AN INVESTIGATION INTO THE STRUCTURE AND ASSEMBLY OF THE HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) CAPSID USING THE BACULOVIRUS EXPRESSION SYSTEM

by

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ABSTRACT

Intermediate (type B) capsids of HSV-1 are composed of seven proteins encoded by six genes. The proteins encoded by UL18 (VP23), UL19 (VP5), UL35 (VP26) and UL38 (VP19C) are components of the outer capsid shell whereas those specified by UL26 (VP21 and VP24) and UL26.5 (pre-VP22a) are involved in the formation of the scaffold which forms the internal core of B capsids. This scaffold plays a pivotal role in capsid assembly and is removed from the capsid concomitant with the packaging of DNA to form mature (type C) capsids. These, upon acquiring tegument and envelope proteins, exit from the cell as infectious virions. In order to gain a comprehensive understanding of the protein interactions that are important for the capsid assembly process, it was necessary to devise a system in which to examine the structures formed by, and the interactions taking place between, the capsid proteins in isolation from other herpesvirus proteins.

The initial aim of this work was to clone and express the four outer shell capsid proteins in baculovirus so that in conjunction with baculoviruses recombinant for the genes UL26 and UL26.5 (which had been made previously by V. Preston) a complete panel of recombinant baculoviruses would exist. Each recombinant produced a protein profile with a unique band of the correct size, as judged by its co-migration with the respective protein in preparations of purified HSV-1 capsids. In addition, the autoprocessing by the protease into VP21 and VP24 and the processing of the scaffolding protein, into VP22a; an event which is an essential requirement for DNA packaging, occurred as in wild type HSV-1.

Co-expression of the six genes in insect cells resulted in the formation of capsids that were indistinguishable in appearance, as viewed in the electron microscope, and protein composition from those made during HSV-1 infection of mammalian cells. This demonstrated that the proteins encoded by the known capsid genes contain all the structural information necessary for capsid assembly and that other virus-encoded proteins are not required for this process.

The requirements for capsid assembly were then analysed in further depth. Omission of single recombinant baculoviruses from this system allowed the role of individual HSV-1 proteins in capsid assembly to be determined. Capsid assembly did not take place in the absence of VP5, VP19C or VP23, whereas lack of VP26 had no discernible effect on capsid formation. These results agreed with findings from the analysis of HSV-1 mutants with lesions in the UL19, UL38 and UL18 genes which had demonstrated that these genes were essential for capsid assembly. Lack of a UL35 mutant virus meant that it was previously not known whether this protein was required for capsid assembly.

Capsids which assembled in the absence of the UL26 gene products had a large-cored phenotype resembling that previously described for the HSV-1 mutant *ts*1201 which has a lesion in this gene. Some apparently intact capsid shells were also made in the absence of the major scaffolding protein, pre-VP22a thereby demonstrating that the outer shell proteins could polymerise into the correct conformation in the absence of this protein. However, in these experiments, VP21 which is identical to VP22a except for a short N-terminal extension, was present and could have compensated for the lack of the scaffolding protein. Indeed when both UL26 and UL26.5-expressing baculoviruses were omitted from the reaction, this resulted in the appearance of large numbers of partial and deformed capsid shells which suggested that the products of one or other of these genes is required to direct correct capsid shell assembly.

Capsids made in the baculovirus system were purified and analysed by collaborators W. Chiu and H. Zhou who had previously performed high resolution (2.6nm) three-dimensional cryo-electron microscopic analysis on wild type HSV-1 capsids. Comparison of the three-dimensional structures of wild type HSV-1 B capsids and of baculovirus generated capsids lacking VP26 revealed the presence of a horn shape mass of density present on all the hexons, but not the pentons, in the wild type capsid which was absent from the hexons in the VP26 negative capsid. Analysis of the difference map of the two capsids revealed a star shape mass of density on the distal tip of the hexons composed of six copies of VP26. In addition to confirming the location of VP26 this also provided information as to the structure of this protein; which appears to consist of a large and small domain which interact with each other and with the underlying VP5 molecules to form bridges between the hexon subunits.

The baculovirus system was also used to examine whether any of the capsid proteins could interact to form discrete sub-capsid structures. Co-infection with the viruses expressing the products of the UL26 and UL26.5 genes resulted in the formation of 40-60nm diameter spheres which resembled the internal scaffold-cores of HSV-1 B capsids. These structures were also present in mixed infections in which one of the outer shell proteins, VP5, VP19C or VP23, was absent. Expression of UL26 in the absence of the other capsid proteins resulted in aggregates of fibrous material whereas expression of UL26.5 resulted in similar material interspersed with particles of a similar size and appearance to scaffolds. Although these scaffold structures were not purified by gradient centrifugation, Western blot analysis identified the presence of VP21/VP22a sedimenting more slowly than capsids.

Co-infection of baculoviruses expressing VP5 and pre-VP22a resulted in the formation of particles which were about 40nm in diameter and had an ill-defined appearance. Some of these structures had the appearance of an inner ring with distinct projections

which could represent VP5 adhering to the outer surface of pre-VP22a scaffolds. Since these particles were not observed in the presence of the protease, it appears that the removal of the C-terminal 25 amino acids from pre-VP22a to form VP22a, prevents this interaction. Together with information from several other studies, this suggests that the C-terminal 25 amino acids of pre-VP22a mediate the interaction of the outer shell proteins with the scaffold during the formation of intermediate B capsids and further suggests that cleavage of the pre-VP22a occurs after capsid assembly or when the protein is in a complex with one or more capsid shell proteins.

When recombinant baculoviruses expressing the VP5 and VP19C capsid proteins were co-infected, densely staining round particles of approximately 70nm in diameter were observed in the electron microscope. When analysed by negative stain, it was apparent that these particles were composed of capsomers and often had the appearance of broken shells, suggesting that they were either less stable than capsids or were incorrectly formed. In the presence of VP23 aberrant capsid shells were formed instead of the densely staining spherical particles formed by VP5 and VP19C alone suggesting that VP23 has a role in modulating the interactions of the outer capsid shell proteins.

Analysis of the capsid structures formed from co-expression of the HSV-1 capsid proteins in baculovirus has provided valuable information as to the role of these proteins and their position in the capsid. The baculovirus system has proved to be a valuable tool for manipulation of proteins and analysis of capsid structure and has opened up a range of exciting possibilities for future studies of capsid structure and morphogenesis.

ABREVIATIONS

A	adenine
aa	amino acid
Ac	*Autographa californica*
ADP	adenosine diphosphate
ala	alanine
APS	ammonium persulphate
Ar^+	argon ion
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BHI	brain heart infusion
BHK	baby hamster kidney cells
bp	base pairs
BSA	bovine serum albumin
BTV	blue tongue virus
C	cytosine
C-	carboxy
^oC	degrees centigrade
^{14}C	carbon 14 radioisotope
CAT	chloramphenicol acetlytransferase
cDNA	complementary DNA
Ci	curie
CIP	calf intestinal phosphatase
CLP	core-like particle
cm	centimeter
CNBr	cyanogen bromide
CPE	cytopathic effect
CsCl	caesium chloride
Da	dalton
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dH_20	distilled water
dTTP	2'-deoxythymidine-5'-triphosphate
dUTP	2'-deoxyuracil-5'-triphosphate
dNTP(s)	2'-deoxynucleoside-5'-triphosphate(s)

DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase 1	deoxyribonuclease 1
DR	direct repeat
ds	double stranded
DTT	dithiothreitol
E. coli	*Escherichia coli*
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethylene-bis (oxyethylenenitrol) tetra-acetic acid
EHV-1	equine herpes virus type 1
EtBr	ethidium bromide
FCS	foetal calf serum
G	guanidine
g	gravity
GuHCl	guanidine hydrochloride
h	hour
HCMV	human cytomegalovirus
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid
HHV	human herpes virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
ICP	infected cell polypeptide
IE	immediate early
Ig	immunoglobulin
IRL	internal long repeat
IRS	internal short repeat
IPTG	isopropylthio-β-D-galactoside
k	kilo (ie. 10^3)
kb/kbp	kilobase pairs
kDa	kilo daltons
L	long segment
l	litre
LAT	latency associated transcript
m	milli (ie. 10^{-3})
M	molar
mAb(s)	monoclonal antibody(s)

mCi	millicurie
MCP	major capsid protein
mg	milligrams
Mg^{++}	magnesium ion
ml	millilitre
mm	millimeter
mM	millimolar
m.o.i.	multiplicity of infection
Mw.	molecular weight
mRNA(s)	messenger RNA(s)
n	nano (ie 10^{-9})
N-	amino
NaCl	sodium chloride
NCS	newborn calf serum
ng	nanogram
nm	nanometer
NP40	nonidet P40
NPT	non-permissive temperature
OBP	origin-binding protein
OBPC	C terminus of OBP
OD	optical density
ORF(s)	open reading frame(s)
ori	origin of replication
oriL	origin of replication in U_L
oriS	origin of replication in U_S
^{32}[P]	phosphorus 32 radioisotope
P22	*Salmonella typhimurium* bacteriophage P22
PAA	Phosphonoacetic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
p.f.u.	plaque forming units
p.i.	post-infection
pI	isoelectric point
pmol	picomolar (10^{-12})
pol	polymerase
PRV	pseudorabies virus

PS	penicillin/streptomycin
PT	permissive temperature
RGB	resolving gel buffer
RNA	ribonucleic acid
RNaseA	ribonuclease A
RNasin	ribonuclease inhibitor
r.p.m.	revolutions per minute
RT	room temperature
S	short segment
^{35}[S]	sulphur-35 radioisotope
SCMV	simian cytomegalovirus
SDS	sodium dodecyl sulphate
ser	serine
Sf21	*Spodoptera frugiperda* insect cell line
SGB	stacking gel buffer
ss	single stranded
SV40	simian virus 40
T	thymidine
T=	triangulation number equal to
T4	*Escherichia coli* bacteriophage T4
TEMED	N, N, N', N', -tetramethlyethylene diamine
TIF	trans-inducing factor
Tris	tris (hydroxymethyl) aminomethane
TK	thymidine kinase
TRL	long terminal repeat
TRS	short terminal repeat
ts	temperature sensitive
u	micro (ie. 10^{-6})
uCi	microcurie
ug	microgram
ul	microlitre
UL	long unique
US	short unique
UV	ultraviolet
V	volts
vhs	virion host shut-off
VLP	virus-like particle

Vmw	apparent molecular weight in kilodaltons of HSV induced polypeptides
VP	virion protein
v/v	volume/volume (ratio)
VZV	varicella-zoster virus
W	watts
wt	wild type
w/v	weight/volume (ratio)
w/w	weight/weight (ratio)
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER 1

INTRODUCTION

1 CLASSIFICATION OF HERPESVIRUSES

There are at least 112 species of the family of *Herpesviridae*, members of which have been isolated from more than 80 vertebrates (Nahmias, 1972; Roizman *et al.*, 1992).

All members of this family conform to a set of morphological characteristics. Herpesvirus virions are enveloped and have been observed to range in size from 150-300nm in diameter. They possess linear ds DNA genomes of Mw. 80-150 x 10^6 of variable (31-75%) G+C content, which are tightly packaged into an icosahedral capsid which is approximately of 100-150nm in diameter. DNA replication and capsid assembly take place in the nucleus from which the packaged capsids leave by budding through the nuclear membrane. In addition to having common morphological characteristics, the herpesviruses share the ability to establish and maintain a latent state in their infected hosts. In cells harbouring latent virus, the viral genome takes the form of closed circular molecules and only a small subset of viral genes is expressed. The wide occurrence of herpesviruses in vertebrates and their high degree of species specificity suggests that they have evolved in close association with their hosts. Historically, they have been classified into three sub-families on the basis of biological properties such as host range, reproductive cycle, cytopathology and characteristics of latent infection. (Roizman *et al.*, 1981; Roizman, 1982; Matthews, 1982; Roizman *et al.*, 1992). However, since each of these biological characteristics is variable, sequence analysis has led to the reclassification of certain herpesviruses and therefore provides a greater understanding of the evolutionary relatedness between the different herpesviruses (McGeoch *et al.*, 1995).

1.1 *ALPHAHERPESVIRINAE*

These viruses have a genome of Mw. 85-110 x 10^6. The reproductive cycle is short (<24h) and results in rapid destruction of the infected cell. Many members of this family are neurotropic and cause acute self-limiting primary skin or respiratory tract diseases in their natural host. The primary infection is often followed by establishment of latent infection, frequently in the sensory ganglia. This sub-family can be further sub-divided into two genera.

The genus *Simplexvirus* includes herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2). In the standard nomenclature of the human herpesviruses (HHV) these are defined as HHV-1 and HHV-2 respectively. HSV-1 is primarily responsible for oral vesicular lesions but also causes ocular keratitis and occasionally encephalitis. HSV-2 which is normally associated with genital lesions, is closely related to HSV-1 and both

viruses have been isolated from both oral and genital sites (Whitley, 1990). Latency is established in the sensory ganglia innervating oral and genital lesions respectively. The cattle virus, bovine mammillitis virus (BMV) is also a member of this group.

The genus *Varicellovirus* includes varicella-zoster virus (VZV) of humans which is also known as HHV-3 (reviewed by Gelb, 1990). Primary infection, usually in childhood, causes chickenpox and is followed by a long period of virus latency in the sensory ganglia. In immunocompromised or elderly individuals, the virus can reactivate, usually from an individual ganglion to produce the condition shingles. Animal herpesviruses which are included in this group are equine herpes virus type one (EHV-1), which causes abortion in pregnant mares and pseudorabies virus (PRV) which causes severe encephalitis in pigs.

1.2 BETAHERPESVIRINAE

Viruses in this subfamily have a larger genome which is in the range of Mw. 110-150 x 10^6. The replication cycle is much slower than that of the alpha herpesviruses and infected cells frequently become enlarged (cytomegalia). Contained in this group is the genus *Cytomegalovirus* which is represented by the human pathogen human cytomegalovirus (HCMV or HHV-5). Although infections in the immunocompetent host are usually asymptomatic, HCMV causes a wide range of chronic infections in the immunocompromised and has been implicated in causing neonatal abnormalities. It has also been found associated with several cancers: cervical carcinoma, adenocarcinoma and Kaposi's sarcoma (reviewed by Alford and Britt, 1990). Other viruses in this sub-family include CMVs isolated from mice, pigs and primates.

Two other newly discovered viruses; HHV-6 and HHV-7 (reviewed by Levine *et al.*, 1992) were initially classified as gamma herpesviruses (see below) as they showed tropism for lymphocytes. However, on the basis of sequence homology and genetic relatedness to HCMV, they have recently been assigned to a different genus of beta herpesviruses, designated *Roseolovirus*. HHV-6 is the causative agent of exanthem subitum (roseola infantum) a common childhood disease characterised by a high fever and a skin rash. This virus has also been associated with a broad range of other illnesses including chronic fatigue syndrome. HHV-7 was isolated in 1990 from $CD4^+$ cells purified from peripheral blood mononuclear cells from a healthy individual (Frenkel *et al.*, 1990). It is molecularly and immunologically related to HHV-6 but the molecular divergence is sufficient to warrant that HHV-7 should not be considered a sub-type of HHV-6 but a separate herpesvirus. Little is known about this virus and its association with a specific disease has yet to be investigated.

Figure 1. Electron micrograph of a frozen hydrated HSV-1 virion

The major structural features are indicated: capsid (C), tegument (T), and envelope with associated glycoprotein spikes (E). The bar represents 50nm. This figure was reproduced with permission of W. Chiu, Baylor College of Medicine, Houston, Texas.

1.3 GAMMAHERPESVIRINAE

Viruses in this group have a genome of Mw. <110 x 10^6 and are specific for either T or B lymphocytes. In the lymphocyte, infection is frequently arrested at a pre-lytic stage with persistance and minimal expression of the viral genome in the cell, or at a lytic stage causing death of the cell without production of complete virions. Latent virus is frequently demonstrated in lymphoid tissue.

This sub-family comprises two genera. *Lymphocryptovirus* includes the B-cell trophic viruses such as the human pathogen Epstein-Barr virus (EBV), as well as several primate herpesviruses. EBV, also known as HHV-4, (reviewed by Miller, 1990) replicates lytically in epithelial cells of the throat before establishing latency in B lymphocytes. It is the agent responsible for infectious mononucleosis, more commonly known as glandular fever and it has also been associated in the aetiology of the malignancy, nasopharyngeal carcinoma and the lymphoblastoid tumour Burkitt's lymphoma. The second genus, *Rhadinovirus* includes T-cell tropic viruses such as herpesvirus saimiri which infects the squirrel monkey.

Recently, DNA sequences from what appears to be a new gamma herpesvirus has been identified in lesions of Kaposi's sarcoma (Chang *et al.*, 1994; Dupin *et al.*, 1995; Huang *et al.*, 1995). However, infectious virus still remains to be purified from these lesions.

1.4 UNCLASSIFIED HERPESVIRUSES

The recent sequencing of the entire genome of Channel Catfish Virus (CCV), which was previously thought to be an alphaherpesvirus as judged by its structural morphology and growth properties, detected no significant homology with any known herpesvirus (Davison, 1992). However, from recent cryoelectron microscopic studies on capsid morphology and identification of several putative gene homologues, it is now suggested that CCV shares a common evolutionary origin with the other herpesviruses but this virus still remains unassigned to a particular herpesvirus family (Davison and Davison, 1995).

2 THE STRUCTURAL FEATURES OF THE VIRION

The herpesvirus virion, as observed in the electron microscope, is composed of three separate components comprising nucleocapsid, tegument and envelope (figure 1). The DNA is contained within an icosahedral nucleocapsid which is surrounded by a layer of

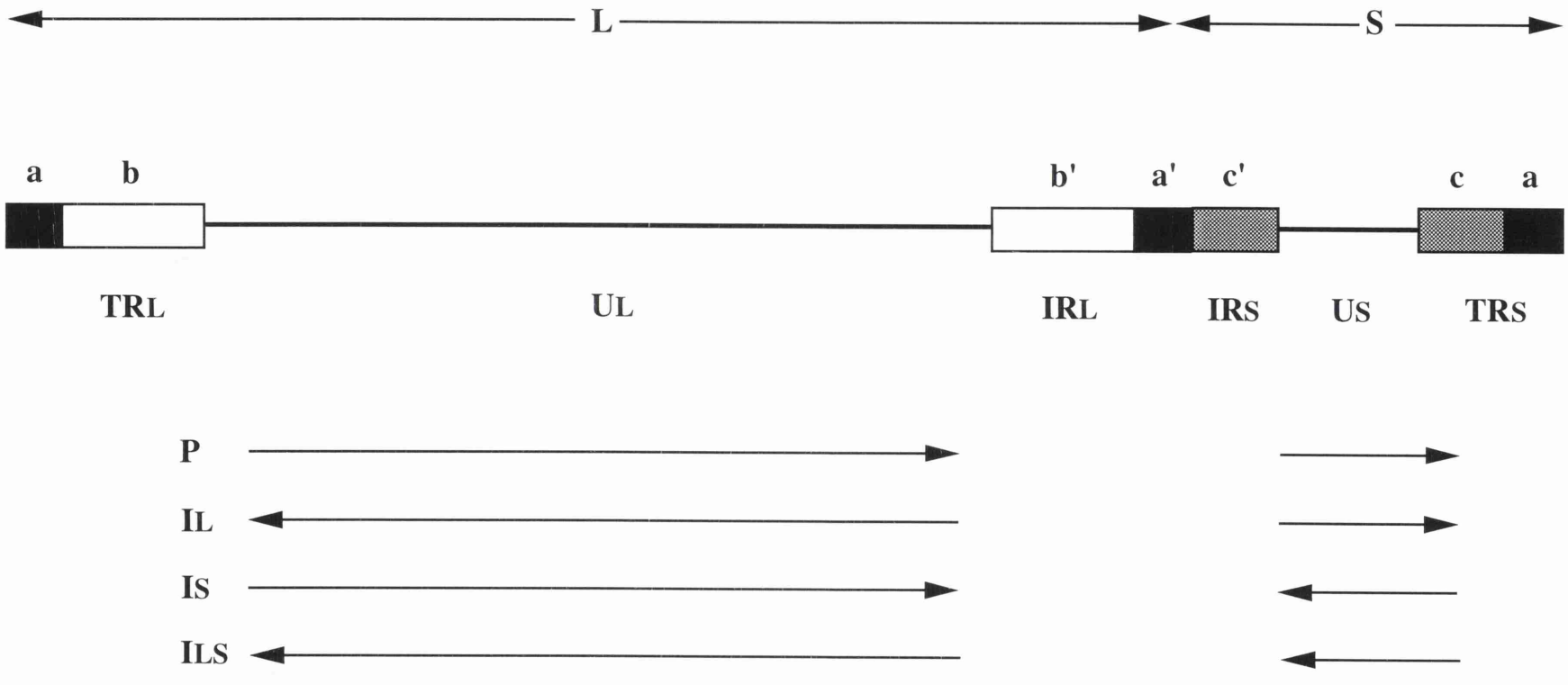
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Figure 2 Structure of the HSV-1 genome

The viral genome which consists of two covalently linked components L and S is shown. Each component contains unique sequences UL and US (solid lines) flanked by terminal and internal inverted repeat elements; TRL and IRL, TRS and IRS respectively (open and shaded boxes). A direct repeat, termed the *a* sequence, is present at each end of the genome and also in an inverted orientation at the L-S junction. Below the genome representation the isomerisation of the HSV-1 genome is illustrated. The four isomers which are present in equimolar amounts are: P (prototype), IL (inversion of L), IS (inversion of S) and ISL (inversion of both L and S).

amorphous material termed the tegument. The tegument is surrounded by a lipid bilayer membrane which contains numerous glycoprotein spikes (Wildy *et al.*, 1960).

