined in terms of antibody production in rabbit sera. The immunization of rabbits with HBc_{3-144}-HCMV produced high levels of HBcAg-specific antibodies, indicating that the fusion of HCMV-gp58 epitope to the carboxyl terminus of HBcAg had not altered the immunogenicity of HBcAg. Although all the immunized rabbits mounted a strong immune response to the core component of HBc_{3-144}-HCMV fusion protein as determined using RIA, the antibody response to the HCMV epitope could not be analyzed or compared similarly because of unavailability of appropriate antigens that could be used in RIA. Therefore, anti-HCMV antibodies were initially checked using Abbott CMV latex agglutination test but the results were non specific (results not shown). This was followed by Immunofluorescence using insect cells infected with a recombinant baculovirus expressing the gB region of HCMV. The presence of anti-HCMV specific antibodies was confirmed using this technique and strong immunofluorescence produced by immune rabbit sera provides the evidence that HCMV epitope is immunogenic. As the insect cells were only expressing the gB region of HCMV, it was only possible to show the presence of

anti-HCMV antibodies specifically directed against the gB/gp58 region.

In a study by Borisova et al [1989] in which several HBcAg carboxyl terminal fusions were constructed (mentioned above) it was noticed that the level of anti-HBc response in all cases corresponded to that obtained in a control after immunization with HBcAg. However, the titre of anti-epitope antibodies were markedly lower. Moreover, the inserted oligopeptides showed different immunogenicity despite the equal HBc immunogenicity of chimeric capsids. Although chimeras present multiple copies of the inserted oligopeptides on their surface, the appropriate immune response in rabbits was noticed to be lower than expected [Borisova et al., 1989]. However, it was not possible to compare the strength of the anti-HBc and anti-HCMV response in rabbits in this study because different assays were used to detect the presence of antibodies.

In another report [Yon et al., 1992] peptides from different regions of simian immunodoficiency virus (SIV) fused to the amino terminus or inserted between amino acid 80 and 81 of the core protein, induced good anti-peptide antibody responses. When the same peptides were fused to the full length HBcAg at the carboxyl terminus, anti-peptide antibody was not detected. This contrasts with the results previously described with carboxyl terminal fusions, where the fusions were found to be immunogenic [Stahl and Murray, 1989; Schodel et al., 1992]. This difference could be explained by the fact that SIV fusions were constructed using the full length core protein which contains the arginine rich region, which is thought to interact with encapsidated nucleic acid in the hepatitis B virion. The presence of this region in core-SIV fusion may be responsible for the lack of immunogenicity of these carboxyl terminal fusions by ensuring that the peptides are on the inside of the particle [Yon et al., 1992]. This suggestion is strengthened by the findings described in this

thesis as the HBcAg/HCMV-gp58 carboxyl terminal fusion did not contain the arginine rich region and was thus found to be antigenic and immunogenic.

CHAPTER IV

GENERAL DISCUSSION AND CONCLUSIONS

The surface and core proteins of HBV have been used as carrier molecules for less immunogenic heterologous epitopes [Valenzuela et al., 1985; Delpeyroux et al., 1986 and 1990; Stahl and Murray, 1989; Francis et al., 1990]. The use of HBcAg for presenting foreign epitopes to the immune system, on the surface of hybrid core particles, has been discussed in chapters II and III. The amino and truncated carboxyl termini of HBcAg have been employed independently for this purpose but comparison of both termini using the same epitope has not been carried out in detail [Clarke et al., 1987; Francis and Clarke, 1989; Stahl and Murray, 1989]. Only recently has this approach been tried using preS epitopes of HBV [Schödel et al., 1992]. The goal of this study was to compare the expression of a foreign epitope inserted into amino or fused to the carboxyl terminus of HBcAg, in a prokaryotic system (E.coli) and in baculovirus expression vector system (BEVS).

The heterologous epitope used in this study was derived from an immunogenic sequence of HCMV glycoprotein. The HCMV gp58 region is known to carry one immunodominant neutralization domain, which is present in 150 bp region that was used here and could serve as a useful immunogen candidate for a CMV subunit vaccine. The investigations carried out involved the following:

1) Insertion of HCMV-gp58 epitope in the amino terminus of HBcAg in the preC region;
2) Fusion of the same epitope to the truncated carboxyl terminus of HBcAg;
3) Expression and Purification of fusion constructs using a prokaryotic system (E.coli);
4) Analysis of antigenicity of both amino and carboxyl terminal HBcAg fusion proteins by dot blot immunoassays and SDS-PAGE/Immunoblotting.
5) Analysis of immunogenicity of carboxyl terminal HBcAg fusion protein by immunization studies in rabbits.