The structure of the HSV-1 virion has been reviewed by Dargan (1986) and is discussed below. Particular attention will be paid to the structure of the capsid as this forms the basis of this thesis.

2.1 THE HSV-1 GENOME

The genome of HSV-1 consists of a double stranded (ds), linear DNA molecule of Mw. 96 x 10^6. The complete genome of HSV-1 strain 17 has been sequenced and consists of 152,260bp and has an overall G+C content of 68.3% (McGeoch *et al.*, 1985; 1986; 1988; Perry and McGeoch, 1988). This value varies for specific regions of the DNA molecule; eg. the short terminal repeat sequence has a G+C content of approximately 78% (Kieff *et al.*, 1972; Davison and Wilkie, 1981; Murchie and McGeoch, 1982).

The genome consists of two unique components designated unique long (UL) and unique short (US) which comprise 82 and 18% of the DNA respectively. Each is bounded and linked by a set of internal (IR) and terminal (TR) repeats; TRL/IRL and IRS/TRS which are also designated ab/b'a' and a'c'/ca (figure 2). A sequence which varies in length between 250-500bp (the *a* sequence) is present as a direct repeat at both termini and in an inverted orientation at the L-S junction (Sheldrick and Berthelot, 1974; Wadsworth *et al.*, 1976; Delius and Clements, 1976; Hayward *et al.*, 1975; Skare and Summers, 1977; Davison and Wilkie, 1981). In the course of infection the L and S components invert relative to each other such that the progeny viral DNA consists of equimolar amounts of four isomers differing from each other solely in the relative orientation of the two components. These isomers are termed: P (prototype), IL (inversion of L), IS (inversion of S) and ISL (inversion of both L and S) (Hayward *et al.*, 1975; Delius and Clements, 1976; Roizman, 1979).

Subsequent studies on a limited number of herpesviruses has established that the herpesvirus genome arrangements fall into six groups based on the presence and location of reiterated sequences containing at least 100 nucleotides (reviewed by McGeoch, 1989; Roizman and Sears, 1990; Roizman *et al.*, 1992).

2.1.1 The *a* sequence

The structure of the *a* sequence is highly conserved between the herpesviruses and can be divided into several elements which consist of both unique (U) and directly repeated (DR) regions. In HSV-1 strain F the structure of the *a* sequence can be represented as

$$DR1\text{-}U_b\text{-}DR2_n\text{-}DR4_m\text{-}U_c\text{-}DR1$$

where U_b and U_c consist of 65 and 58 base pair unique sequences and DR1, DR2 and DR4 are direct repeats containing 20, 12 and 37 base pairs respectively (Mocarski and Roizman, 1982). DR2 varies from 19 to 22 copies and DR4 from 2 to 3 copies per *a* sequence. The difference in size between *a* sequences is due to a variation in the copy number of these direct repeats and this variation exists both interstrain and intrastrain. DR1, which flanks each copy of an *a* sequence, is shared by adjacent *a* sequences. Thus, for every 2 copies of the *a* sequence there are 3 DR1 sequences. Linear virion DNA contains asymmetric ends with the terminal *a* sequence of the L component ending with 18 base pairs of DR1 and that of the S component ending with only one base pair of DR1. A single 3' residue overhang occurs at both genome termini. The number of *a* sequences at the L-S junction and at the L terminus is variable, although the S terminus only has a single *a* sequence (Wagner and Summers, 1978; Locker and Frenkel, 1979; Davison and Wilkie, 1981; Mocarski and Roizman, 1981, 1982).

The *a* sequence appears to mediate a number of important functions. It is involved in the inversion of the L and S segments, in circularisation of the DNA following infection and in site specific recombination (Davison, 1983; Poffenberger and Roizman, 1985; Mocarski and Roizman, 1981;1982). The role of the *a* sequence in the cleavage and packaging of the viral DNA will be discussed in detail later.

2.2 THE GENETIC CONTENT OF HSV-1

The 72 potential ORFs originally identified in the sequence of HSV-1 strain 17 were predicted to encode 70 proteins. One gene, UL15, is spliced and the intron contains both the UL16 and UL17 ORFs. Initially 56 ORFs, UL1-UL56, were identified within the UL region of the genome and 12 ORFs, designated US1-US12, within the US region while the immediate-early genes 110 and 175 (IE-110 and IE-175) were mapped to the RL and RS repeat sequences and are thus represented twice (McGeoch *et al.*, 1988).

10 additional ORFs have since been identified. Five of these were identified within the UL region. A second, in-frame transcriptional unit mapping within the UL26 ORF was designated UL26.5 (Liu and Roizman, 1991a) and a gene situated between the UL49 and UL50 ORFs was designated UL49A (Barker and Roizman, 1992; Barnett *et al.*, 1992) The UL8.5 ORF lies between and is 3'-co-terminal with the UL8 and UL9 ORFs and is predicted to encode a 486 amino acid protein designated origin binding protein C (Baradaran *et al.*, 1994). An ORF, designated UL12.5, has been identified as encoding a N-terminally truncated 60kDa protein which shares its carboxy terminus with the alkaline nuclease protein encoded by UL12 (Martinez *et al.*, 1996). The most recently identified ORF, UL43.5 maps antisense to the UL43 gene (Ward *et al.*, 1996). A

Gene	Protein	Proposed function	Status
ORF P[a]		Function unknown, only expressed in absence of functional ICP4	-
RL1	ICP34.5	Neurovirulence factor	ne
RL2	ICP0, Vmw110	IE tegument phosphoprotein; regulator of transcription	ne
UL1	gL	Envelope glycoprotein, involved in penetration of host cell membrane; complexes with gH	e
UL2		Uracil-DNA glycosylase	ne
UL3		Function unknown	ne
UL4		Function unknown	ne
UL5		Component of DNA helicase-primase complex; possesses helicase motifs	e
UL6[b]		Associated with capsid; functions in cleavage and packaging of viral DNA	e
UL7		Function unknown	-
UL8		Component of DNA helicase-primase complex	e
UL9		*Ori*-binding protein; DNA helicase	e
UL10	gM	Envelope glycoprotein; function unknown	ne
UL11[c]		Myristylated tegument protein; role in envelopment and transport of nascent virions	ne
UL12		Alkaline nuclease; role in maturation and packaging of viral DNA	e
UL12.5[l]		Function unknown	-
UL13[d]	VP18.8	Protein kinase, major substrate is the UL49 protein, VP22; involved in shut-off of host protein synthsis	ne
UL14		Function unknown	-
UL15[e]		Cleavage and packaging of viral DNA	e
UL16		Function unknown	ne
UL17		Function unknown	e?
UL18	VP23	Component of capsid triplexes	e
UL19	VP5	Major capsid protein; constitutes both the hexons and pentons	e
UL20		Integral membrane protein; role in egress of nascent virions	e/ne
UL21		Function unknown	-
UL22	gH	Envelope glycoprotein; forms hetero-oligomer with gL; involved in penetration of host cell membrane	e
UL23		Thymidine kinase	ne
UL24		Function unknown	ne
UL25		Cleavage and packaging of viral DNA; penetration of cells	e
UL26	VP24	Encoded by N-terminal portion of gene; serine protease which processes pre-VP22a	e
	VP21	Encoded by C-terminal portion of gene; involved in capsid assembly	
UL26.5[f]	VP22a	Processed form of the scaffolding protein of intermediate (B) capsids	e

Gene	Protein	Proposed function	Status
UL27	gB	Envelope glycoprotein, exists as a dimer; involved in penetration of host cell membrane and in cell fusion	e
UL28		Role in DNA packaging	e
UL29	ICP8	Single-stranded DNA binding protein	e
UL30		Catalytic subunit of replicative DNA polymerase; complexes with UL42 protein	e
UL31[g]		Nuclear phosphoprotein; function unknown	-
UL32		Function unknown	-
UL33		Cleavage and packaging of viral DNA	e
UL34		Membrane-associated phosphoprotein; substrate for US3 protein kinase	e
UL35	VP26	Present on tips of capsid hexons	-
UL36	VP1/2, ICP1/2	Tegument phosphoprotein; release of viral DNA from capsid; cleavage and packaging of DNA	e
UL37[h]		Tegument phosphoprotein; function unknown	-
UL38	VP19C	Component of capsid triplexes	e
UL39	ICP6/R1 Vmw136	Ribonucleotide reductase large subunit	e/ne
UL40	Vmw38/R2	Ribonuclease reductase small subunut	e/ne
UL41	vhs	Virion host shutoff protein; decreases stability of host mRNA; inhibits host cell translation	ne
UL42		Subunit of replicative DNA polymerses; increases processivity; complexes with UL30 protein	e
UL43		Probable integral membrane protein; function unknown	ne
UL43.5[k]		Possible accessory role in assembly of viral particles	ne
UL44	gC/VP8	Envelope glycoprotein; role in attachment to host cells	ne
UL45[i]		Membrane protein; mediator of cell fusion	ne
UL46	VP11/12	Tegument phosphoprotein; modulates activity of αTIF (UL48)	ne ne
UL47	VP13/14	Tegument phosphoprotein; modulates activity of αTIF (UL48)	ne ne
UL48	VP16/αTIF/ Vmw65	Tegument protein; transactivates IE genes	e
UL49	VP22	Basic tegument phosphoprotein	-
UL49A		Possible membrane glycoprotein	e?
UL50		Deoxyuridine triphosphatase (dUTPase)	ne
UL51		Function unknown	e/ne
UL52		Component of DNA helicase-primase complex	e
UL53	gK	Envelope glycoprotein; involved in cell fusion	ne
UL54	ICP27/Vmw63	IE protein; post-translational regulator of gene expression	e
UL55		Function unknown	ne
UL56		Function unknown	ne
LAT		Family of transcripts, some extending into RS, expressed in latency; function unknown; protein coding capacity uncertain	ne

Gene	Protein	Proposed function	Status
RS1	ICP4/Vmw175	IE tegument phosphoprotein; regulator of transcription	e
US1	ICP22/Vmw68	IE protein; function unknown	e/ne
US2		Function unknown	ne
US3		Protein kinase; phosphorylates UL34 protein	ne
US4	gG	Envelope glycoprotein; function unknown	ne
US5	gJ	Envelope glycoprotein; function unknown	ne
US6	gD/VP18	Envelope glycoprotein; involved in penetration of host cell membrane; involved in neuroinvasion	e
US7	gI	Envelope glycoprotein; complex with gE forms receptor for Fc of IgG	ne
US8	gE	Envelope glycoprotein; complex with gI forms receptor for Fc of IgG	ne
US8.5[j]		Function unknown	-
US9		Phosphorylated tegument protein; function unknown	ne
US10		Virion protein; function unknown	ne
US11		Basic protein; binds to RNA, associated with the 60S ribosomal subunit	ne
US12	ICP47/Vmw12	IE protein; function unknown	ne

TABLE 1: THE GENES AND PROTEIN PRODUCTS OF HSV-1 STRAIN 17

Where appropriate, the more commonly used names for the proteins are given; this is not a comprehensive list due to the many different designations given to HSV-1 proteins: virion protein (VP), immediate early proteins (IE), infected cell polypeptides (ICP), apparent molecular weight of HSV-induced polypeptide, latency associated transcript(LAT).

The status of genes known to be essential for virus growth in cell culture is indicated as essential (e) and non-essential (ne). e? indicates that the data regarding status is not conclusive. e/ne indicates that necessity depends on culture conditions or temperature.

References are ommitted because of space constrictions; recently referenced lists of HSV-1 functions have been published (McGeoch; 1989; McGeoch and Schaeffer, 1993; Haarr and Skulstad, 1994). Recent references not included in these reviews: [a]Lagunoff and Roizman (1994); [b]Patel and MacLean (1995); [c]Baines *et al.* (1995); [d]Coulter *et al.*, 1993; Purves *et al.*, 1993; Overton *et al.* (1994); [e]Poon and Roizman (1993); [e]Baines *et al.* (1994); [f]Matusick-Kumar *et al.* (1994); [g]Chang and Roizman (1993); [h]Schmitz *et al.* (1995); [i]Haanes *et al.*, (1994); [j]Georgopoulou *et al.* (1993); [k]Ward *et al.* (1996); [l]Martinez *et al.* (1996b).

thirteenth ORF in the US region of the genome lies between and overlaps in part with the US8 and 9 ORFs and has been designated US8.5 (Georgopoulou *et al.*, 1993). A second ORF within RL was first described for HSV-1 strain F and encodes the protein ICP34.5 (Chou and Roizman, 1986; Chou *et al.*, 1990). The presence of this gene in strain 17 was confirmed by Dolan *et al.* (1992). An ORF of 248 codons, ORF P that is expressed under conditions in which ICP4 is not functional, has been identified that is coincident with but antisense to the ICP34.5 gene (173 codons); only 8 codons of ORF P are not antisense to ICP34.5 which has 23 codons that are not antisense to ORF P (Lagunoff and Roizman, 1994). Both these genes are represented twice. Thus this brings the total number of unique genes to 78.

HSV-encoded proteins play a role in regulation of transcription, DNA replication and in virion structure and assembly. Many of these are dispensable for growth of the virus in tissue culture and a number of the gene products and functions remain to be characterised. The present understanding of the genes and their functions has been reviewed (McGeoch *et al.*, 1993; Haarr and Skulstad, 1994) and is summarised in table 1.

2.3 THE ENVELOPE

The envelope which is composed of lipids, has a mean diameter of 180nm and exhibits spikes 5 to 14nm long projecting from its suface. These spikes consist of glycoproteins that are present in structures that differ in size, morphology and distribution in the envelope (Wildy *et al.*, 1960; Asher *et al.*, 1969; Ben-Porat and Kaplan, 1971; Stannard *et al.*, 1987; Szilagyi and Berriman, 1994).

The envelope is thought to be acquired by virus particles budding across areas of the inner nuclear membrane, which have been altered after infection of the cell (Darlington and Moss, 1968; Ben-Porat and Kaplan, 1971). However an alternative pathway has been proposed in which enveloped capsids are deenveloped in the perinuclear space (reviewed in Rixon, 1993). Final modification of the envelope glycoproteins are thought to take place by budding through the golgi apparatus (van Genderen *et al.*, 1994).

Eleven glycoproteins, designated gB to gM (with the exception of gF), have been identified to date (reviewed by Spear, 1993; Haarr and Skulstad, 1994). Originally, a glycoprotein, gA, was described that was present in infected cells but absent from virions (Spear, 1976) but it has since been shown that gA represents a form of gB, differing in the degree of glycosylation and that both gA and gB are synthesised from a common precursor (Eberle and Courtney, 1980). Balachandran *et al.* (1981) detected a new glycoprotein, designated gF, in HSV-2. However, since this protein was

determined to be the homologue of gC in HSV-1, it was later named gC-2 (Zezulak and Spear, 1984). None of these glycoproteins are known to be required for virion assembly although four (gB, gD, gH and gL) are essential for infectivity.

Glycoprotein B (gB) is encoded by the HSV-1 gene UL27 (Bzik *et al.*, 1984; DeLuca *et al.*, 1984; McGeoch *et al.*, 1988). It is synthesised as monomers and is converted to oligomers postranslationally; being present as dimers both in infected cells and in the virion (Sarmiento and Spear, 1979; Highlander *et al.*, 1991). Identification of *ts* mutants (Sarmiento *et al.*, 1979; Haffey and Spear, 1980; Little *et al.*, 1981) and the construction of null mutants (Cai *et al.*, 1987; 1988) that do not express gB have demonstrated that this protein is not essential for production of enveloped virions although it is essential for infectivity. gB plays a role in fusion of the virion to the host cell membrane since virions of mutants lacking gB were able to absorb to the host cell membrane but could only infect the cell after treatment with PEG; a membrane fusion agent. Further evidence for the role of gB in membrane fusion and penetration arises from the identification of several mutants that either induce cell fusion (syncytial mutants) or enter cells more rapidly (Manservigi *et al.*, 1974; Cai *et al.*, 1988). Antibodies mapping to different domains of gB blocked one or other of these functions demonstrating that fusion and penetration are characterised by different domains (Navarro *et al.*, 1992).

Glycoprotein C (gC) is encoded by the UL44 gene (Frink *et al.*, 1983; McGeoch *et al.*, 1988). It is not required for the production of infectious virions (Draper *et al.*, 1984) and gC negative mutants have been isolated from humans (Hidaka *et al.*, 1990; 1991). gC has been shown to bind to heparin sulphate moieties (Trybala *et al.*, 1993); a process which is involved in the attachment of virus to cells (WuDunn and Spear, 1989; Shieh *et al.*, 1992). gC also appears to have a role in modifying infection by regulating membrane fusion (Manservigi *et al.*, 1977) and mAbs against gC were found to inhibit adsorption (Fuller and Spear, 1985). HSV-1 gC, but not HSV-2 gC, acts as a receptor for the C3b component of complement (Friedman *et al.*, 1984) thus modulating the immune response by protecting against complement-mediated neutralisation and thereby decreasing the efficiency with which infected cells are lysed. Four distinct C3b-binding regions of HSV-1 gC have been identified (Hung *et al.*, 1992).

Glycoprotein D (gD) is encoded by the US6 gene (Watson *et al.*, 1982; McGeoch *et al.*, 1988) and is essential for virion infectivity (Ligas and Johnson, 1988; Johnson and Ligas, 1988). gD has been suggested to have a role in the rounding up of infected cells Norrild *et al.* (1983) and has also been implicated in receptor-binding (Johnson *et al.*, 1984) and in adsorption (Fuller and Spear, 1985). The major role for this protein appears to be in the penetration of host cells since gD negative mutants were able to adsorb to cells but could not penetrate without the addition of PEG. (Johnson and Ligas, 1988; Ligas and Johnson, 1988). Expression of HSV-1 gD renders cells resistant to infection with HSV-1 (Campadelli-Fumi *et al.*, 1988; Johnson and Spear, 1989) as

does pre-treatment of cells with either HSV-1 or -2 soluble gD (Johnson *et al.*, 1990) Similarly, mAb to gD inhibited penetration at the level of fusion (Noble *et al.*, 1983; Highlander *et al.*, 1987).

Glycoprotein E (gE) has been mapped to the US8 ORF (Lee *et al.*, 1982; Para *et al.*, 1982b; McGeoch *et al.*, 1988) and is dispensible for virus growth in tissue culture (Longnecker *et al.*, 1987). It has been shown to function as a receptor for the Fc portion of IgG (Baucke and Spear, 1979; Para *et al.*, 1980;1982a).

Glycoprotein I (gI) is encoded by the US7 gene and is dispensible for virus growth in tissue culture (McGeoch *et al.*, 1985; Longnecker *et al.*, 1987; Johnson *et al.*, 1988). gI forms a complex with gE on the surface of infected cells to form a Fc receptor of much higher affinity for IgG than either gE or gI alone (Johnson and Feenstra, 1987; Johnson *et al.*, 1988a; Bell *et al.*, 1990).

Glycoprotein G (gG) has been identified as the product of US4 and is not essential for virus infectivity (McGeoch *et al.*, 1985; 1987; Ackerman *et al.*, 1986; Richman *et al.*, 1986). The function of this protein remains to be determined although it appears to be a target for antibody-mediated complement-dependent virus neutralisation (Sullivan and Smith, 1987).

Glycoprotein H (gH) was first described by Buckmaster *et al.* (1984) and is encoded by the UL22 gene (Gompels and Minson, 1986; McGeoch *et al.*, 1988). gH negative mutants are able to attach to cells but are unable to penetrate demonstrating that gH is essential for infectivity via membrane fusion (Desai *et al.*, 1988; Forrester *et al.*, 1992). Virus particles treated with anti-gH will bind to cells but will not penetrate thus neutralising virion infectivity (Buckmaster *et al.*, 1984; Fuller *et al.*, 1989).

Glycoprotein L (gL) which is encoded by the gene UL1 (Hutchinson *et al.*, 1992a) forms a hetero-oligomer with gH. It is essential for infectivity as, like gH, it is involved in cell fusion and penetration (Roop *et al.*, 1993). gH and gL are dependent on each other for normal folding and posttranslational folding and are transported to the cell surface as a heterodimer (Gomples and Minson, 1989; Foa-Tomasi *et al.*, 1991; Forrester *et al.*, 1991; Roberts *et al.*, 1991; Hutchinson *et al.*, 1992a)

Glycoprotein K (gK) is encoded by the UL53 gene and is not essential for virus infectivity (Hutchinson *et al.*, 1992b; Ramaswamy and Holland, 1992). It is thought to play a role in regulating membrane fusion since syncitial mutants have been mapped to this region (Ruyechan *et al.*, 1979; Bond and Person, 1984; Pogue-Geile *et al.*, 1984).

Glycoprotein M (gM) encoded by the UL10 gene (Baines and Roizman, 1993), is the most recently identified glycoprotein. Its function remains to be characterised although it is non-essential for growth in tissue culture (Baines *et al.*, 1991; MacLean *et al.*, 1993).

Glycoprotein J (gJ) is predicted to be encoded by the US5 ORF (McGeoch *et al.*, 1993) but as yet the identity of this protein has not been confirmed. This gene is non-essential for virion assembly.

2.4 THE TEGUMENT

The HSV tegument appears in electron micrographs as an amorphous layer between the envelope and nucleocapsid and accounts for over 50% of the volume of the virion. The thickness, which varies amongst different herpesviruses, is genetically determined (Nazerian and Witter, 1970; McCombs *et al.*, 1971). The tegument is the most poorly characterised part of the virion and proteins are usually assigned to the tegument on the basis that they are neither capsid nor envelope proteins.

The tegument of HSV-1 comprises at least 15 virus-encoded proteins and many of the genes specifying these polypeptides have been identified. These include: VP1/2 (UL36), VP11/12 (UL46), VP13/14 (UL47), VP16 (UL48), VP22 (UL49), two phosphoproteins encoded by US9 and UL37, a protein kinase (UL13), virion host shut-off (vhs) protein (UL41), a myristylated protein (UL11) and, depending on cell type, the IE proteins Vmw175 (RS1) and Vmw110 (RL2). Although the functions of many tegument proteins are not clear several of them, in addition to being structural components, have regulatory functions which start immediately after infection to enhance the yield of the lytic cycle (reviewed by Haarr and Skulstad, 1994). The only known tegument protein that is required for virion assembly is VP16 which is a structural protein that *trans*-induces HSV-1 IE gene transcription (Post *et al.*, 1981; Batterson and Roizman, 1983; Campbell *et al.*, 1984) and is required for viral gene expression and replication (Ace *et al.*, 1988; 1989). A second function in virion assembly has been reported for this protein after the observation that several deletion mutants had a reduced ability to encapsidate DNA and produce mature virions (Weinheimer *et al.*, 1992).

The assembly of the tegument does not appear to be totally controlled as demonstrated by the number of proteins which can be dispensed with for growth in tissue culture (table 1). This supports the view that many tegument proteins have a role as auxillary helper functions rather than as essential structural proteins. Leslie *et al.* (1996) have determined that the abundance of the major tegument protein VP22 (UL49) in the tegument can be augmented by increasing its level of expression. This however resulted in a decrease in the VP13/14 protein demonstrating that the amount of protein incorporated into the tegument is finite but not necessarily specific. Conversely, increasing levels of synthesis of the UL37 tegument protein had no effect on the level of its incorporation (J. McLauchlan; personal communication).

PROTEIN	GENE	APPROX Mw. (kDa)	PRESENCE IN CAPSIDS	LOCATION IN CAPSID
VP5	UL19	155	A, B, C	Hexamers & pentamers
VP19C	UL38	53	A, B, C	Triplexes
VP23	UL18	33	A, B, C	Triplexes
VP26	UL35	12	A, B, C	Tips of hexomers
VP22a	UL26.5	38	B	Internal Scaffold
VP21	UL26*	42	B	Internal
VP24	UL26*	25	A, B, C	Internal

Table 2:- The capsid proteins of HSV-1

*The product of UL26 is a protease which self cleaves to generate VP21 and VP24

The structural integrity of the tegument has been shown to be independent of either the capsid or the envelope (McLauchlan and Rixon, 1992). Thus when L-particles (described below) were treated with detergent to remove the envelope, the residual tegument material retained its shape and stability.

2.4.1 Light particles

Non-infectious particles, termed L-particles, have been isolated from HSV-1-infected cells (Szilagyi and Cunningham, 1991). These consist solely of envelope and tegument and can be produced in the absence of virion maturation (Rixon *et al.*, 1992). They are able to attach to and fuse with cells but due to their lack of DNA they are unable to initiate infection (McLauchlan *et al.*, 1992). L-particles appear to contain the viral-encoded transactivator protein Vmw175 which is not found in virions made in certain cell types (Szilagyi and Cunningham, 1991; McLauchlan and Rixon, 1992; Yang and Courtney, 1995). This may suggest that these particles could serve to provide an additional reservoir of helper proteins to enhance the efficiency of infection. The quantity of L-particles produced is dependent on cell type (Yang and Courtney, 1995) and it is not yet known whether they are produced during the course of natural infection.

2.5 THE HSV CAPSID

Three types of capsids which differ in their protein composition and DNA content have been isolated from infected cells (Gibson and Roizman, 1972; Cohen *et al*, 1980). The nomenclature of capsids has been confused and as a result capsids have been given several different names. Initially, capsids isolated from nuclei were named A (top) and B (bottom) bands corresponding to their relative positions after sedimentation on sucrose gradients. B capsids were originally described as containing 10x as much DNA as A capsids and having two structural proteins, VP21 and VP22a, absent from A capsids. A third category of capsid, designated type C, was obtained by removal of the envelope and tegument from virions isolated from the cytoplasm and these banded at a position below B capsids. C capsids contain DNA as well as all the A capsid proteins (table 2) (Gibson and Roizman, 1972). C capsids have subsequently taken on a broader definition as capsids containing the genome. Electron microscopy of infected cells revealed the presence of 3 capsid types and provided further information as to the identity of the different capsid types. Empty (type A) capsids lack internal structure whereas intermediate (type B) capsids have a proteinaceous core (comprising of VP22a) which is not found in empty or full capsids. Full (type C) capsids contain the viral DNA. B capsids were at first described as comprising 6 proteins (Gibson and Roizman; 1972) until Heilman *et al.* (1979) identified a seventh capsid protein p12 which is also

present in type A and C capsids. This protein is now referred to as VP26 (Newcomb and Brown; 1991). The nomenclature of the capsid proteins follows that of their first description by Spear and Roizman (1972) and Gibson and Roizman (1972) when they were preceded with VP (virion protein) and numbered in order of decreasing size as determined by electrophoresis of virions. The protein composition of each capsid type is shown in table 2 and their properties and the genes encoding them are discussed in turn as is their involvement in the capsid assembly process.

2.5.1 Proteins of the capsid

i) VP5

The 155kDa structural protein VP5 (Spear and Roizman, 1972) was identified as a componant of the capsid by Gibson and Roizman (1972) and has since been referred to by a number of different names by other workers. These include: ICP5 (Honess and Roizman; 1973), VP1 (Powell and Watson, 1975), ICP155 (Powell and Purifoy; 1976), Vmw155 (Marsden *et al.*, 1976), p155 (Zweig *et al.*, 1979b), NC-1 (Cohen *et al.*; 1980) and p5 (Newcomb *et al.*, 1989). VP5, which accounts for 60-70% of the capsid mass of type A capsids (Gibson and Roizman, 1972, Newcomb *et al.*, 1989) is generally referred to as the major capsid protein (MCP). This protein is non-glycosylated (Heine *et al.*, 1974) and the HSV-2 counterpart has been suggested to be linked by disulphide bonds to VP19C (Zweig *et al.*, 1979a). VP5 of HSV-1 and -2 have been implicated in DNA binding (Powell and Purifoy, 1976; Purifoy and Powell, 1976). Other researchers were unable to determine any DNA binding activity of this protein (Bayliss *et al.*, 1975; Blair and Honess, 1983; Braun *et al.*, 1984a) and it seems highly unlikely in view of its known function. A VP5 null mutant virus (Desai *et al.*, 1993) and a temperature sensitive mutant *ts*G8 (Weller *et al.*, 1987) which failed to replicate on non-complementing cells were unable to form capsids. Although normal amounts of DNA were produced, mutant viruses were not able to process concatamers into genome unit length molecules. The fact that VP5 is an essential protein is consistent with its role as the major protein in the capsid.

The location of the major capsid protein gene (UL19) was originally determined by Morse *et al.* (1978) and Marsden *et al.* (1978) from studies of intertypic recombinants between HSV-1 and HSV-2. Costa *et al.* (1981) characterised a 6kb leftward orientated mRNA which mapped in this region and which had previously been determined to be the most abundant transcript associated with polyribosomes at late times in infection (Anderson *et al.*, 1979). The 6kb mRNA maped between 0.231 and 0.266 map units and was unspliced (Costa *et al.*, 1981). When this mRNA was translated *in vitro* it produced a protein with an apparent molecular weight of 155kDa which reacted with a

polyclonal antibody to the major capsid protein, VP5 (Costa *et al.*, 1984) thereby confirming the location of the VP5 ORF; designated UL19 in the nomenclature of McGeoch *et al.*, 1888). According to this numbering of the HSV-1 sequence the 5' end of the UL19 mRNA is at position 40768 and there is a TATATAA promoter sequence beginning at 40796 (Costa *et al.*, 1984; Dennis and Smiley, 1984; Davison and Scott, 1986b). Transcription terminates at one of two AATAAA polyadenylation sequences beginning at positions 35032 and 36028 respectively. There is a possible third polyadenylation sequence at 36405 which may result in the polyadenylation of a minor proportion of the VP5 transcript (Costa *et al.*, 1984; Davison and Scott, 1986b; McGeoch *et al.*, 1988). The promoter and other sequences influencing transcription (eg. TATA box, SP1-binding site and *cis*-acting elements) are further discussed by Blair and Wagner (1986) and Huang and Wagner (1994). The initiation codon for the start of translation of VP5 is at position 40528 and the stop codon, TAA at position 36406 resulting in an ORF of 4122bp (McGeoch *et al.*, 1988). The predicted molecular weight of the product of UL19 is 149,075 Da which is in close agreement to the observed size of VP5 made in HSV-1 infected cells (Gibson and Roizman, 1972; Killington et *al.*, 1977; Zweig *et al.*, 1979b; Cohen *et al.*, 1980; Rixon *et al.*, 1990) and suggests that extensive posttranslational modification does not occur. UL19 is widely regarded as an early-late ($\beta\gamma$) gene since the production of UL19 mRNA is greatly reduced in the presence of inhibitors of DNA synthesis (Harris-Hamilton and Bachenheimer, 1985) and only reduced levels of the VP5 protein are made in cells infected with a DNA$^-$ *ts* mutant at the NPT (Conley *et al.*, 1981). Thus the expression of UL19 is increased by viral DNA replication although it is not dependent on it. Regulatory elements important in maintaining the $\beta\gamma$ kinetics of expression of UL19 are discussed by Huang *et al.* (1993).

ii) VP19C

Gibson and Roizman (1972) first identified a 53,000 molecular weight capsid protein which they named VP19 after the virion protein of the same name which had an identical electrophoretic mobility (Spear and Roizman, 1972). This protein was later renamed VP19C to distinguish it from a glycosylated protein which co-migrated with the capsid protein (Heine *et al.*, 1974). VP19C has also been referred to as ICP31 (Honess and Roizman, 1973); Vmw53 (Marsden *et al.*, 1976); p50 (Zweig *et al.*, 1979a); NC-2 (Cohen *et al.*, 1980). VP19C is non-glycosylated (Heine *et al.*, 1974) and has been shown to be linked by disulphide bonds to VP5 in HSV-2 (Zweig *et al.*, 1979a). VP19C of both HSV-1 and -2 has been implicated in DNA binding (Braun *et al.*, 1984b). This has been contested by Nicholson (1992) who was unable to determine DNA binding activity in VP19C expressed by a recombinant vaccinia virus.

There has been a lot of confusion as to the location of the gene encoding VP19C. In the first instance Costa *et al.* (1983) suggested that this gene was located between 0.16 and 0.19 map units of the viral genome. This group had used S1 nuclease analysis to map four mRNAs to this region and had subsequently determined that a polyclonal antiserum against VP19C reacted weakly with one of the *in vitro* translated products of these mRNAs. Their tentative suggestion that the VP19C mRNA mapped in this region was based on their incorrect interpretation of the work of Lemaster and Roizman (1980) who by using intertypic recombinants had mapped the gene encoding a 50,000 molecular weight structural protein to between 0.15 an 0.18 map units. Lemaster and Roizman (1980) designated this protein as VP18.8 and suggested that it might be a phosphorylated form of a polypeptide which co-migrated with VP19C in virion protein profiles but which was readily distinguished from the capsid protein VP19C. From studies of polypeptides made by HSV-1/-2 recombinants, Braun *et al.* (1984a) suggested that VP19C was derived from a DNA-binding protein ICP32 and also mapped within the coding region (between 0.58 and 0.60 map units) specifying ICP32. Pertuiset *et al.* (1989) investigated the properties of a temperature sensitive mutant (*ts*2) which neither assembled capsids nor processed replicated DNA into unit-length molecules at the NPT. This mutant mapped within the region 0.553-0.565 map units which was designated ORF.553 and which was predicted to encode a polypeptide of molecular weight 50,175Da. Sequencing within this region determined that ORF.553 corresponded to the ORF UL38 described by McGeoch *et al.* (1988) and placed the gene product on the other side of the ribonucleotide reductase complex than had previously been described for VP19C (Braun *et al.*, 1984a). However, the protein encoded by ORF.553 was not identified which made it difficult for the authors to conclusively determine a role for the ORF.553 gene product in capsid morphogenesis; whether it was a integral capsid component or a non-structural protein required for capsid assembly. The possibility of this protein being VP19C was ruled out in light of the results of Braun *et al.*, (1984a). Further characterisation of the product of the UL38 ORF/ORF.553 was obtained by Yei *et al.* (1990) who identified the map position of the HSV-2 homologue of this gene. Antisera directed against this region reacted with both HSV-1 and -2 VP19C/ICP32 polypeptides in infected cells which suggested that the UL38 ORF/ORF.553 encoded the VP19C capsid protein. The genes suggested to encode ICP32 (HSV-2) and VP19C (HSV-1) share 78% amino acid homology (Yei *et al.*, 1990). The identity of the gene encoding VP19C was not finally resolved until Rixon *et al.* (1990) approached the problem from the opposite direction and sequenced the amino-terminus of VP19C purified from capsids. The sequence obtained aligned precisely with the amino terminus of the UL38 ORF described McGeoch *et al.* (1988) and thus confirmed that VP19C was the protein product of the UL38 ORF. This was later confirmed by Davison *et al.* (1992) who determined the amino acid sequence of fragments of VP19C which had been generated from partial cleavage by CNBr.

The UL38 gene is contained within the HindIII *k* fragment which is located between 0.527 and 0.592 map units (Wilkie, 1976; Skare and Summers, 1977; Anderson *et al.*, 1981). UL38 is transcribed from left to right on the prototype arrangement of the DNA; the translation initiation codon is at position 84531 and the TGA stop codon at position 85926 (McGeoch *et al.*; 1988). The 1395 ORF is predicted to encode a protein with a molecular weight of 54,000Da. The observed sizes of VP19C: 53,000Da (Gibson and Roizman, 1972; Zweig *et al.*, 1979a; Cohen *et al.*, 1980);. 50,000Da (Zweig *et al.*, 1979a) and 54,000Da (Rixon *et al.*, 1990), suggest that extensive posttranslational modification does not take place. Yei *et al.* (1990) have reported that the electrophoretic mobility of HSV-2 VP19C increases as infection progresses suggesting that this protein is processed to the smaller form which has been identified as a component of the capsid. No change in electrophoretic mobility of the HSV-1 VP19C has been observed. Anderson *et al.* (1980; 1981) identified a 54kDa polypeptide that was encoded by both a 7kb mRNA and a 1.9kb mRNA and mapped these mRNAs to between 0.552-0.598 and 0.552- 0.563 map units respectively. These workers also determined that neither species contained introns. The UL38 promoter has been characterised. Flanagan *et al.* (1991) determined that both the mRNAs initiate at position 84373 and that only 45 bases of the DNA sequence 5' of the UL38 mRNA cap is required to direct reporter gene expression in recombinant virus; as few as 30 bases of the 5' sequence were necessary for promoter activity. The 5' ends of the mRNAs are located 16bp 3' of a possible TATA homology (TATA) and 28bp 3' of a more striking homology (TTTAAA). Guzowski and Wagner (1993) have further characterised the promoter and have identified other regulatory elements (such as a downstream activation sequence) required for full levels of transcription. Possible polyadenylation sites (AATAAA) have been identified beginning at positions 86016 and 90983 (McGeoch *et al.*, 1988). It is now recognised that the UL38 gene is encoded by the 1.9kb mRNA transcript and that the 7kb mRNA represents a transcriptional readthrough product (Flanagan *et al.*; 1991). The polyadenylation site at 90983 marks the shared 3' termini of the UL39 and UL40 genes which are transcribed from 5.0 and 1.2kb mRNAs respectively (McLauchlan and Clements, 1983; McGeoch *et al.*, 1988). The UL38 ORF has been designated as a true late (γ_2) gene and as such depends on viral DNA synthesis for expression (Anderson *et al.*, 1981, Holland *et al.*, 1980; Yei *et al.*, 1990). However, capsids are made by mutants defective in production or processing of DNA (Schaffer *et al.*, 1974; Sherman and Bachenheimer, 1987;1988; Weller *et al.*, 1987).

iii) VP23

A 33,000 molecular weight capsid protein was described by Gibson and Roizman (1972) and was named after its virion counterpart VP23 (Spear and Roizman (1972). Other workers have referred to this protein as: ICP40 (Honess and Roizman, 1973); Vmw37 (Marsden *et al.*, 1976); p38 (Marsden *et al.*, 1978); p32 (Zweig *et al.*, 1979b;

NC-5 (Cohen *et al.*, 1980) and ICP39 (Braun *et al.*, 1984a). There has been confusion as to the nature of VP23. Lemaster and Roizman (1980) determined that VP23 was phosphorylated by the HSV-1 protein kinase which they found to partition with capsid-tegument structures. That this bound phosphate could be transferred *in vitro* suggested a role for VP23 as either a component of, or a substrate of the protein kinase. This idea was substantiated by Preston and Notarianni (1983) who identified a 38kDa protein with a similar electrophoretic mobility to VP23 that was poly-ADP-ribosylated; a modification which has been suggested to affect protein kinase activity. Subsequently Braun *et al.* (1984a) determined that VP23 (ICP39), which migrated as two species on two-dimensional gel electrophoresis and was not phosphorylated, had been confused with a protein of similar electrophoretic mobility but which consisted of numerous phosphorylated forms that differed in their isoelectric properties. This protein, which is a component of the tegument, has been named ICP39.3 to distinguish it from the capsid protein VP23.

Rixon *et al.* (1990) purified VP23 from capsids and by sequencing the N-terminal amino acids determined that VP23 was encoded by the gene UL18. This gene is leftward orientated; the initiation codon for the start of translation of UL18 is at position 36051 and the stop codon, TAA, at position 35097. The size of the ORF is 954bp and is predicted to encode a protein of molecular weight 34,268Da (McGeoch *et al.*, 1988). Since this is in close agreement with the estimated size of the protein in purified capsids: 34kDa (Rixon *et al.*, 1990), 32kDa (Zweig *et al.*, 1979b) and 33kDa (Gibson and Roizman, 1972; Cohen *et al.*, 1980) it is unlikely that VP23 is extensively modified post-translationally. Costa *et al.* (1984) detected a 1.5 kb mRNA which was colinear with the 3' end of the 6kb (UL19) mRNA but whose product, a 35kDa protein, was non-reactive with the VP5 antiserum. This mRNA is unspliced and it initiates at approximately position 36250 and like the 6kb mRNA terminates at either the polyadenylation site at position 35032 or that at 36028 (Costa *et al.*, 1984; McGeoch *et al.*, 1988). The UL18 mRNA was reported to be regulated with early-late ($\beta\gamma$) kinetics in lytic infections (Costa *et al.*, 1985). This has been confirmed by Nicholson (1992) who determined that VP23 was produced at late times in infection and that its production was independent of DNA synthesis.