   AND

6) Expression of HBcAg and HBsAg in BEVS.

IV.A. Expression of HBcAg/HCMV-gp58 Fusion Proteins in a Eukaryotic System (BEVS)

As described in chapter II, two recombinant baculoviruses containing genes encoding HBcAg and HBsAg were isolated by dot blot hybridization method. Unfortunately, standardization of this control system for isolation and purification of a recombinant baculoviruses proved to be a time consuming task. Therefore, it was not possible to utilize the dot blot hybridization method for purification of baculovirus recombinants containing HBcAg/HCMV-gp58 fusions and further work was only carried out in the prokaryotic system.

IV.B. Expression of HBcAg/HCMV-gp58 Fusion Proteins in a Prokaryotic System

As outlined in chapter III and above, two separate fusions were constructed between HCMV-gp58 and the amino and carboxyl termini of HBcAg. When expressed in E.coli HBc antigenicity was retained only by the HBcAg carboxyl terminal fusion and HCMV antigenicity could not be detected for either fusion. Thus it seems that under similar circumstances the constraints imposed on polypeptide folding may be more severe in the case of HBcAg amino terminal fusion. However, the characteristics of foreign epitopes are also important in determining the overall antigenicity of the fusion proteins.

The immunogenicity of HBcAg carboxyl terminal fusion was determined in terms of antibody production in rabbits. The results demonstrate that immunization of rabbits with the fusion protein resulted in generation of HBcAg-specific and HCMV-specific antibodies. The HBcAg-spcific antibodies were produced only a few days after the primary immunization whereas anti-HCMV response was delayed. Nevertheless this indicates that fusion of an HCMV-gp58 epitope at the carboxyl

terminus of HBcAg does not alter the antigenicity or immunogenicity of HBcAg.

IV.C. Conclusions

In conclusion, the fusion of the HCMV gp58 epitope at the carboxyl terminus of HBcAg was soluble and could be purified on the basis of its particulate nature, whereas insertion of the same epitope in the amino terminus of HBcAg altered the physical nature of the fusion protein in such a way that it was not possible to detect its antigenicity. Because hybrid core particles were detected for the amino terminal fusion it is likely that the protein was aggregated. The immunization work was thus carried out only for the carboxyl terminal fusion. The HBc_{3-144}-HCMV elicited high levels of anti-HBc antibody, indicating that the fusion of a 50 amino acid foreign epitope at the carboxyl terminus of HBcAg does not change the antigenicity and immunogenicity of HBcAg. In addition, presence of anti-HCMV antibodies directed against the epitope of interest indicates that the HBcAg can serve as a carrier for presentation of foreign epitopes to the immune system in an effective manner and also emphasizes the potential value of this epitope as a candidate for subunit HCMV vaccine.

IV.D. Future Directions

It would be interesting to carry out further studies on following aspects:

1) Constructing a fusion by using a middle loop region of HBcAg as used by Schödel et al., [1992]. By using this approach, it is possible that gp58 epitope might be presented at the surface of hybrid particle, in a different conformation.

2) To perform the immunization studies in mice using HBc_{3-144}-HCMV and comparing the immune response with that produced in rabbits.

3) To analyze the specificity of anti-HCMV response by setting up an ELISA and/or RIA, using appropriate HCMV antigens and finally

4) To perform HCMV neutralization studies using rabbit sera.

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APPENDIX: SOLUTIONS AND MEDIA

Acrylamide, 30%

29 gm Acrylamide
1 gm Methylenebisacrylamide
Dissolved in 60 ml of DW, at 37°C on a heated stirrer. Volume adjusted to 100 ml and the solution filtered and stored in a bottle covered with aluminium foil at 4°C.

Acrylamide, 40%

380 gm Acrylamide
20 gm Methylenebisacrylamide
Dissolved in 600 ml of DW, and prepared exactly as above Volume adjusted to 1 litre with DW.

Ammonium persulphate (10%)

0.1 gm Ammonium persulphate
1 ml DW

Ampicillin (25 mg/ml)

25 mg ampicillin (sodium salt) in 1 ml DW. Filtered and stored at 4°C.