The construction of a null mutant which did not express VP23 demonstrated that this protein is essential for capsid assembly (Desai *et al.*, 1993).

iv) VP26

A protein of this size (12,000Da) was not detected in capsids by Gibson or Roizman (1972) nor was it detected in virions (Spear and Roizman, 1972). It was first recognised as a component of capsids by Heilman *et al.* (1979) and was named p12 by virtue of its

size. Cohen *et al.* (1980) named this protein NC-7 and Newcomb and Brown (1991) have since designated it VP26.

Davison *et al.* (1992) purified VP26 from capsids and by sequencing fragments produced by CNBr partial digestion mapped this protein to the UL35 ORF. This gene was predicted to encode a protein of molecular weight 12,095 (McGeoch *et al.*, 1988) which is in close agreement with the 12,000 molecular weight of VP26 determined by SDS-PAGE of purified capsids (Zweig *et al.*, 1979b; Heilman *et al.*, 1979; Cohen *et al.*, 1980; Rixon *et al.*, 1990). McNabb and Courtney (1992a) independently determined that VP26 was the product of the UL35 gene. This group constructed a UL35 fusion protein which cross-reacted with an antiserum against NC-7 (Cohen *et al.*, 1980). Antiserum produced against this chimeric protein recognised a 12,000 molecular weight protein associated with capsids. This gene designation for VP26 does not agree with the earlier suggestion that VP26 is encoded by the UL49.5 gene (Barker and Roizman, 1992) which probably encodes a transmembrane protein (Barnett *et al.*, 1992). McNabb and Courtney (1992b) determined from computer analysis of the amino acid sequence of UL35 that the gene product would have a pI of 11.6. This would suggest that VP26 is equivalent to the basic protein BP2 found associated with chromatin (Knopf and Kaerner, 1980) and the 12,000Da DNA binding protein, BP15, described by Bayliss *et al.* (1975). VP26 is non-glycosylated but is subject to posttranslational phosphorylation at serine and threonine residues. VP26 can be resolved by acid-urea electrophoresis into three electrophoretically distinct species designated a-c; the faster migrating species c represents the unphosphorylated precursor of the a and b forms (Knopf and Kaerner, 1980; McNabb and Courtney, 1992b). It has been suggested that the greater abundance of VP26, in particular species b and c, in virions than in isolated capsids may be explained by the DNA binding properties of this protein since the less basic nature of these forms may provide a stronger affinity for DNA present in the virion (McNabb and Courtney, 1992b). Cell fractionation and immunofluoresence studies have determined that VP26 is localised to specific regions in the nucleus of HSV-1 infected cells. It has not been determined whether or not these regions are associated with the viral DNA (McNabb and Courtney, 1992b).

The 336bp UL35 ORF is transcribed from left to right on the prototype arrangement of HSV-1 DNA and is located on the KpnI *c* restriction fragment (Preston *et al.*, 1978; McGeoch *et al*, 1988). The translation initiation codon is located at position 70566 and the TGA stop codon at position 70902 and corresponds to the region encompassing 0.463-0.465 map units (McGeoch *et al.*, 1988). Analysis of HSV-1 transcripts located within this region of the viral genome suggest that UL33, UL34 and UL35 are transcribed late in infection as a nested set of 3' co-terminal mRNAs (Wagner, 1985). However precise mRNA mapping studies have not been performed. The polyadenylation signal, AATAAA, for these three genes is at position 70938 (McGeoch

et al., 1988). The transcription start site has not yet been identified. UL35 is expressed as a true late (γ_2) gene since it had a stringent requirement for DNA synthesis and was not expressed in the absence of DNA replication (McNabb and Courtney, 1992a).

v) VP21

There has been a lot of confusion as to the exact nature and identity of VP21. Gibson and Roizman (1972) identified it as a minor protein present in what they termed full capsids but absent from A capsids and virions and suggested that it was the sole protein associated with the DNA 'core' of the capsid. Like VP22a, VP21 has been shown to be transiently associated with intermediate capsids and is removed during the process of DNA encapsidation and capsid maturation (Rixon, 1993). Antisera prepared against VP22a was shown to cross-react with VP21 and confirmed that they were related (Zweig *et al.*, 1979b). Subsequently, Braun *et al.* (1984a) proposed that VP21 was a slowly migrating form of VP22a and an unprocessed member of the ICP35 family represented in virions as VP22. It is now accepted that VP21 is encoded by the UL26 ORF and represents the carboxy terminal cleavage product (Nb) of the full length UL26 gene product (Liu and Roizman, 1991a; Davison *et al.*, 1992; Person *et al.*, 1993; Deckman *et al.*, 1992; Preston *et al.*, 1992; DiIanni *et al.*, 1993; Weinheimer *et al.*, 1993). Other names given to this protein include: ICP34 (Honess and Roizman; 1973), Vmw43 (Marsden *et al.*, 1976), p45 (Zweig *et al.*, 1979b) and NC-3 (Cohen *et al.*, 1980). A protein of a similar size to VP21 has been shown to bind DNA (Bayliss *et al.*, 1975; Powell and Purifoy, 1976).

vi) VP24

The 25kDa molecular weight polypeptide VP24 is a minor protein present in the interior of intermediate capsids (Newcomb and Brown, 1989). In contrast to VP21 and VP22a, this protein is also a component of A and C capsids as well as virions (Gibson and Roizman; 1972). VP24 has also been described as having a molecular weight of 24kDa (Rixon et *al.*, 1990) and has been referred to as the 26kDa protein NC-6 (Cohen *et al.*, 1980), the 25kDa protein p45 (Zweig *et al.*, 1979b; Heilman *et al.*, 1979) and the 25kDa ICP45 (Honess and Roizman, 1973). It has protease activity and corresponds to No, the N-terminal cleavage product of the UL26 gene product (Liu and Roizman, 1991b; Davison *et al.*, 1992; Person *et al.*, 1993; Deckman *et al.*, 1992; DiIanni *et al.*, 1993; Weinheimer *et al.*, 1993). The relationship of this protein with VP21 and VP22a is discussed below.

vii) VP22a

The 38,800 molecular weight VP22a protein was first identified as a component of B capsids by Gibson and Roizman (1972). It was so named because it did not have an electrophoretically identical counterpart in virion protein profiles. It was originally

thought to be a precursor to the 37kDa virion protein VP22 due to the similarity in electrophoretic, staining and radiolabelling properties of the two proteins (Gibson and Roizman, 1972; Spear and Roizman, 1972; Gibson and Roizman, 1974). VP22 is now recognised as a component of the tegument and is encoded by the gene UL49 (Elliott and Meridith, 1992).

Heilman *et al.* (1979) characterised a monospecific antiserum that reacted with a protein p40, that was believed to be equivalent to VP22a by virtue of its size (40kDa) and its presence in full, but not empty capsids. Monoclonal antisera to p40 was found to immunoprecipitate four antigenically related forms of this protein which ranged in molecular weight from 39 to 45kDa as well as an 80kDa protein (p80) that was absent from nucleocapsid preparations. Pulse-chase experiments determined that p80 was not a precursor to p40 (Zweig *et al.*, 1980). Braun *et al.* (1984a), using a monoclonal antibody to ICP35, identified a family of highly processed proteins which have molecular weights ranging from 37-50kDa and named them ICP35a-f. Twenty forms were identified by two-dimensional electrophoresis. Most of these forms were found to be phosphorylated, a property which might explain the formation of the doublets cd and ef. Their electrophoretic mobilities after a pulse chase suggested that posttranslational processing, including cleavage, occurred. Thus, the slower migrating species, ICP35cd represented the unprocessed forms of ICP35ef which are found in B capsids (Braun *et al.*, 1984a; Sherman and Bachenheimer, 1988). Other researchers have assigned this protein different names which include the 38kDa protein NC-4 (Cohen *et al.*, 1980), the 40kDa Vmw40 (Preston *et al.*, 1983) and the 38kDa 22a (Desai *et al.*, 1994). For simplicity, pre-VP22a is used to describe the slower migrating forms (ICP35cd) whereas VP22a is used throughout to refer to the processed forms (ICP35ef) found in B capsids.

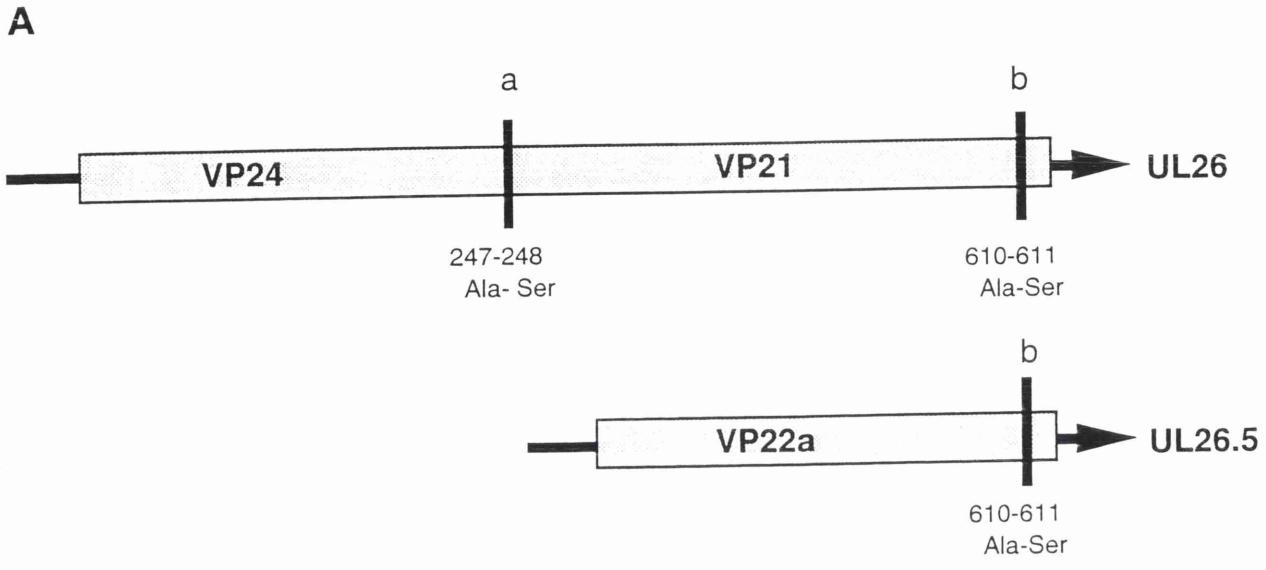
VP22a is transiently associated with capsids at an early stage of their development and is lost during the process of DNA packaging (Rixon *et al.*, 1988). Mutants in viral DNA processing and packaging have shown that association of pre-VP22a with capsids and processing of VP22a from precursor forms can occur independently of DNA packaging. This suggests that the association of VP22a with capsids may be prerequisite to subsequent cleavage/packaging events (Rixon *et al.*, 1988; Sherman and Bachenheimer, 1988). However, studies with the *ts*1201 mutant have determined that processing of pre-VP22a to VP22a is required for DNA packaging and capsid maturation. This virus is unable to process pre-VP22a at the NPT and results in the formation of intermediate capsids that have a larger size core than wt B capsids (Preston *et al.*, 1983) The importance of pre-VP22a to capsid assembly has been demonstrated with the construction of a null mutant which does not express this protein (but does express the UL26 gene product) and which was only able to form capsids and produce infectious virus inefficiently (Matusick-Kumar *et al.*, 1994). The role of VP22a in capsid assembly and in packaging of DNA will be discussed in a later section.

The genes encoding VP21, VP22a and VP24

The gene encoding pre-VP22a was initially mapped by analysis of intertypic recombinants to the region between 0.3-0.38 map units (Marsden *et al.*, 1978). Braun *et al.* (1983) mapped the gene specifying pre-VP22a on the basis of intertypic recombinants to a sequence located between the genes specifying thymidine kinase and glycoprotein B ie between map units 0.32- 0.36. On the basis of the mapping of a *ts* mutation, *ts*1201, which fails to process pre-VP22a at the NPT, VP22a was assigned to the UL26 ORF which is located between bases 50809 and 52714 (0.331-0.335 map units) on the prototypic arrangement of HSV-1 DNA (Preston *et al.*, 1983; McGeoch *et al.*, 1988). A second in-frame ATG codon was identified located 27 nucleotides downstream from the first initiation codon, at position 50836. This initiation codon functions in the absence of the first ATG but was not the preferred translation start site (McGeoch *et al.*, 1988; Preston *et al.*, 1992).

Since the predicted product of the 635 amino acid UL26 ORF is considerably larger than that required to encode ICP35, this suggested that ICP35 did not map to this gene. Liu and Roizman (1991a) mapped the nucleotide sequence that encodes ICP35 and determined that it overlapped and was in frame with the UL26 ORF identified by McGeoch *et al.* (1988). This second transcriptional unit was predicted to encode a protein of 329 amino acids and was designated UL26.5. Translation was initiated from a methionine initiation codon located 307aa (at position 51727 of the genome) downstream of the preferred UL26 ATG (Liu and Roizman, 1991a).

Three rightward orientated mRNAs of sizes 5.6, 5.2 and 4.4kb were mapped to this region as well as the 5' portion of 1.4 and 2.4kb mRNAs. Since the size predicted for a polypeptide of 38-40kDa is less than 1.5kb, it was suggested that the 1.4kb mRNA encoded pre-VP22a. The 2.4kb mRNA, the transcript for the UL26 gene, is also present in this region and is 3' co-terminal with the 1.4kb mRNA (Liu and Roizman, 1991a). Both the UL26 and UL26.5 ORFs share a common TGA termination codon at position 52714 and the polyadenylation sequence, AATAAA for both transcripts begins at position 52766 of the genome (McGeoch *et al.*, 1988; Liu and Roizman, 1991a; Preston *et al.*, 1992). The transcription initiation site of the UL26 mRNA is approximately 180 nucleotides upstream of the ATG although no sequences corresponding to a TATA box or other regulatory elements have been detected (Liu and Roizman, 1991a). The promoter of the UL26.5 transcript is embedded in the 5' terminal domain of the coding sequences of the UL26 ORF. The mRNA initiates approximately 99 nucleotides upstream of the ATG; +1000 from the UL26 initiation site (Liu and Roizman, 1991a; 1991b). Other potential regulatory elements in this region are at position 51604 (TATAA), 51572 (GGGCGG) and 51561 (CAAAAT) (McGeoch *et al.*, 1988). Differential expression from the 2 promoters explains the higher abundance of VP22a (approximately 1000 to 1500 copies per capsid) compared to that of the UL26 gene


A
a
b
VP24
VP21
UL26
247-248
Ala- Ser
610-611
Ala-Ser
b
VP22a
UL26.5
610-611
Ala-Ser

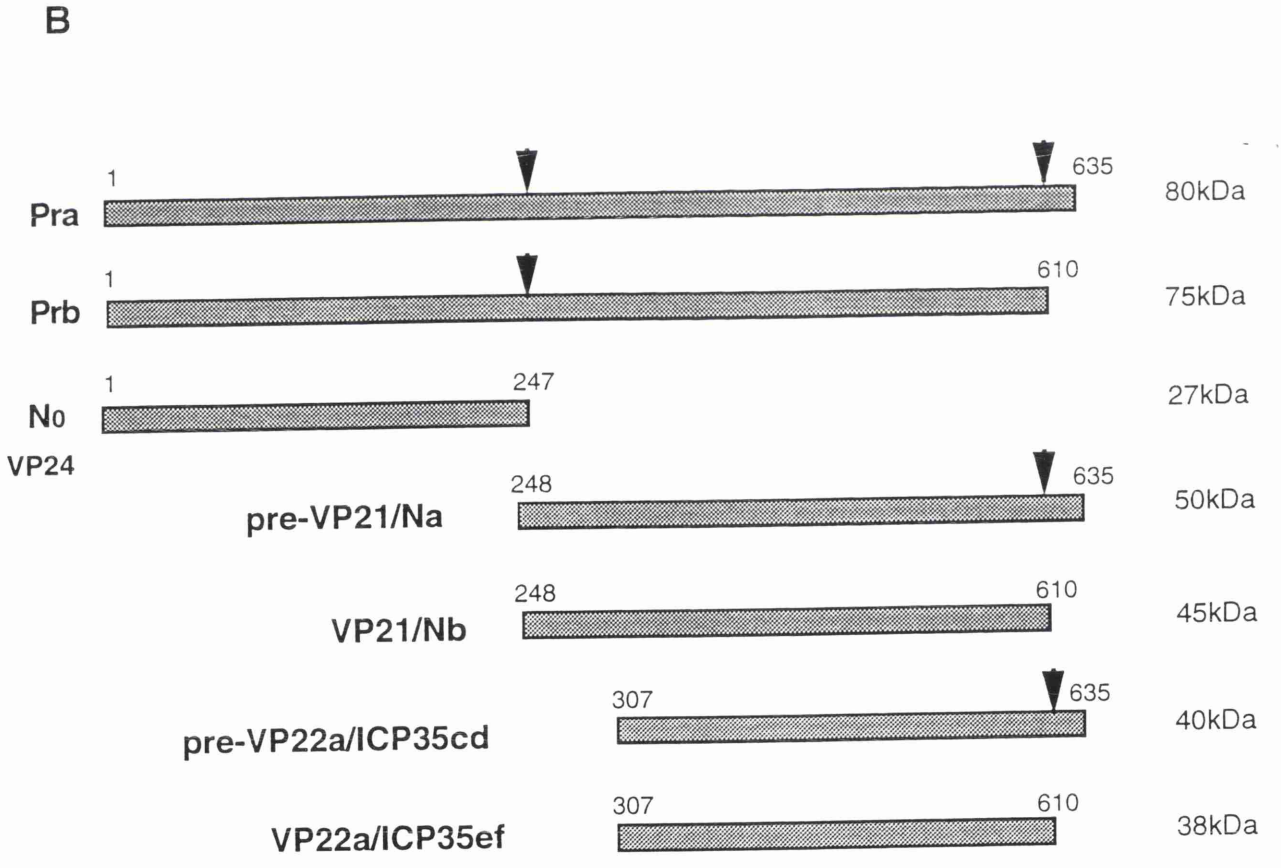

B
1
635
Pra
80kDa
1
610
Prb
75kDa
1
247
N0
27kDa
VP24
248
635
pre-VP21/Na
50kDa
248
610
VP21/Nb
45kDa
307
635
pre-VP22a/ICP35cd
40kDa
307
610
VP22a/ICP35ef
38kDa

Figure 3. Organisation of the HSV-1 genes UL26 and UL26.5

A The 3' coterminal transcripts are shown by arrows which are overlaid by open boxes indicating the positions of the open reading frames specifying the UL26 and UL26.5 gene products. Proteolytic cleavage sites (cleavage takes place between alanine and serine residues) *a* and *b* are indicated as are their codon positions. Cleavage of the UL26.5 gene product at site *b* gives rise to VP22a whilst cleavage of the protease (UL26) at sites *a* and *b* releases the capsid proteins VP21 and VP24. This figure was reproduced from Rixon (1993).

B The genesis of HSV-1 protease (Pra), substrate (ICP35cd) and cleavage products (Prb, No, Na, Nb and ICP25ef), which are indicated by the shaded boxes, are described in the text. HSV-1 cleavage sites are indicated with arrowheads and the UL26 amino acid numbers of amino and carboxy termini of each protein are indicated. Estimated molecular weights are shown at the right. The VP nomenclature used for the capsid proteins is also shown. This figure was reproduced from Weinheimer *et al.* (1993).

products, VP21 and VP24 which are present in approximately 100 copies per capsid (Liu and Roizman, 1991b; Newcomb *et al.*, 1993).

The 1.4kb and 2.4kb mRNAs are regulated with βγ and γ kinetics respectively (Holland *et al.*, 1984). This agrees with the designation by Braun *et al.* (1984a) of the ICP35 family as late polypeptides which were not stringently dependent on viral DNA replication for their synthesis.

In summary, the genes UL26 and UL26.5 encode two related proteins of molecular weights 80,000 and 40,000 respectively, specified by overlapping transcripts with unique 5' ends but a shared 3' end (Liu and Roizman 1991a). The relationship between the genes and their protein products is as shown in figure 3a.

The protein products

The 80,000 molecular weight product of UL26 is a serine protease which catalyses cleavage of pre-VP22a at its C-terminus (Liu and Roizman, 1991b; Liu and Roizman, 1992; Preston *et al.*, 1992). Pre-VP22a is posttranslationally cleaved by the protease into VP22a, an event which is not dependent on capsid assembly (Liu and Roizman, 1991b; Preston *et al.*, 1992). In addition to processing pre-VP22a, the protease cleaves itself autoproteolytically at two sites; the C-terminal site is the same as that of pre-VP22a and is shown in figure 3b. To differentiate the full length protease from the shorter processed form they have been named Pra and Prb respectively.

Several expression studies have localised the proteolytic domain of the protease to the N-terminal half of Pra. For example, expression of the protease in *E.coli* determined that the first autoproteolytic cleavage site within Pra was N-terminal to the initiation codon of pre-VP22a (Deckman *et al.*, 1992). Similarly, *in vitro* expression of plasmids containing various deletions or insertions upstream of Ala-247 resulted in the elimination of protease activity whereas truncations downstream of the pre-VP22a initiation codon retained protease activity (Liu and Roizman, 1991b; 1992). Expression of the 247 amino acid catalytic domain, which has been designated No, in *E. coli* and in mammalian cells determined that this region was sufficient to direct the cleavage of ICP35cd, thus indicating that this region contained the protease activity (Weinheimer *et al.*, 1993; Liu and Roizman, 1992). A series of C'-terminal truncations of the UL26 ORF expressed in *E. coli* has further showed that autoprocessing of the full length protease was unnecessary for cleavage of pre-VP22a (Weinheimer *et al.*, 1993). DiIanni *et al.* (1993) expressed the protease in *E.coli* and by direct amino acid sequencing of the isolated proteolytic products, identified the positions of the cleavage sites; the carboxy-terminal cleavage occurs 25 amino acids from the carboxy-terminus of both the protease and pre-VP22a between the alanine and serine residues at position 610/611, whereas the amino-terminal cleavage occurred between Alanine-247 and Serine-248. The 50kDa molecular weight C-terminal cleavage product of Pra has been named Na and the

capsid protein equivalent	Approximate molecular weight of capsid protein (kDa)							
	HSV-1	HSV-2	EHV-1	SCMV (Colburn)	HCMV	EBV	PRV	VZV
VP5	155	155	148	145	153	160	150	155
VP19C	53	53	59			52	63	57
VP21	42	44		45,39,38	37	46	41,38	38,36,34
VP22a	38	38	46	37	36	40	35,32	32
VP23	33	33	36	34	34	37	27	34.5
VP24	25	25	30	28	28	28	22.5	31.5
VP26	12	12	12	12	11	18		17.5
Ref.	a	b	c	d	e	f	g	h

TABLE 3. CAPSID PROTEIN EQUIVALENTS OF HERPESVIRUSES

[a] Gibson and Roizman (1972), Rixon *et al.* (1990)
[b] Gibson and Roizman (1972), Heilman *et al.* (1979)
[c] Perdue *et al.* (1975; 1976), Newcomb *et al.* (1989)
[d] Gibson (1981)
[e] Irmiere and Gibson (1985)
[f] Dolyniuk *et al.* (1976), Van Grunsven *et al.* (1993)
[g] Stevely (1975), Ladin *et al.* (1982)
[h] Zweerink and Neff (1981), Friedrichs and Grose (1986)

smaller (45kDa) form lacking the terminal 25 amino acids is referred to as Nb (figure 3b). On the basis of their similarity in size and immunoreactivity to ICP35 antisera it has been suggested that they are analogous to ICP35ab, the two members of the ICP35 family that migrate more slowly than full length ICP35cd (pre-VP22a) in SDS-PAGE (Braun *et al.*, 1984a; Weinheimer *et al.*, 1993).

The releationships of these forms to the capsid proteins was established when Davison *et al.* (1992) determined that fragments of VP24 exhibited amino acid sequence identity to the predicted product of UL26 (upstream of Ala-247) and migrates in SDS-PAGE with the predicted size of No. Similarly, the 363 residue C-terminal product was equivalent to VP21 (Nb), a protein which was originally proposed to be a slowly migrating form of VP22a (Braun *et al.* 1984a Davison *et al.*, 1992; Person *et al.*, 1993). The presence of these other protease autoprocessing products in addition to VP22a in intermediate B capsids suggests a key role for the proteolytic maturation of the protease and pre-VP22a in capsid assembly.

2.5.2 THE CAPSID PROTEINS OF OTHER HERPESVIRUSES

Capsids have been described from other herpesviruses which are structurally similar to those of HSV and contain between 4 and 7 proteins which vary in their immunogenic cross-reactivity and in their molecular weights. Since herpesviruses capsids closely resemble each other in structure and composition the work on HSV-1 capsids presented in this thesis is likely to throw light onto the structure and assembly of the capsid of other herpesviruses. Therefore, the protein constituents of capsids from a number of other herpesviruses are briefly reviewed below with particular reference to the similarities and dissimilarities to HSV.

i) CMV

The best studied herpesvirus apart from HSV is the betaherpesvirus HCMV and there exist some interesting differences between the two viruses. Differences also exist between HCMV strains and those that have been most extensively studied are strain AD169 and strain Colburn; which although isolated from a human patient, shows more similarity to SCMV than to HCMV (Gibson, 1983) and is hence discussed as an example of SCMV.

As in HSV, 3 types of capsid also named A, B and C capsids, have been observed in HCMV-infected cells. HCMV B capsids contain 6 proteins (table 3). The 153 kDa MCP, a 34kDa minor capsid protein, a 28kDa protein and a small (11kDa) protein are present in all three capsid types and are components of the virion, whereas the 36kDa protein is unique to B capsids. A 37kDa minor protein has also been described as being unique to B capsids and presumably represents the HSV-1 VP21 equivalent (Irmiere and

Gibson, 1985). The MCP of SCMV is 8kDa smaller than the HCMV counterpart whereas the other capsid proteins (table 3) are closer in size (Gibson, 1981; Irmiere and Gibson, 1985). Although the 28kDa protein was present in all three capsid types it was absent from SCMV virions and it has been suggested that this protein contibutes to the structural integrity of capsids (Gibson, 1981). SCMV B capsids contain two major additional species; a 37kDa major component and a minor component of approximately 45kDa. Several other proteins have observed migrating below the 37kDa protein in preparations of B capsid proteins (Gibson, 1981) and two other minor proteins of molecular weights 39kDa and 38kDa have been described (Irmiere and Gibson, 1985).

The 36kDa protein of HCMV and the 37kDa SCMV protein share similar properties with the 38/40kDa capsid assembly protein of HSV; they are present only in B capsids, are phosphorylated and share similar staining properties (Gibson and Roizman, 1974; Gibson, 1981; Gibson, 1983; Irmiere and Gibson, 1983) thus supporting their relationship to the assembly protein of HSV.

Further similarities are apparent in the manner in which these proteins are processed from precursor proteins. It has been shown by tryptic peptide analysis and immunological cross-reactivity, that the 45, 39 and 38kDa proteins described above for SCMV are related to the 37kDa assembly protein (Gibson *et al.*, 1990). The 37kDa assembly protein is derived from a 39/40kDa precursor by processing that involves elimination of 32 amino acids from the C-terminus of the precursor. It has been suggested that the 38kDa minor protein represents a partially processed form of the precursor (Robson and Gibson, 1989; Gibson *et al.*, 1990; Schenk *et al.*, 1991). Similarly, the 45kDa protein is formed from the processing of a 48kDa precursor via a 47kDa putative processing intermediate and represents a N-terminal extended form of the assembly protein. Although the precise role of these intermediate proteins has not yet been determined it is apparent that they are analogous to the HSV ICP35 family of proteins described by Braun *et al.* (1984a).

The gene arrangement of CMV assembly proteins is similar to that of HSV and is organised as a nested set of four (as compared to two in HSV-1) in-frame overlapping 3' co-terminal genes. These are referred to as the assembly protein nested genes (APNG) for SCMV and correspond to the HCMV UL80 set of genes (Robson and Gibson, 1989; Welch *et al*, 1991a). The sequence encoding the assembly protein precursor was designated APNG.5 (UL80.5) and is the 3' half of the larger APNG1 (UL80a) gene, the N-terminus of which is responsible for the protease activity which processes the precursor proteins (Schenk *et al.*, 1991; Welch *et al.*, 1991a; 1991b). The sequence of the assembly genes on the DNAs of HCMV and SCMV show positional alignment and also share nucleotide homology with UL26.5 of HSV-1. The most conserved region between assembly proteins is at the carboxy-terminus, in particular the cleavage domain which is conserved amongst the herpesviruses (Robson and Gibson, 1989; Welch *et al.*,

1991b). Another well conserved site within the protease gene is at the release site between the VP21 and VP24 equivalents (Welch *et al.*, 1991b; Liu and Roizman, 1992). The similarity between the proteinases of these two viruses has been demonstrated by (Burck *et al.*, 1994) who expressed the HCMV proteinase in bacteria and determined that it was able to process the peptide homologs of the HSV-1 protease cleavage sites. However, the HSV-1 proteinase does not appear able to cleave the corresponding CMV substrates (Welch *et al.*, 1995).

The gene encoding the MCP of HCMV, UL89, was identified by sequence homology to the sequences of other human herpesviruses and the assignment confirmed immunologically. HCMV UL89 shares 25, 29, and 23% homology to the sequences encoding the MCP of HSV-1, EBV and VZV respectively and is predicted to encode a protein of molecular weight 153,875 (Chee *et al.*, 1989; 1990). Sequence homology has suggested that the HCMV VP23 equivalent is encoded by the UL85 gene (predicted molecular weight 34,596).(Chee *et al.*, 1990). A recently identified ORF, designated UL49A, is predicted to encode a protein of molecular weight of 8,480Da and has been suggested to encode the 11kDa VP26 HCMV equivalent (Davison *et al.*, 1992). The most striking difference between the capsids of HSV and CMV is the lack of a protein of comparable size to HSV-1 VP19C (Gibson, 1981; Irmiere and Gibson, 1985). This is surprising considering the essential structural role of this protein in HSV-1 capsids (Pertuisset *et al.*, 1989) and indicates a significant difference between alpha and beta herpesviruses. However, the HCMV UL46 gene is positionally related to the suggested HSV-1 UL38 counterparts, EBV BORF1 and VZV gene 20, and although there is no apparent sequence homology between these genes, it has been suggested that this gene could represent the HCMV gene homologue (Pertuisset *et al.*, 1989; Chee *et al.*, 1990).

ii) VZV

VZV, like HSV, is an alphaherpesvirus and as such would be expected to share a large degree of similarity in structural composition. Indeed, as is discussed below, there is a modest degree of homology between the capsid genes. However, although the genome has been completely sequenced (Davison and Scott, 1986a), less work has been done on the characteristics of the individual capsid proteins.

Capsids isolated from the cytoplasm of infected cells were found to contain a number of proteins some of which appeared to be contaminating glycoproteins. Of these proteins, the 155kDa MCP and three minor capsid proteins of sizes 34.5, 31.5 and 17.5kDa were determined to be integral components of the capsid. A 57kDa protein which reacted with antibodies raised against VZV capsids is of a similar size to VP19C (Zweerink and Neff, 1981) may thus represent the homologue of this HSV-1 capsid protein. Friedrichs and Grose (1986) described a group of proteins, the p32/36 complex, that was a component of both empty and full capsids. Other immunologically related proteins, of

molecular weights 34 and 38kDa, may be precursor forms of the p32 protein that were enzymatically cleaved before incorporation into the capsid and it is possible that this set of proteins represent assembly protein equivalents (Friedrichs and Grose, 1986).

The genome of VZV is co-linear with the IL and ISL genome arrangement of HSV-1 and many counterparts of HSV-1 genes have been identified (Davison and Wilkie, 1983; Davison and Scott, 1986a; McGeoch *et al.*, 1988). The MCP, predicted molecular weight of 154,971Da, is encoded by gene 40 and shares 50% homology with the corresponding HSV-1 gene (Davison and Scott, 1986b). The region of the genome homologous to that encoding the assembly proteins of other herpesviruses stands out as being potentially the most complicated with at least eight possible 3' co-terminal genes. The largest ORF, designated gene 33, is predicted to encode a protein of molecular weight 66,043Da and shares 34% identity with the HSV-1 UL26 gene (Davison and Scott, 1986a; Welch *et al.*, 1991a). The VP19C homologue is suggested to be encoded by gene 20 and displays 29% identity whereas gene 41 shares 43% identity with the HSV-1 UL18 gene (Davison and Scott, 1986a; Yie *et al.*, 1990). VZV gene 33, which is predicted to encode a protein of 24,416Da, is positionally conserved and shows only 15% identity with the HSV-1 gene UL35 due to a C-terminal extension (Davison *et al.*, 1992).

iii) EHV-1

Another example of an alphaherpesvirus is the well studied EHV-1. Three types of capsid, designated light (L), intermediate (I) and heavy (H), which correspond to HSV-1 A, B and C capsids, have been purified from EHV-1 infected cells (Perdue *et al.*, 1974; 1975). H capsids are comprised of 6 major proteins as shown in Table 3. (Perdue *et al.*, 1975; 1976; Newcomb *et al.*, 1989). I capsids contain all these proteins although the 30kDa protein (the VP24 equivalent), was found to be present in much lesser amounts than in H capsids (Perdue *et al.*, 1975). Although the 46kDa and 30kDA proteins were absent from L capsids they were both present in H capsids. If they represent homologues of the HSV-1 VP22a and VP24 proteins respectively, then this indicates a marked difference in protein composition between the H and C capsids of EHV-1 and HSV-1. However, in view of what is known about the role of the scaffolding protein, then it is likely that the observation of this protein in EHV-1 H capsids is a result of contamination. As in HSV, both the 46 and 30kDa proteins were heavily phosphorylated. Thus, it would appear that in EHV-1, the assembly protein is larger, 46kDa and that there is no VP21 counterpart (Perdue *et al.*, 1974; 1975; Newcomb *et al.*, 1989). The 12kDa VP26, which was originally identified as an 18kDa protein by Perdue *et al.*, (1974) was found to be phosphorylated only in virions (Perdue *et al.*, 1975). It is known that this protein is also phosphorylated in HSV-1 (McNabb and Courtney, 1992b). Several other proteins have been routinely found within capsid

preparations and are present only in minor amounts (Kemp *et al.*, 1974; Perdue *et al.*, 1974; 1975).

The genome of EHV-1 is co-linear with the IL and ISL genome arrangement of HSV-1 (Davison and Wilkie, 1983) and sequence comparison has enabled the genes encoding the EHV-1 capsid proteins to be identified (Telford *et al.*, 1992). The MCP is encoded by gene 42 and the VP23 equivalent by gene 43. These genes are predicted to encode proteins of molecular weights 152,175 and 33,840 respectively. Gene 35.5 is predicted to encode the 34,769Da assembly protein and the difference between the predicted and apparent molecular weight (46kDa) of this protein may be due to phosphorylation (Perdue *et al.*, 1975). The size of the protease which is encoded by gene 35 (Telford *et al.*, 1992) is predicted to be 68,576. Gene 22 is homologous to the HSV-1 UL38 gene and encodes a protein of molecular weight 51,304Da. Gene 25, which is predicted to encode a 13,596Da molecular weight protein (Telford *et al.*, 1992) is the homologue of the HSV-1 UL35 gene.

iv) PRV

Although, unlike the other herpesviruses described in this section, the PRV genome has not been fully sequenced, it is included as another example of an alphaherpesvirus since, as a result of the large amount of research that has been done investigating the effect of mutants on capsid assembly, this virus is frequently referred to in later sections.

Ladin *et al.* (1982) identified five proteins whereas Stevely (1975) identified the six proteins indicated in Table 3 as well as proteins of molecular weight 82 and 120kDa, in preparations of non-DNA containing capsids. The 35kDa protein is greatly underrepresented in mature virions and is immunologically related to 41, 42, and 44kDa molecular weight proteins that have been detected in infected cells and which are thought to represent precursor forms of a 32kDa protein also present in capsids. The 41kDa protein as well as a 38kDa protein were detected in capsids in minor amounts (Ladin *et al.*, 1982).

The genome of PRV is co-linear with the IL and ISL genome arrangement of HSV-1 except that the region between 0.1 and 0.4 appears to be inverted (Davison and Wilkie, 1983). Lomniczi *et al.* (1987) translated *in vitro* four mRNAs whose protein products reacted with antiserum against purified capsids and thus localised the coding regions of the 150, 62 and 32kDa capsid proteins. The MCP has been shown to have a small degree of antigenic cross-reactivity with the MCP of HSV-1 (Yeo *et al.*, 1981) and the gene encoding it shares 58% homology with the HSV-1 and VZV MCP genes and 27, and 24% homology with the corresponding ORFs of EBV, and CMV respectively (Yamada *et al.*, 1991).

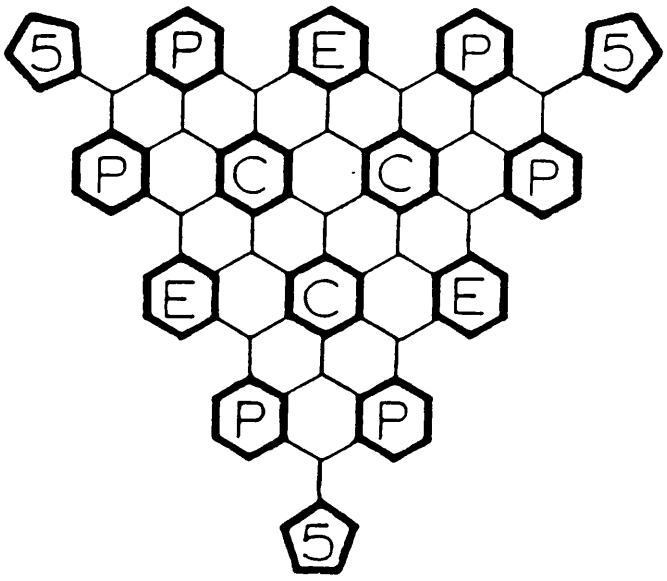
v) EBV

EBV represents the third group of herpesviruses, the *Gammaherpesvirinae* and like the other herpesviruses discussed above, would be expected to show some dissimilarlty to HSV. Nucleocapsids prepared by detergent treatment of extacellular virions were found to contain five possible capsid proteins (Table 3) as well as two other major proteins of 275kDa and 144kDa. Nine other proteins were found associated with capsids and possibly represent contaminating tegument proteins (Dolyniuk *et al.*, 1976). Van Grunsven *et al.* (1993) identified two additional capsid proteins of molecular weights 18kDa and 40kDa. The 40kDa was found to be expressed as a complex of proteins of different isoelectric points and probably represents the equivalent of the HSV VP22a family. The size and basic properties of the 18kDa protein suggests it represents the VP26 equivalent.

Comparisons of the nucleotide sequence of HSV-1 (McGeoch *et al.*, 1988) with that of EBV (Baer *et al.*, 1984) has identified possible capsid gene counterparts. The major capsid protein is the product of the BcLF1 gene which was predicted to encode a protein of molecular weight 153,916Da and shared 27% and 24% homology with the HSV-1 and VZV MCP genes respectively (Baer *et al.*, 1984; Davison and Scott, 1986b). The coding region of the assembly protein homologues, like that of HSV-1, contains two potential in-frame, 3'-coterminal genes collectively referred to as the BVRF2 gene family (Baer *et al.*, 1984; Welch *et al.*, 1991a). BVRF2a which represents the larger ORF is predicted to encode a protein with a molecular weights of 64,102 and corresponds to the UL26 gene of HSV-1. The gene encoding the 40kDa protein was identified as the BdRF1 gene (Van Grunsven *et al.*, 1993) which had previously been reported to encode a protein that was 100% identical to the C' terminus of the protein encoded by the BVRF2 gene (Farrell, 1989). Donaghy and Jupp (1995) have demonstrated that the product of BVRF2 is a polyprotein capable of proteolytic cleavage of itself and the product of the BdRF1 gene in a similar manner to the HSV-1 protease and assembly protein gene. The exact relationships between these proteins remains to be determined. The BORF1 gene, predicted to encode a protein of 39,101Da, shares 20.2% homology to HSV-2 gene encoding VP19C but appears to be completely missing part of the N-terminus, a domain which shows the greatest degree of variation amongst other human herpesvirus homologues (Baer *et al.*, 1984; Pertuisset *et al.*, 1989; Yei *et al.*, 1990). However, this gene may be too small to encode the 52kDa VP19C equivalent (Dolyniuk *et al.*, 1976). The BGLF2 gene which encodes a 36kDa protein reactive against viral capsid antigen-expressing cells possibly represents the VP23 homologue (Chen *et al.*, 1991). Van Grunsvan *et al* (1993) using antisera to the 18k protein, identified the BFRF3 gene as the UL35 conterpart.