$CaCl_2$, 1 M

147 gm $CaCl_2$ dissolved in DW and volume adjusted to 1000 ml

$CaCl_2$-Tris Buffer

50 mM $CaCl_2$
10 mM Tris HCl pH8.0

Denhardts Solution, 50x

1% (w/v) BSA
1% (w/v) Ficoll
1% (w/v) PVP (polyvinyl pyrollidone)

DTT, 1 M

3.09 gm	DTT
20 ml	NaAc pH5.2 0.01 M

Filtered and stored at -20°C

Extraction Buffer

100 mM	Tris HCl pH7.4
90 mM	EDTA
200 mM	KCl
DW to	4.5 ml

EDTA, 0.5M (Ph8.0)

186.1 gm of the salt in 800 ml of DW. The pH adjusted to 8.0 with NaOH pellets and sterilized by autoclaving.

Hepes-buffered Saline, 2x

1.6 gm	NaCl
0.074 gm	KCl
0.027 gm	Na_2HPo_4
0.2 gm	Dextrose
1 gm	Hepes
90 ml	DW

The pH adjusted to 7.05 with NaOH (0.5M). Volume made upto to 100 ml with DW. Filtered and stored at -20°C in 5 ml aliquots.

IPTG

2 gm of IPTG dissolved in 8 ml of DW and volume adjusted to 10 ml. Filtered through 0.22 micron filter and stored in 1 ml aliquots at -20°C.

LB Broth

10 gm	Bactotryptone
5 gm	Bacto yeast extract
10 gm	NaCl

Dissolved in 950 ml of DW and the pH adjusted to 7.0-7.5 with 5 M NaOH. Volume made up to 1000 ml with DW and autoclaved.

Lysis Buffer A

10%	N-Lauroylsarcosine sodium salt
10 mM	EDTA

Lysis Buffer B

30 mM	Tris HCl pH7.5
10 mM	Magnesium acetate
1%	Nonidet P40

$MgCl_2$, 1 M

203.3 gm of the salt dissolved in 800 ml of DW. Volume adjusted to 1 litre before sterilization.

Nick Translation Buffer (10x)

0.5 M	Tris HCl pH7.5
0.1 M	$MgSO_4$
1 mM	DTT
500 µg/ml	BSA

Phenol Chloroform

Equal volume of phenol and chloroform were mixed, and equilibrated with several volumes of 0.1 M Tris Hcl pH7.6. The mixture was stored under an equal volume of 0.01M Tris-Hcl pH7.6, in dark glass bottles at 4°C.

Phenol-Chloroform-Isoamylalcohol (25:24:1)

The mixture containing chloroform and isoamylalcohol (24:1) does not require any treatment and is mixed with equilibrated phenol. It may be stored under 100 mM Tris HCl pH8.0.

PBS

PBS tablet dissolved in 100 ml of DW and the solution autoclaved for sterilization.

Sodium Acetate, 3 M (pH5.2)

408.1 gm of the salt was dissolved in 800 ml of DW. The pH was adjusted to 5.2 with acetic acid, the volume made upto 1 litre and the solution autoclaved.

Sodium Chloride, 5 M

292.2 gm of salt dissolved in 800 ml of DW. Volume adjusted to a litre and the solution autoclaved.

SDS, 10%

100 gm of SDS dissolved in 900 ml of DW at 68°C. The pH adjusted to 7.2 by adding few drops of concentrated HCl and the volume made to 1 litre.

Solution I (alkali lysis)

50 mM	Glucose
25 mM	Tris HCl pH8.0
10 mM	EDTA

Solution II (alkali lysis)

0.2 M	NaOH
1%	SDS

Solution III (alkali lysis)

60 ml	Potassium acetate (5M)
11.5 ml	Acetic acid glacial
28.5 ml	DW

Prepared fresh before use and stored on ice.

STE Buffer

100 mM	NaCl
10 mM	Tris HCl pH8.0
1 mM	EDTA

TAE, 50x

242 gm	Tris base
57.1 ml	Acetic acid glacial
100 ml	EDTA, 0.5 M (pH 8.0)

TBE, 5x

54 gm	Tris base
27.5 gm	Boric acid
20 ml	0.5 M EDTA (pH 8.0)

Tris EDTA Buffer (TE)

pH7.4		
	10 mM	Tris HCl pH7.4
	1 mM	EDTA pH8.0
pH7.6		
	10 mM	Tris HCl pH7.6
	1 mM	EDTA
pH8.0		
	10 mM	Tris HCl pH8.0
	1 mM	EDTA pH8.0

TM Buffer

100 mM	Tris HCl pH8.5
50 mM	$MgCl_2$

YT Broth

16 gm	Bactotryptone
10 gm	Bacto yeast extract
5 gm	NaCl

Solutes dissolved in 900 ml of DW, pH adjusted to 7.0 with 5 M NaOH and the solution autoclaved.