The capsids of other herpesviruses also contain proteins homologous to those of HSV-1 capsids and are described elsewhere: HHV-6 (Lawrence *et al.*, 1990; Gomples *et al.*,

A


5
P
E
P
5
P
C
C
P
E
C
E
P
P
5

B

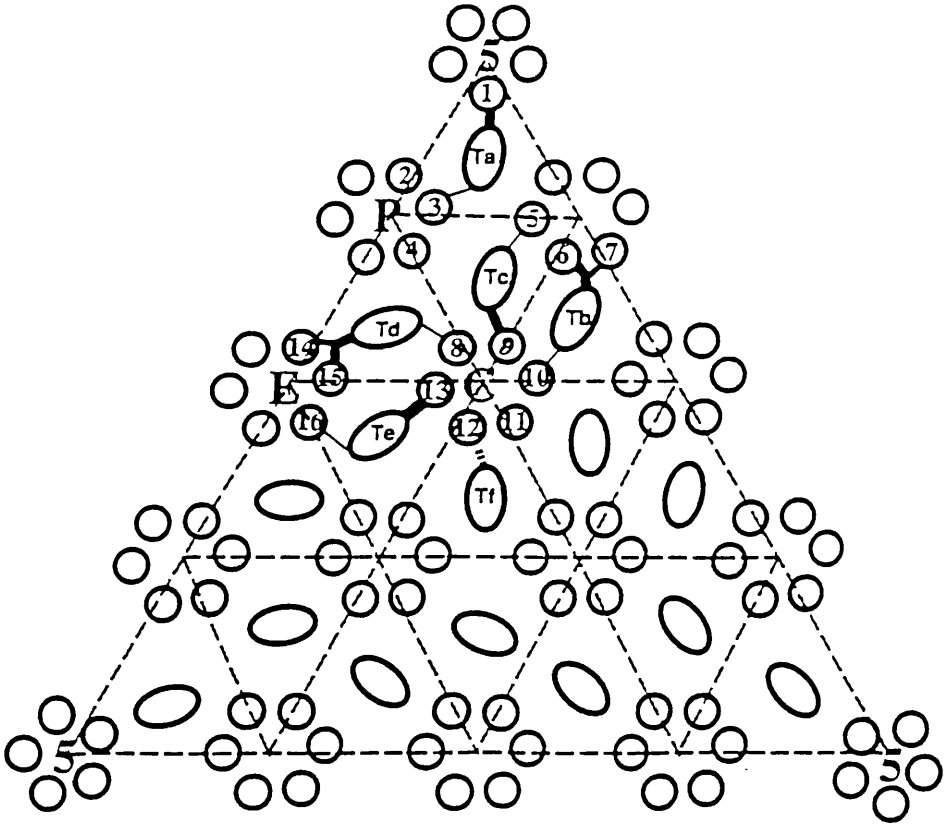

5
1
Ta
2
3
P
5
4
6
7
Tc
Tb
Td
8
9
14
15
13
E
C
10
16
Te
12
11
Tf
5
5

Figure 4. Schematic representation of a single triangular face of a herpesvirus capsid

A:- Illustration of the bonding relationships between the capsomers. The quasi-equivalent hexons are labelled P, E and C. The relative positions of the pentons (5) and hexons; P (peripheral), E (edge) and C (central) are indicated. This figure is reproduced with permission from Schrag *et al.* (1989).

B:- Illustration of the relative locations of the various structural components and the quasi-equivalent subunits in a triangular face of the T=16 icosahedral lattice (broken lines). The quasi-equivalent subunits of penton and hexons in one of the asymmetric units are labelled 1 to 16 and the quasi-equivalent triplexes are labelled Ta through Tf. The more extensive molecular interactions between the triplexes and their neighbouring capsomeric subunits are shown with thick lines and the less extensive ones with thin lines. This figure was reproduced with permission from Zhou *et al.* (1994).

1995), EHV-2 (Caughman *et al.*, 1984; Telford *et al.*, 1995), EHV-3 (Allen and Bryans, 1976) and HVS (Blair and Honess, 1983; Randall *et al.*, 1983).

2.5.3 THE STRUCTURE OF THE HERPESVIRUS CAPSID OUTER SHELL

The morphology of the capsid is a characteristic of herpesviruses and will be discussed using HSV-1 as an example but drawing on information from the other herpesviruses where appropriate.

The capsid of HSV-1 is 125nm in diameter and icosahedral in shape displaying 2:3:5-fold axial symmetry. The shell is organised into 162 capsomers which comprise 150 hexavalent capsomers (hexons) and 12 pentavalent capsomers (pentons). The capsomers lie on a T=16 icosahedral lattice with the pentons located at the vertices and hexons occupying the capsid faces and edges (Wildy *et al.*, 1960; Schrag *et al.*, 1989; Baker *et al*, 1990). Cryoelectron microscopy and three-dimensional computer reconstructions of capsids have identified three layers of increasing density in the outer capsid shell totalling 15nm in thickness.

i) The outer layer

The outermost layer consists of hexon and penton capsomers which protrude 5nm above the midlayer of density (Baker *et al.*, 1990). Vernon *et al.* (1974) examined negatively stained capsids which had been digested with trypsin to produce a flattened sheet of capsomers, and suggested that the hexons were trimers. Further ultrastructural studies demonstrated that the hexons, as viewed in two-dimensions in the electron microscope, have six-fold symmetry (Palmer, 1975; Furlong, 1978; Steven *et al.*, 1986). Three-dimensional imaging demonstrated that the hexon is made up of 6 subunits (Schrag *et al.*, 1989; Baker *et al.*, 1990; Booy *et al.*, 1991, Zhou *et al.*, 1994). Each hexon is cylindrically shaped and has a diameter of 17nm and a height of 14nm (Steven *et al.*, 1986; Schrag *et al*, 1989; Zhou *et al.*, 1994). They are each penetrated by a channel which is 5nm wide at the outer surface and which tapers progressively (to about 2nm in diameter) towards the base of the capsomer forming a direct pathway connecting the inside of the capsid with the external environment (Zhou *et al.*, 1994). Although no significant structural differences has been observed between the 150 hexons, they occupy different bonding (quasi-equivalent) environments and have been divided into three types P, E and C, depending on their positions on the surface lattice (figure 4a) (Steven *et al.*, 1986). Each of the sixty peripentonal (P) hexons are immediately adjacent to a pentavalent capsomer, whereas each of the sixty central (C) hexomers lie in the centre of a facet, surrounding the icosahedral 3-fold axis. The thirty edge (E)

hexomers lie on the icosahedral 2-fold axes and are located on the edge of a triangular face in a position which is not adjacent to a vertex (Steven *et al.*, 1986; Schrag *et al.*, 1989). From studies on EHV-1, Baker *et al.* (1990) observed that the three types of hexon differ both in their relative orientations and in the shapes of their channels; which were widest in type E and narrowest in type P. These researchers suggested that these differences were generated by slew displacement between the six subunits that surround each channel.

Each penton is composed of five subunits and forms a cylindrical protrusion 14.5nm in diameter and 14nm in height (Zhou *et al.*, 1994). Thus the pentons are slightly narrower than the hexons (Baker *et al.*, 1990; Booy *et al.*, 1994) and have a wider (~5nm) axial channel running through them (Zhou *et al.*, 1994). They appear to be less tightly integrated into the capsid structure since they were removed from capsids by treatment with either 6M urea or 2M GuHCl (Newcomb *et al.*, 1993) or EDTA/trypsin (Palmer *et al.*, 1974; Steven *et al.*, 1986).

Penton subunits are composed of three domains; an upper, diamond-shaped domain, a middle, stem-like domain and a lower anchoring domain. The structural similarity between the hexon and penton subunits suggested that they are composed of the same protein (Zhou *et al.*, 1994). The major difference observed between the two is a horn-shaped mass of density on the tips of the hexons which is absent from the pentons (Zhou *et al.*, 1994). The reason for this difference is described below. In addition, the pentons lack a small globular density at the central domain that protrudes out toward the central axis of the hexon subunit. It is possible that this density difference is caused by mass translocation: it is tucked inward in the penton subunit whereas it protrudes outward in the hexon subunit. It was suggested by Zhou *et al.* (1994) that this difference could be the result of covalent modifications such as acetylation or phosphorylation. The integrity of the capsomers is maintained by disulphide and hydrogen bonding (McCombs and Williams; 1973).

ii) The midlayer

The additional density of the midlayer is due to the triplexes which connect neighbouring capsomers. Fibrils, approximately 2.5nm in diameter connecting three adjacent capsomers were first observed in negatively stained preparations of capsids (Vernon *et al.*, 1974; Palmer *et al.*, 1975; Almeida *et al.*, 1978). Three dimensional computer reconstructions initially portrayed them as Y-shaped densities (Schrag *et al.*, 1989; Baker *et al.*, 1990; Booy *et al.*, 1991). However, higher resolution studies have revealed structural differences between triplexes located at the various quasi-equivalent sites (Zhou *et al.*, 1994). At this resolution it is apparent that the triplexes are not solid Y-shaped mass as previously thought. As diagramatised in figure 4b, some triplexes connect only two adjacent capsomers whereas others connect three; the connecting arms

vary in density and there appears to be holes under the triplexes formed by their arch-shaped appearance (Zhou *et al.*, 1994). Differences in the bonding strengths of the triplexes have also been suggested based on the extent of their interactions. For example, treatment of capsids with 2M GuHCl removed 120 of the 320 triplexes along with the pentons although the rest of the capsid shell remained structurally intact. The triplexes removed were the ten closest to each capsid vertex, that is, those closest to the pentons were the most easily extracted (Newcomb *et al.*, 1993). Zhou *et al.* (1994) identified 6 types of triplex which they designated Ta-Tf based on their relative locations within the icosahedral lattice (figure 4b) Ta-Te triplexes are asymmetric, each having two 'legs' that are connected to the floor of the capsid and an upper domain which has a tail and two 'arms' that interact with two or three adjacent capsomer subunits. The Tf triplex is located at the strict three-fold axis of symmetry and appears to be three-fold symmetric although it cannot be discounted that this appearance is artifactual and imposed on the structure by the method of reconstruction. These differences in conformation may explain the selective removal from the capsid of the triplexes (Ta) adjacent to the pentons.

iii) The innermost layer

The innermost layer (between the radii of 47.5nm and 50nm) of the shell is a continuous 2.5nm thick sheet of protein that is punctured by the channels of the hexons and pentons and also by the holes below the triplexes. This layer is predominately composed of the closely interacting mass densities from the lower portions of the hexameric and pentameric subunits as well as from the triplexes (Zhou *et al.*, 1994). In addition, the inner surface of the floor would be expected to contain regions of contact with the scaffolding protein of B capsids and the DNA of C capsids. Zhou (personal communication) at a resolution of 1.9nm has identified a density of protein which lines up beneath the channel of the pentons that is present in B capsids but is absent in A capsids. It is possible that this is due to the scaffold protein interacting with the inner surface of the shell.

The surface features of empty, intermediate and full capsids are indistinguishable at a resolution of 2.6nm (Schrag *et al.*, 1989; Booy *et al.*, 1991). The difference between capsid types arises from the absence of a core or DNA in empty capsids. In contrast to the distinct proteinaceous core in HSV-1, intermediate capsids of EHV were seen to have one or more internal components with dimensions of 20-30nm. This could imply that the distribution of material was not icosahedrally symmetric or constant from particle to particle in these reconstructions and it was suggested that these structures consisted of aggregates of scaffolding protein which might be remnants of cores retained in capsids interrupted in the process of DNA packaging (Baker *et al.*, 1990). In

addition, two and not three layers of densities were resolved in the capsid shell of EHV-1.

2.5.4 THE LOCATIONS OF THE CAPSID PROTEINS IN THE CAPSID STRUCTURE

The assembly of the capsid involves specific interactions between the capsid proteins and is dependent on specific properties of the proteins including their relative amounts in the capsid. Studies using antibodies directed against individual capsid proteins and techniques involving sequential labelling or degradation of the capsid have contributed to existing knowledge on capsid assembly. More recently, computer reconstruction techniques have enabled the structure of the individual capsid components to be visualised. Work with the baculovirus expression system has enabled the interaction of the capsid proteins and their contribution to the capsid structure to be studied in more detail. Since this contributed to the major bulk of work in this thesis, results obtained using this system will be examined in the Discussion chapter and only information obtained from other sources will be discussed below.

i) VP5 constitutes the hexons and pentons

The first report of the location for this protein came from Powell and Watson (1975) who determined that VP5 was present on the surface of capsids since antiserum against VP5 resulted in the agglutination of capsids. Surface iodination of capsids labelled VP5 (Braun *et al.*, 1984a) and erosion of the capsid by Ar^+ ions resulted in rapid loss of VP5 (Newcomb *et al.*, 1989) confirming that VP5 is exposed on the outer surface of the capsid. Similarly, immune electron microscopic analysis of capsids incubated with low concentrations of antiserum demonstrated that VP5 was present all over the surface of the capsid and was consistent with the idea that VP5 is the major component of the hexamers (Vernon *et al.*, 1981). The calculated mass densities of the hexons is sufficient to accommodate six copies of the 155kDa MCP. The calculated mass density of the penton has been determined to closely match the expected molecular mass of 5 copies of VP5 (Schrag *et al.*, 1989; Zhou *et al.*, 1994). For VP5 to make up both hexons and pentons would require that VP5 is able to alter its bonding state in a manner that allows formation of both hexamers and pentamers. Trus *et al.* (1992) mapped two separate VP5 mAbs; one to the distal tips of the hexon protrusions and the other to a different site on the hexon, neither of which bound to pentamers. This confirmed that the hexons were composed of VP5 but suggested that the pentons were either composed of some other protein(s) or that they contained VP5 in a different conformation. A third antibody raised in the same way, bound pentons but not hexons and supported the latter suggestion. Treatment of capsids with either 6M urea or 2M GuHCl was found to cause

the removal of pentons, but not hexons, from capsids. This penton removal correlated with loss of a quantity of VP5 that was in agreement with the amount expected if each penton was composed of five copies of VP5 (Newcomb and Brown, 1991; Newcomb *et al.*, 1993). Zhou *et al.* (1994) have provided structural evidence that the hexons and pentons are composed of the same protein and have determined that these structures extend from the outer surface through to the inner face of the shell floor. Assigning VP5 to the pentons as well as the hexons requires 960 copies of VP5 per capsid (Stevens *et al.*, 1986; Schrag *et al.*, 1989). This is within the range of the estimated number of copies of VP5 per capsid which was detemined quantitatively to be ~960 (Newcomb *et al.*, 1993). The transcapsomeric channels are suggested to be the port of entry for the genomic DNA so that the location of VP5 might have implications for its role in binding of DNA as reported by Braun *et al.* (1984a). However it is more likely that a protein on the exterior of the capsid is responsible of this since it would be in close proximity with the DNA.

ii) The triplexes are composed of VP19C and VP23

VP23 has been deemed an exposed component of the capsid shell since it could be labelled by iodination (Braun *et al.*, 1984a) and was easily etched from capsids by a beam of Ar^+ ion plasma (Newcomb and Brown, 1989). The position of VP19C was initially subject to controversy. Antiserum against VP19C localised to the capsid vertices and lead Vernon *et al.* (1981) to suggest that this protein was a constituent of the pentamers. To support this external location, Newcomb and Brown (1989) determined that VP19C was as rapidly lost from capsids by erosion with an Ar^+ ion plasma beam as were VP23 and VP5. Conversly, Braun *et al.* (1984a) demonstrated that VP19C was not accessible for radiolabelling which suggested that it was an internal component of the capsid. Studies with CMV provided evidence which suggested that VP19C was unlikely to constitute the pentamers since CMV B capsids were found to have morphologically normal pentons despite the fact that they lack a protein analogous to VP19C (Gibson, 1983; Irmiere and Gibson, 1985). Since treatment of capsids with 2M GuHCl completely removed the pentons but did not eliminate VP19C and VP23 these proteins were ruled out as components of the pentons (Newcomb and Brown, 1991). The partial loss of some of VP19C and VP23 (unlike VP21, VP22a, VP24 and VP26 which were completely removed) upon 2M GuHCl extraction correlated with the loss 120 of the triplexes and implicated these as the triplex proteins (Newcomb *et al.*, 1993). The relative quantities removed were compatible with the idea that each triplex is a heterotrimer consisting of one copy of VP19C and two copies of VP23. This would give 320 copies of VP19C and 640 copies of VP23. This ratio is in approximate agreement with the measured copy numbers of these proteins which have been estimated to be between 353-397 and 505-639 (Newcomb *et al.*, 1993) for VP19C and VP23 respectively. Zhou *et al.* (1994), based on the structures of the triplex which they

observed from 3 dimensional reconstructions of capsids, suggested that VP23 could constitute the two 'legs' of each triplex whereas the tail together with the upper domain would represent the VP19C molecule (Zhou *et al.*, 1994). VP19C is able to form disulphide bonds with VP5 (Zweig *et al.*, 1979a) and thus may have a role in holding the hexamers together. Possible locations for such linkages have been suggested to be at either the arms that connect the VP5 sub-units with the triplexes or in the floor of the capsid (Zhou *et al.*, 1994). The observation that part of the triplex domains comprise the floor of the capsid (Zhou *et al.*, 1994) has implications for the possible DNA binding domain of VP19C described by Braun *et al.* (1984b). Thus VP19C could serve both to anchor the capsomers on the outer side of the capsid shell whilst conferring a strong affinity for DNA to the interior surface of the capsid which would be important for the process of viral DNA packaging. Less is known about the properties of VP23.

iii) VP26 is present on the tips of the hexons but is absent from the pentons

Until recently, the position of VP26 could only be speculated upon as this protein had been less extensively investigated. VP26 is not essential for capsid stability as treatment of capsids with 2M GuHCl resulted in the loss of this protein, The concomitant removal of the pentons led to the suggestion that VP26 was a component of the pentons. However the ability of this protein, but not the pentons, to reattach to capsids after dialysis to remove the GuHCl discounted this theory (Newcomb and Brown, 1991). Similarly VP26 was solublised by 3M urea whereas the pentons and adjacent triplexes remained intact thus ruling out these structures as the location of VP26 (Newcomb *et al.*, 1993). The number of copies of VP26 per capsid has been estimated as ~1470 (Person *et al.*, 1993) and ~952 (Newcomb *et al.*, 1993) and is consistent with the idea that VP26 could be involved in capsomer structure in a 1:1 complex with the 960 copies of VP5 (Newcomb *et al.*, 1993). Cryo-electron microscopy and three-dimensional image reconstructions have localised VP26 to outer tip of each hexon projection (Booy *et al.*, 1994; Zhou *et al.*, 1994). Comparison of the three-dimensional density maps (at a resolution of ~3nm) between capsids treated with 2M GuHCl to remove VP26 and capsids containing VP26 have determined that VP26 subunits are distributed symmetrically around the outer tip of each hexon protrusion although the possibility that VP26 might also form the pentons could not be disproved since this treatment removed the pentons (Booy *et al.*, 1994). Examination of capsid reconstructions at an even higher resolution (2.6nm) and comparison by superimposition of the hexons and pentons has identified the presence of a horn-shaped mass of density, of about 15kDa, at the distal end of each hexon subunit which was not present on the penton subunits (Zhou *et al.*, 1994). Since both penton and hexon subunits are comprised of VP5 this strongly suggested that VP26 was present on the hexon but not on the pentons. The presence of VP26 on the hexons but not the pentons would partially explain the difference in antigenic character between the hexons and pentons observed by Trus *et al.* (1992);

either by direct masking of some antigenic sites on the hexon or by introducing a conformational change between the hexon subunits. By virtue of its external location, VP26 is an obvious candidate to form specific interactions with the tegument proteins during capsid maturation. A protein which may represent VP26, has been reported as associating with DNA (Bayliss *et al.*, 1975; Knopf and Kaerner, 1980; McNabb and Courtney, 1992b) and this may suggest a role for this protein in the initial association of the viral genome with the capsid prior to packaging of the DNA and maturation of the capsid.

iv) VP22a: the scaffolding protein

The location of VP22a in relation to the capsid was initially the subject of some debate. Gibson and Roizman (1972) suggested that this protein was located on the surface of DNA-containing capsids. However, this was due to a misbelief that B capsids contained DNA. Likewise, Braun *et al.* (1984a) suggested that ICP35a-d were the cytoplasmic precursors of ICP35ef which were found in the nucleus and iodination experiments implicated ICP35ef as being bound to the surface of DNA containing capsids. These observations suggest a role for VP22a in the encapsidation of DNA. Vernon *et al.* (1981), using antibodies to VP21/VP22a determined, from the observation that the intercapsomeric spaces were obscured by binding of this antibody, that either or both these proteins were located interior to the major capsid protein. An internal location for VP22a was later confirmed by immunoelectron microscopy when VP22a was observed to lie along the inner margin of intermediate capsids (Rixon *et al.*, 1988). Shenk *et al.* (1988) also described a monoclonal antibody against this protein which localised on capsids within the nucleus of infected cells but they did not distinguish between capsid types. VP22a is transiently associated with capsids at an early stage of their development and is lost during the process of DNA packaging (Rixon *et al.*, 1988). Mutants in viral DNA processing and packaging have shown that the processing of VP22a from precursor forms and its association with capsids can occur independently of DNA packaging (Rixon *et al.*; 1988, Sherman and Bachenheimer; 1988). It is now generally accepted that VP22a constitutes the scaffold core that is present only in B capsids, thus explaining the absence of this protein in A and C capsids. In addition, VP22a has been observed to have an intrinsic ability to self-assemble into 60nm core-like structures (Newcomb and Brown, 1991). With reference to its role in guiding capsid assembly VP22a is frequently referred to as the 'assembly protein' and the 'scaffolding protein'. The equivalent protein of EHV-1, VP22, contributes to the additional mass found in intermediate capsids (Newcomb *et al.*, 1989) and has the appearance of clumps of aggregates of VP22 distinct from the spherical core of HSV-1 (Baker *et al.*, 1990).

v) VP21 and VP24

Together these proteins account for less than 5% of the B capsid mass and are present in about 100 and 150 copies respectively (Newcomb *et al.*, 1993). Erosion of capsids with Ar^+ ion plasma suggested that VP21 and VP24 are inside the capsid cavity since these proteins were only lost after the other shell proteins had been eroded away (Newcomb and Brown, 1989). VP21 was originally suggested to form the main component of the capsid core (Gibson and Roizman, 1972) but it is now believed to associate with VP22a in the scaffold. In contrast to VP21 and VP22a, VP24 is retained after DNA encapsidation and is thus a component of C capsids and virions. Considering the relationship between VP21 and VP24, it seems probable that VP24 is targeted to assembling capsids, prior to N-terminal cleavage, via the VP21 domain which would then direct its assembly into scaffolds together with pre-VP22a (Davison *et al.*, 1992; Weinheimer *et al.*, 1993). The importance of the cleavage events and the role of these proteins in capsid assembly is discussed in light of the results obtained in the course of, and subsequent to this thesis, in the Discussion.

vi) Summary

Based on our understanding of the capsid structure, the protein composition of B capsids is as follows: the 12 pentons are each composed of 5 copies of VP5; the 150 hexons are each composed of 6 copies of VP5 and 6 copies of VP26; the 320 triplexes are each composed of 1 copy of VP19C and 2 copies of VP23. Thus the total number of copies of VP5 equals 960, of VP19C equals 320, of VP23 equals 640 and of VP26 equals 900. VP22a, VP21 and VP24 are each found in the internal cavity of the capsid in 1250, 100 and 150 copies respectively.

2.6 THE STRUCTURE OF ENCAPSIDATED DNA

The DNA of herpesviruses is tightly packaged into the capsid as a structure that is generally referred to as the core. However, as is discussed below, this implies the presence of a distinct entity which is no longer thought to be the case.

Microscope studies of DNA containing nucleocapsids have resulted in a variety of structures being suggested for the DNA (Furlong *et al.*, 1972; Nazerian, 1974; Friedmann *et al.*, 1975; Hagueriau and Michelson-Fiste, 1975). Many of these observations were in agreement with Furlong *et al.* (1972) who proposed that the viral DNA was spooled round a cylindrical structure with spacings of 4-5nm between DNA strands generating a toroid arrangement of DNA in the core. In HSV-1 the candidate for the core protein was suggested to be the capsid protein VP21 (Gibson and Roizman, 1972).

Most of the variability in core structure which is evident in electron micrographs has been attributed to differences in the preparation of specimens, such as fixing, staining and dehydration techniques (Puvion-Dutilleul *et al.*, 1987) as well as to the plane of sectioning (Perdue *et al.*, 1976). However, some of the different structures observed may represent different stages in capsid morphogenesis (Friedmann *et al.*, 1975; Perdue *et al.*, 1976) or indeed might represent intermediate maturational stages before the scaffolding protein is fully expelled and the DNA fully packaged (Sherman and Bachenheimer, 1988; Rixon *et al.*, 1988). A T=4 lattice structure present as an inner shell has been described from electron micrograph 3-D reconstruction studies of capsids by Schrag *et al.* (1989) but is now thought not to exist.

The major inconsistencies in these models for the DNA core is that it does not take into account the mass of DNA that has to be accommodated. This is such that the volume of the internal cavity is barely sufficient to accomodate a protein plug (Booy *et al.*, 1991). In addition, our current understanding of capsid structure and composition does not suggest a candidate for a plug protein. Using cryo-electron microscopy and three-dimensional image reconstruction, Booy *et al.* (1991) determined that the packaged DNA formed a uniformly dense ball extending right up to the inner surface of the surrounding icosahedral shell. Thus they saw no evidence of a cylinder structure. Interduplex spacing was calculated to be ~2.6nm and the DNA strands were depicted as patterns of striations forming a fingerprint motif (Booy *et al.*, 1991). Not only did this demonstrate the organised state of the packaged DNA but it suggested that it is packaged as locally ordered liquid crystalline bundles of parallel DNA strands in a similar manner to the packaged bacteriophage T4 and lambda genomes (Lepault *et al.*, 1987).

3 THE LYTIC CYCLE

3.1 INITIAL STAGES OF INFECTION

At present the pathway by which HSV-1 gains entry into the host cell is not completely clear. A model for entry has been proposed which involves a series of interactions between the viral envelope and the cell plasma membrane which trigger membrane fusion, nucleocapsid penetration and virion disassembly (reviewed by Roizman and Sears, 1990; Spear, 1993).

The initial attachment of HSV-1 to cells has been shown to involve the binding of viral glycoproteins to heparin sulphate proteoglycans on the host cell surface (WuDunn and

Spear, 1989; Shieh *et al.*, 1992). Following attachment, the virion adsorbs to the cell surface and then penetrates the host cell by fusion of the virion envelope with the cell membrane (Morgan *et al.*, 1968; Abodeely *et al.*, 1970). These three processes are mediated by viral glycoproteins which protrude from the surface of the virion envelope; the functions of which were discussed earlier. The product of the UL45 gene, a non-glycosylated membrane protein has also been implicated as a mediator of cell fusion (Haanes *et al.*, 1994) and the UL25 protein has been implicated in penetration (Addison *et al.*, 1984).

Subsequent to penetration, the nucleocapsid is transported through the cytoplasm to the nucleus where capsids are thought to release their DNA without themselves entering the nucleus (Morgan *et al.*, 1968; Batterson and Roizman, 1983). Several researchers described the disintegration of the capsid prior to release of the DNA as a solid 'core' (Morgan *et al.*, 1968; Hummeler *et al.*, 1969). However Miyamoto and Morgan (1971) subsequently suggested that DNA is extruded slowly without damage to the capsid. Newcomb and Brown (1994) have induced extrusion of DNA from capsids *in vitro* upon treatment with 0.5M GuHCl. They observed the DNA exiting in the form of thick strands or fibres from discrete sites which appeared to coincide with capsid vertices. Analysis of the *ts*B7 mutant which accumulates nucleocapsids at the nuclear pores and fails to release DNA until downshift to the PT has indicated a role for a virion protein in DNA release (Batterson *et al.*, 1983). The lesion in *ts*B7 has since been mapped to the UL36 gene (McGeoch *et al.*, 1988) which encodes the very large tegument protein VP1 (McNabb and Courtney, 1992c).

3.2 EFFECT OF HSV-1 INFECTION ON HOST CELL MACROMOLAR SYNTHESIS

Infection of cells with HSV-1 results in a rapid decrease in host protein synthesis and in induction of viral gene expression (reviewed by Fenwick, 1984).

Host shut-off occurs in two stages. Early shut-off (virion host shut-off) is a constituent of the infecting virus particle and does not require *de novo* protein synthesis. The activity of this virion function is associated with the disaggregation of host polyribosomes and with the degradation of host mRNA (Sydiskis and Roizman, 1967; Fenwick and Walker, 1978; Fenwick *et al.*, 1979; Schek and Bachenheimer, 1985; Kwong and Frenkel, 1987).

A late secondary function (delayed shut-off) requires prior expression of viral genes and involves enhanced degradation of host mRNA thereby completing the inhibition of host-protein synthesis (Nishioka and Silverstein, 1978; Fenwick and Clark, 1982; Read and

Frenkel, 1983). Most HSV-2 strains inhibit synthesis of both cellular DNA replication and protein synthesis more rapidly than HSV-1 strains (Fenwick *et al.*, 1979).

The viral component responsible for virion host shut-off (vhs) activity has been mapped to the UL41 ORF (Kwong *et al.*, 1988; McGeoch *et al.*, 1988). This gene encodes a 53kDa virion protein which is phosphorylated in infected cells (Fenwick and Everett, 1990; Smibert *et al.*, 1992) and is a component of the tegument (McLauchlan *et al.*, 1992). The 57kDa protein product of the UL13 gene (which encodes the protein kinase) is also necessary to produce the virion host shut-off effect (Overton *et al.*, 1992; 1994). The manner in which it does this is not understood but this protein has been implicated in the phosphorylation of VP22 (Purves and Roizman, 1992; Coulter *et al.*, 1993; Purves *et al.*, 1993).

In contrast to the shut-off of host protein synthesis described above, the synthesis of some cellular polypeptides, including cellular stress and heat shock proteins are stimulated during HSV-1 infection (LaThangue *et al.*, 1984; Kemp *et al.*, 1986; Patel *et al.*, 1986).

3.3 HSV-1 DNA REPLICATION

HSV-1 DNA replication occurs in the nucleus and is first detected 3h p.i., peaks at about 8h p.i. and is virtually complete by 16h p.i. (Wilkie, 1973). The initial sites of DNA replication appear to be virus specific structures induced by infection and have been called replication compartments (Quinlan *et al.*, 1984) although as infection proceeds the entire nucleus becomes involved (Rixon *et al.*, 1983). Since the majority of progeny viral DNA exists as large concatemeric molecules it has been hypothesised that viral DNA replication occurs by the rolling circle mechanism (Jacob *et al.*, 1979; Poffenberger and Roizman, 1985).

DNA replication begins at three *cis*-acting origins of replication (ori) whose sequences were originally identified as those required for the replication of defective genomic DNA (Frenkel *et al.*, 1976; Schroder *et al.*, 1975). OriS is located in the inverted repeat sequence flanking the short component of the genome and is therefore present in the genome in two copies (Stow, 1982; Stow and McMonagle, 1983). OriL is located near the middle of the long unique component of the genome (Gray and Kaerner, 1984; Weller *et al.*, 1985). Both oriS and oriL contain an extensive inverted repeat sequence although that of oriL is longer (Murchie and McGeoch, 1982; Weller *et al.*, 1985). The minimum sequences required for the function of oriS has been identified and coincides with the region of highest similarity with oriL indicating that oriS and oriL are functionally equivalent (Lockshon and Galloway, 1988).

The complete set of viral genes that are required for HSV DNA replication was identified by means of a transient complementation assay in which cloned segments of HSV DNA were tested for the ability to support the replication of co-transfected plasmids containing oriS or oriL (Challberg, 1986; Wu *et al.*, 1988). Seven genes (UL5, UL8, UL9, UL29, UL30, UL42 and UL52) were found to be both necessary and sufficient for origin dependent DNA synthesis. Similar results were obtained in insect cells transfected with an oriS-containing plasmid and superinfected with a mixture of 7 recombinant baculoviruses expressing individual HSV-1 replication proteins (Stow, 1992). These genes are all essential for virus replication and DNA synthesis in tissue culture (Weller, 1991). The involvement of the protein products in HSV DNA replication is described in greater detail than is presented here in the review by Challberg (1991).

The initial event in the activation of a HSV-1 origin of replication is likely to be the sequence specific binding of an origin binding protein (OBP). The product of the UL9 gene has been found to bind specifically to both oriL or oriS sequences (Olivio *et al.*, 1988). The sequence of events following UL9 binding are unclear but the two parental DNA strands must be unwound before replication is initiated. This function may also be mediated by the UL9 protein since it has been shown to have helicase activity (Bruckner *et al.*, 1991). Unwinding occurs in the 3' to 5' direction on the single-strand to which the enzyme is bound (Bruckner *et al.*, 1991; Stow, 1992; Stow *et al.*, 1993). An associated DNA-dependent nucleoside 5'-triphosphatase (ATPase) activity has also been described (Crute *et al.*, 1988; Bruckner *et al.*, 1991).

The DNA polymerase (pol) which is encoded by UL30, has an intrinsic 3'-5' exonuclease activity which probably serves a proof-reading function to increase the fidelity of DNA replication (O'Donnell *et al.*, 1987; Powell and Purifoy, 1977) and a 5'-3' exonuclease/ribonuclease H activity similar to that of *E. coli* Pol I (Crute and Lehman, 1989). This latter enzymic activity may play a role in the removal of RNA primers present on the 5' Okazaki fragments which are made on the lagging strand during semi-discontinuous synthesis. Pol exists as a heterodimer with the accessory protein (UL42) (Gallo *et al.*, 1988; Crute *et al.*, 1989). One known function of the accessory protein (encoded by UL42) is to increase the ability of pol to synthesise longer DNA products by acting to increase processivity of polymerisation (Hernandez and Lehman, 1990; Gottlieb *et al.*, 1990). This function may be facilitated by its ability to bind non-specifically to ds DNA (Gallo *et al.*, 1988; Vaughn *et al.*, 1985). Thus the role of UL42 in the DNA polymerase complex may be keep the polymerase from dissociating from the template after each cycle of catalysis (Challberg, 1991).

Infection of cells with HSV induces novel helicase and primase activities (Crute *et al.*, 1988) which are both components of a complex formed by the products of the UL5, UL8 and UL52 genes (Crute *et al.*, 1988; 1989; Wu *et al.*, 1988; McGeoch *et al.*, 1988;

Dodson *et al.*, 1989; Crute and Lehman, 1991). The helicase component unwinds duplex DNA substrates in a 5' to 3' direction on the strand to which it is bound while the primase component synthesises oligoribonucleotide primers. Thus the net function of the complex is to prime lagging-strand synthesis as it unwinds DNA at the viral replication fork (Crute *et al.*, 1988; Crute and Lehman, 1991). The precise role which each subunit plays in these activities is unclear but a complex consisting of only the UL5 and UL52 subunits *in vitro* exhibited all the known activities of the three component complex. In addition, purified UL5 has not been shown to act as a helicase in the absence of UL52 (Calder and Stow, 1990; Dodson and Lehman, 1991). Calder *et al.* (1992) used immunofluoresence to demonstrate that the UL8 protein is important for efficient nuclear uptake of the helicase-primase complex. UL8 in addition acts to increase the efficiency of primer utilisation by stabilising the association between nascent oligoribonucleotide primers and template DNA (Sherman *et al.*, 1992). However no binding of purified UL8 to either ss DNA, ds DNA or to a DNA/RNA hybrid has been demonstrated (Parry *et al.*, 1993) suggesting that UL5 and/or UL52 are required for binding of UL8 to nucleic acid.

The major ss DNA binding protein (ICP8) is encoded by the UL29 gene (Conley *et al.*, 1981; Quinn and McGeoch, 1985) and has many of the properties that are characteristic of helix destabilising proteins. It binds preferentially and co-operatively to ss DNA templates in a sequence specific dependent manner and serves to hold ss DNA in a extended configuration (Ruyechan, 1983; Ruyechan and Weir, 1984). In addition, ICP8 enhances the denaturation of poly dA and poly dT duplexes (Powell *et al.*, 1981) and promotes DNA strand displacement in either direction (Boehmer and Lehman, 1993). Thus it is likely that ICP8 has a function analogous to that of the *E. coli* ss DNA binding protein, functioning to bind to and destabilise duplex DNA during origin unwinding and to facilitate the use of these strands for DNA polymerase. ICP8 has been shown to stimulate the DNA-dependent ATPase and helicase activities of the viral DNA polymerase and is required for the synthesis of long DNA strands (Boehmer *et al.*, 1993; Dodson and Lehman, 1993).

ICP8 has also been shown to form intracellular complexes with DNA (Lee and Knipe, 1983). In the absence of viral DNA replication, ICP8 is found at discrete pre-replicative sites throughout the nucleus whereas in the presence of viral DNA replication it is localised to replication compartments (Quinlan *et al.*, 1984; deBruyn Kops and Knipe, 1988; Bush *et al.*, 1991). Together with evidence that ICP8 interacts specifically with other replication proteins (Vaughn *et al.*, 1984; Bush *et al.*, 1991; McLean *et al.*, 1994), it has been suggested that ICP8 plays a role in organising a multi-protein complex at the replication fork by providing specific contacts with the other replication proteins.

3.3.1 Enzymes indirectly involved in DNA replication

There are many other HSV-encoded enzymes not required for DNA synthesis that are, nevertheless, involved in nucleic acid metabolism.

The HSV UL23 gene encods a pyrimidine deoxyribonuclease kinase enzyme, referred to as **thymidine kinase** (TK) phosphorylates thymidine (Dubbs and Kit, 1964) deoxycytidine (Jamieson *et al.*, 1974) and thymidylate (Chen and Prusoff, 1978). It is not essential for virus growth in exponentially growing cells (Jamieson *et al.*, 1974) but is required for normal virus multiplication *in vivo* (Tenser and Dunstan, 1979). It is thought to be important for the reactivation of the virus from latency (Coen *et al.*, 1989; Efstathiou *et al.*, 1989).

The HSV-1 UL2 ORF encodes a **uracil DNA glycosylase** (Caradonna and Cheung, 1981; Cardonna *et al.*, 1987; McGeoch *et al.*, 1988) which is non-essential for growth in tissue culture (Mullaney *et al.*, 1989). This enzyme functions in DNA repair and proof-reading by removing uracil residues from DNA created either by the deamination of cytosine or the incorporation of dUTP into DNA. The extremely high G+C content of HSV DNA makes this enzyme an important element of error correction in HSV DNA replication.

An HSV encoded **deoxyuridine 5' triphosphatase** (dUTPase) has been identified (Cardonna and Cheung, 1981) which catalyses the hydrolysis of dUTP to dUMP and pyrophosphate, leading to a reduction in the intracellular concentration of dUTP, thereby reducing the incorporation of uridine into DNA and providing a pool of dUMP for conversion into dTMP by thymidylate synthase (Wohlrab and Francke, 1980). The dUTPase (encoded by UL50) is not essential for viral replication *in vitro* (Preston and Fisher, 1984; Fisher and Preston, 1986). Recent studies have indicated that the dUTPase may be important for neurovirulence and neuroinvasiveness as well as virus reactivation from latency (Pyles *et al.*, 1992).

Ribonucleotide reductase catalyses the reduction of the four ribonucleotides to the corresponding deoxyribonucleotides, creating a pool of substrates for DNA synthesis. The enzyme is composed of a large (Mw. 144kDa; UL39) and a small (Mw. 38kDa; UL40) subunit which are both required for full enzymatic activity (Preston *et al.*, 1984; Bacchetti *et al.*, 1986; Ingemarson and Lankinen, 1987) Although this enzyme has an essential role in DNA synthesis, it is not essential for virus growth in actively dividing cells at low temperatures which suggests that the host cell ribonucleotide reductase can substitute under these conditions (Dutia, 1983; Goldstein and Weller, 1988a,b; Preston *et al.*, 1988).

A virus specific **topoisomerase** has also been reported (Muller *et al.*, 1985). This enzyme, which serves to break the phosphodiester backbone of DNA and then to reseal

the free DNA ends allows for strand passage and alterations in DNA topology and was later shown not to be virally encoded (Bapat *et al.*, 1987).

Another HSV-1-encoded enzyme, **alkaline nuclease** (UL12), appears to be involved not in DNA synthesis but in processing and packaging the DNA into capsids that are able to mature into the cytoplasm (Weller *et al.*, 1990; Shao *et al.*, 1993). It has been suggested that this enzyme is required for the efficient processing of viral DNA replication intermediates (Martinez *et al.*, 1996a).

3.4 REGULATION OF HSV-1 GENE EXPRESSION

Herpesvirus DNA is transcribed in the nucleus by cellular DNA dependent RNA polymerase II but with the participation of viral factors at all stages (Wagner and Roizman, 1969; Costanzo *et al.*, 1977). In common with most eukaryotic mRNAs, HSV-1 transcripts tend to be capped at their 5'-termini and methylated at internal nucleosides (Moss *et al.*, 1977). The viral proteins are synthesised in the cytoplasm and many undergo some form of post-translational processing (reviewed by Roizman and Sears, 1990) such as cleavage, myristylation, phosphorylation, poly(ADP)ribosylation and sulphation.

Virus specific polypeptides have been classified according to their temporal order of synthesis into three broad groups; immediate-early (IE or α), early (β) and late (γ) The sequential appearance of these proteins is regulated in a cascade pattern; the IE proteins, by allowing DNA replication, are required for the induction of the early proteins which in turn are required to facilitate late protein synthesis (Honess and Roizman, 1973;1974;1975; Swanstron and Wagner, 1974; Roizman *et al.*, 1975; Clements *et al.*, 1977; Sacks *et al.*, 1985).

The properties and functions of the IE polypeptides have been reviewed by Everett, 1987). They are encoded by the IE mRNAs which can be expressed abundantly without *de novo* protein synthesis (Kozak and Roizman, 1974; Clements *et al.*, 1977; Jones *et al*, 1977). They have regulatory functions and are required for the synthesis of subsequent polypeptide groups. IE protein synthesis is detectable within 1h p.i. and peaks at 3-4 h p.i. after which it declines (Honess and Roizman, 1974; Harris-Hamilton and Bachenheimer, 1985). However, in the case of the IE110 gene, accumulation of the protein product has been shown to continue late in infection (Everett, 1991; Everett and Orr, 1991) although the functional significance of this is not understood.

Recent work on the control of gene expression has resulted in division of early (β) and late (γ) genes each into two subsets (Pereira *et al.*, 1977; Wagner, 1985). β proteins reach their peak rate of synthesis between 5 and 7 h p.i. after which synthesis

subsequently declines. β1 genes appear earlier in infection and have previously been mistaken for IE proteins (Clements *et al.*, 1977). Unlike β2 genes they were found to be expressed in the presence of the arginine analogue canavanine (Pereira *et al.*, 1977). However, neither β1 nor β2 genes are expressed in the absence of competent IE proteins (Preston, 1979; Dixon and Schaeffer, 1980; O'Hare and Hayward, 1985; Quinlan and Knipe, 1985). The appearance of early proteins signals the onset of viral DNA synthesis and most viral genes involved in viral nucleic acid metabolism appear to be in this group.

The late (γ) genes form a continuum differing in their timing and requirement for DNA synthesis and most encode structural polypeptides. γ1 genes are expressed relatively early in infection, reach their peak between 8-10h p.i. and persist for the remainder of the lytic cycle (Honess and Roizman, 1974; Harris-Hamilton and Bachenheimer, 1985). These genes are only partly affected by inhibitors of DNA synthesis and are also known as βγ (or leaky late) genes. Conversely, γ2 genes are expressed late in infection only following the onset of DNA synthesis and thus are very poorly expressed in the presence of DNA inhibitors (Honess and Roizman, 1974; Holland *et al.*, 1980; Conley *et al.*, 1981; Pederson *et al.*, 1981; Johnson and Everett,1986a).

The switching on of IE genes has been shown to be dependent on a viral protein, VP16 (also known as αTIF and Vmw65), the product of the UL48 gene (Moss *et al.*, 1979; Campbell *et al.*, 1984; Ace *et al.*, 1988). This protein has been shown to induce expression of IE genes prior to *de novo* viral protein synthesis (Batterson and Roizman, 1983). IE proteins do not bind directly with the DNA promoter sequence (Marsden *et al.*, 1987) but participate in the formation of a multiprotein complex, involving host proteins (Preston *et al.*, 1988), which initiates trancription. In addition to Vmw65, the IE proteins Vmw110 and Vmw175 also appear to be involved in the regulation of gene expression (Everett, 1984; 1986; Gelman and Silverstein, 1986; 1987; O'Hare and Hayward, 1985; 1987).

Gene expression, as mentioned above, is regulated at a transcriptional level although mechanisms operating at a post-transcriptional level play an important role in regulating the cascade pattern of protein synthesis (Weinheimer and McKnight, 1987). Distinct promoters and regulatory domains are important in determining the kinetic class of the HSV-1 genes (Silver and Roizman, 1985; Wagner, 1985; Johnson and Everett, 1986b; Roizman and Spears, 1990). The role of the immediate early genes in regulation of transcription is reviewed in detail by Everett (1987), Roizman and Sears (1990), and Hayward (1993).

3.5 PACKAGING AND ENCAPSIDATION OF REPLICATED DNA

Shortly after the virus enters the cell and the subsequent release of DNA from the capsid into the nucleus, the HSV-1 genome circularises (Poffenberger and Roizman, 1985) and the DNA is replicated via a rolling circle mechanism forming head to tail concatemars (Jacob *et al.*, 1979). This results in the generation of a novel *a-a* joint fragment in which tandem *a* sequences share a DR1 (direct repeat 1) element, the position where cleavage of the concatemeric DNA into unit-length DNA molecules takes place (Mocarski and Roizman, 1981; 1982). The *a* sequence has been shown to contain the *cis*-acting elements required for site specific cleavage and packaging (Vlazney and Frenkel, 1981; Mocarski and Roizman, 1982; Stow *et al.*, 1983; Spaete and Mocarski, 1985). Stow *et al.* (1987) have shown that plasmid DNA containing an *a* sequence along with the HSV-1 origin of replication could be packaged and propagated as a defective genome.

The signals for cleavage and packaging have been localised to two highly conserved regions, designated *Pac-1 and Pac-2*, within the Ub and Uc sequence (described on page 4) across the *a-a* junction of concatemeric DNA. Thus cleavage generates two new termini, ba and ac, each possessing a copy of the packaging signal (Varmuza and Smiley, 1985; Deiss *et al.*, 1986; Nasseri and Mocarski, 1988; Smiley *et al.*, 1992). Dalziel and Marsden (1984) identified two viral proteins of molecular weights 21kDa and 22kDa that bound to the HSV-1 *a* sequence. Two different proteins (>250kDa and 140kDa) have been identified which form a sequence specific complex with *Pac-2* (Chou and Roizman, 1989). However, these proteins are now thought to bind RNA.

Only genomic DNA molecules that are processed into unit lengths are packaged into viral capsids. Cleavage and packaging of DNA are linked processes and several different models have been proposed (Deiss *et al.*, 1986; Deiss and Frenkel, 1986; Roizman and Sears, 1990) Basically, during replication a putative cleavage/packaging protein complex recognises and cleaves an *a-a* junction within the concatemeric molecules of viral DNA only when it occurs in a particular orientation with regards to the flanking unique and direct repeat elements. Cleavage at the next similarly orientated junction will therefore generate a unit length genome with an *a* sequence at both termini. Cleavage of the viral DNA at an *a* sequence is thought to initiate packaging by enabling insertion of a free terminus into a pre-formed type B capsid. The DNA may then be continuously packaged into this structure until the next identically orientated *a* sequence is recognised and cleaved.

The precursor-product relationship between type B and type C capsids has lead to the proposal that DNA is packaged into type B capsids and concomitant with entry of DNA is the loss of the scaffolding protein and the appearance of type C capsids (Ladin *et al.*, 1980; Perdue *et al.*, 1976; Lee *et al.*, 1988; Sherman and Bachenheimer, 1988; Rixon *et*

al., 1988). Similar models have been proposed for the packaging of concatemeric DNA into the preformed proheads of bacteriophage T4 (reviewed by Black and Showe, 1983) and P22 (King *et al.*, 1973).

DNA is thought to enter the capsid at one discrete site. The suggested site for entry and exit of DNA is at a capsid vertex although no morphological difference between the 12 vertices has been observed (Newcomb and Brown, 1994; Zhou *et al.*, 1994). Several studies have revealed electron-dense nucleoprotein fibres penetrating HSV-1 nucleocapsids which may represent DNA in the process of passing from the nucleoplasm into the interior of capsids (Nii *et al.*, 1968a; Friedmann *et al.*, 1975, Luetzeler and Heine, 1978).

An alternative model was presented by Pignatti and Cassai (1980) who proposed that newly replicated viral DNA is packaged into nucleoprotein complexes (NPC) which consisted of the capsid proteins VP5, VP19C and VP24 as well as several minor polypeptide species. These NPC were thought to represent an intermediate stage in capsid assembly and addition of further structural polypeptides would produce mature nucleocapsids. However, in view of what is now proposed about packaging ie. that DNA is packaged into preformed capsids, this model seems unlikely.

The maturation of viral DNA is a complex process involving multiple viral gene products and is dependent on capsid formation. Thus *ts* mutants in the HSV-1 UL18, UL19 and UL38 genes, which encode proteins essential for capsid assembly, neither form capsids nor process viral DNA into genome length molecules at the restrictive temperature (Weller *et al.*, 1987; Pertuisset *et al.*, 1989; Desai *et al.*, 1993). The mutant *ts*1201 which fails to process the precursor form of the scaffolding protein at the NPT, forms capsids with a larger core morphology is unable to cleave and package DNA until downshift to the PT. *De novo* protein synthesis is not required and this demonstrates the importance of the maturation of the scaffolding protein to DNA packaging (Preston *et al.*, 1983).

Other viral proteins which are not required in the assembly of type B capsids are required for the formation of full capsids. Mutants in UL6, UL15, UL25, UL28, UL32 and UL33 (Sherman and Bachenheimer 1988; Addison *et al.*, 1984; 1990; Al-Kobaisi *et al.*, 1991; Poon and Roizman, 1993; Tengelson *et al.*, 1993; Baines *et al.*, 1994; Patel *et al.*, 1996) synthesised near wild type levels of DNA but were unable to cleave and package the viral DNA and thus accumulated only B type capsids. The effect of the *ts* mutation was normally reversible after downshift to the PT but only after new proteins and capsids were synthesised. This indicates the tight coupling of cleavage and packaging events. Recently, the UL6 protein has been identified as being closely associated with capsids (Patel and McLean, 1995) which might be expected if it formed part of a packaging complex.

3.6 ASSEMBLY OF CAPSIDS; A HISTORICAL PERSPECTIVE

Capsid assembly takes place in the nuclei of infected cells (Morgan *et al*, 1954; 1959) and not surprisingly, capsid proteins have been detected by immunofluorscence in the nuclei of infected cells (Heilman *et al.*, 1979; Cohen *et al.*, 1980). Small electron-dense granules consisting of clumps of intranuclear amorphous or granular material were observed in HSV-1-infected cells tagged with immunoferritin prepared against the virus. These were suggested to represent a subunit of either the capsid or the core which simultaneously came together in the correct spatial position and condensed to form a core-containing nucleocapsid (Nii *et al.*, 1968c; Miyamoto, 1971).

Powell and Watson (1975) using antiserum prepared against the major capsid protein (MCP) VP5, determined that this protein was present as large aggregates in the nucleus of infected cells. VP5 only appeared in the nucleus after a lag period and it was suggested that this lag was caused by the assembly of a cytoplasmic multi-component complex before translocation to the nucleus (Knipe and Spang, 1982). A similar lag in transport for the MCP has been reported for HCMV. Transport was inhibited in the presence of cycloheximide (added 72h p. i.) which suggested that transport to the nucleus of MCP required the continuous synthesis of proteins (Yamauchi *et al.*, 1985). Recently it has been shown that VP5, expressed in the absence of other viral proteins, does not localise to the nucleus suggesting that it lacks a nuclear localisation signal and requires to be chaperoned to the nucleus by another protein (Nicholson *et al.*; 1994). Pre-VP22a and VP19C were both found to be capable of relocating VP5 to the nucleus (Nicholson *et al.*, 1994; Dr F. Rixon, personal communication).

As early as 4 hours post-infection some viral proteins, in particular VP5, have been found associated with the nuclear matrix. At late stages in infection (15 hours post-infection), capsids devoid of DNA were observed bound to the filamentous networks of the nuclear matrix (Bibor-Hardy *et al.*, 1982a; 1982b; 1985; Tsutsui *et al.*, 1983). This suggested an important role for the nuclear matrix in nucleocapsid morphogenesis. Bibor-Hardy *et al.* (1985) observed that VP5 was synthesised on polyribosomes associated with the cytoskeleton. VP5 was then observed entering via a nuclear pore and accumulating in the centre of the nucleus within the non-chromatin network where capsids were rapidly formed.

In PRV-infected cells which were depleted of arginine, migration of structural polypeptides from the cytoplasm to the nucleus did not occur (Mark and Kaplan, 1971). These researchers postulated that an arginine-dependent protein acted to condense structural proteins in the nucleus thus creating reduced levels of these proteins free in the nucleus, leading in turn to a flow of capsid proteins from the cytoplasm to the nucleus. Analysis of *ts* mutants of PRV defective at the NPT both in processing of the 35kDa capsid protein (the counterpart of HSV-1 pre-VP22a) from higher molecular

weight forms and in capsid assembly, suggested that these events were interdependent. The unprocessed higher molecular weight forms were found in the cytoplasm as were the major capsid polypeptides (Ladin *et al.*, 1982). This supported the theory that removal of uncomplexed proteins from the nucleus as a result of capsid assembly provided a gradient for the movement of proteins from cytoplasm to nucleus. In HSV-1-infected cells Braun *et al*, (1984a) detected the unprocessed forms of VP22a partitioning in the cytoplasm and the processed forms partitioning in the nucleus and hence suggested that processing was a prerequisite for nuclear localisation. This theory was disputed by studies of the HSV-1 mutant *ts*1201 which fails to process VP22a from the higher molecular weight precursor polypeptide at the NPT. Unprocessed VP22a was found to localise to the nucleus and type B capsids with the large core phenotype were formed (Preston *et al.*, 1983; Rixon *et al.*, 1988). Further evidence that capsid formation is not required for nuclear localisation of this protein was provided by the presence of VP22a in the nucleus of cells infected with the mutant *ts*2 which is defective in capsid assembly at the restrictive temperature (Preston *et al.*, 1992). Definitive confirmation that neither capsid assembly nor processing is required was obtained by expressing this protein from a recombinant vaccinia virus and from a plasmid (Nicholson *et al.*, 1994). Under these conditions, nuclear localisation of unprocessed VP22a could be observed in the absence of any other viral proteins.

In the model for nucleocapsid assembly proposed by Rixon (1993), the capsid proteins assemble via unknown intermediates into the large cored capsids characteristic of *ts*1201. The outer icosahedral shell is formed from VP5, VP19C, VP23 and VP26 whereas the protease and pre-VP22a make up the internal scaffold. The ability of these proteins to self-assemble into cores similar to those found in B capsids has suggested that they may function *in vivo* as a scaffold around which other proteins could condense to form the capsid shell (Newcomb and Brown, 1991; Preston *et al.*, 1994). Processing of the protease and pre-VP22a occurs, forming small cored B capsids into which the DNA genome is inserted with concomitant removal of VP21 and VP22a and the formation of C capsids.

A capsids are thought to be generated as a result of abortive DNA packaging which causes loss of the scaffold. It has been suggested that they are not precursors of DNA-containing capsids since they are not detected in mutant-infected cells in which only B capsids accumulate until temperature downshift results in the formation of A and C capsids (Rixon *et al.*, 1988; Sherman and Bachenheimer, 1988). Nor are they detected in the presence of certain DNA synthesis inhibitors which block the encapsidation of DNA (Friedmann *et al.*, 1975; Lee *et al.*, 1988).

The importance of capsid protein-protein interactions and the identification of intermediate structures is fundamental to understanding the sequence of events

occurring during capsid assembly and will by discussed further in light of the results presented later in this thesis.

3.7 ENVELOPMENT AND EGRESS

The maturation of nucleocapsids to virions requires the addition of tegument and envelope which are acquired *en route* from the nucleus to the outside of the cell. It is not clear whether the tegument is acquired in the nucleus or the cytoplasm or partly in both. Evidence favours the cytoplasm since capsids lacking tegument and envelope are frequently observed in the cytoplasm. In HHV-6, the addition of tegument appears to take place within a specific region of the cell termed the tegusome which is an invagination of the cytoplasm into the nucleus (Roffman *et al.*, 1990). In thin sections of these structures tegument can be clearly recognised under the electron microscope.

The primary site for envelopment is the inner lamella of the nuclear membrane (Nii *et al.*, 1968a; Darlington and Moss, 1968; Roizman and Furlong, 1974). The mechanism appears to be a budding process whereby nucleocapsids interact with areas of the nuclear membrane lacking the normal complement of cellular polypeptides (Asher *et al.*, 1969; Ben-Porat and Kaplan, 1971; Spear and Roizman, 1972). These areas may correspond to the distorted or duplicated nuclear membrane which are a common feature of herpesvirus infected cells (Nii *et al.*, 1968c). It has been shown that both the envelope acquired by HSV at the inner nuclear membrane and the inner membrane itself contain the glycoprotein precursors and that during the transit to the extracellular space the viral glycoproteins are processed to the mature forms (Torrisi *et al.*, 1992). Thus immature virions are required to pass through the Golgi apparatus for the maturation of their glycoproteins. Disruption of the Golgi apparatus with Brefeldin A (which inhibits transport between the endoplasmic reticulum and golgi apparatus) results in the accumulation of enveloped virions between the inner and outer nuclear membranes as well as the presence of large numbers of unenveloped capsids in the cytoplasm. Conversley, treatment with monensin (an ionophore which blocks the transport of membrane vesicles from the golgi apparatus to the cell surface) resulted in accumulation of enveloped capsids in intracytoplasmic vesicles and inhibiton of the egress of virions so preventing production of infectious virus. Glycoprotein maturation is also inhibited by monensin (Johnson and Spear, 1982; Cheung *et al.*, 1991; Whealy *et al.*, 1991; Eggers *et al.*, 1992). This suggested a multi-step model of nucleocapsid envelopment involving the initial acquisition of a membrane by budding of capsids through the inner nuclear membrane into the perinuclear space followed by deenvelopment and release of these capsids into the cytoplasm. Capsids are then transported, either freely or enclosed within a vacuole, to the Golgi apparatus where they acquire a bilaminar double envelope

containing mature glycoproteins. The mature virion finally leaves the cell by exocytosis from Golgi-derived vesicles (reviewed by Rixon, 1993). That the virions acquire their envelope from Golgi-derived structures is also supported by ultrastructural studies on HCMV (Haguenau and Michelson-Fiske, 1975; Severi *et al.*, 1988) and EBV (Seigneurin *et al.*, 1977). Recent work by van Genderen *et al.* (1994) also supports this pathway. These workers found that the phospholipid composition of virions differs from that of the host cell nuclei and contained lipids that are typically enriched in the golgi and plasma membrane thus suggesting that immature virions pass through these during their maturation.

The alkaline nuclease encoded by the UL12 gene may play a role in the efficient egress of capsids from the nucleus since a UL12 null mutant virus produced few mature capsids in the cytoplasm and hence reduced amounts of infectious virus. This was despite the production and encapsidation of wild type levels of viral DNA (Weller *et al.*, 1990; Shao *et al.*, 1993). The product of the UL20 gene appears to be involved in egress of the virus since deletion of this gene resulted in the accumulation of enveloped and unenveloped nucleocapsids in the perinuclear space (Baines *et al.*, 1991). This protein appears to be membrane associated but although the mechanism of its action is not clear, some cell lines are to be able to complement its function. It has also been suggested that the product of the UL11 gene, a myristylated tegument protein (MacLean *et al.*, 1987; 1992), plays a role in envelopment and release of nascent virions (Baines *et al.*, 1995).

4. AIMS OF PROJECT

This thesis describes the cloning of the capsid genes UL18, UL19, UL35 and UL38 into the appropriate baculovirus transfer vector, their transfection into parental baculovirus and the selection and characterisation of the recombinant viruses. These viruses, designated according to the capsid gene which they express are:- AcUL18, AcUL19, AcUL35 and AcUL38. Seed stocks of AcUL26 and AcUL26.5 were provided by Dr. V. Preston. These viruses were used to study interactions between the capsid proteins and to determine the protein requirements for capsid assembly. The different structures observed from coinfection of combinations of the recombinant viruses are described as are the purification and further characterisation of some of the resulting capsid forms.

CHAPTER 2

MATERIALS AND METHODS

1 MATERIALS

1.1. Chemicals

The chemicals used were of analytical grade and most were supplied by either BDH Chemicals or Sigma Chemical Co. except for the following:

Agar Scientific Ltd.	gluteraldehyde (25% EM grade)
Beecham Research Laboratories Ltd.	ampicillin (Penbritin)
Bio-Rad Laboratories Ltd.	ammonium persulphate, TEMED, Coomasie Brilliant Blue R250.
Biocell International	Fish gelatin (45% solution), gold conjugate (protein A conjgated to 20nm gold particles), silver enhancing kit
Boehringer Mannheim	Tris [2-amino-2 (hydroxymethyl)-1,3-propandiol]
Citifluor Ltd.	Citifluor
Difco Laboratories	bactopeptone, brain heart infusion (BHI), sabouraud medium (SAB), trypsin, tryptose phosphate broth
Du Pont Ltd.	En^{3}hance autoradiography enhancer.
Fisons	Ammonia solution (primar grade)
FMC Bioproducts Ltd.	NuSieve^{TM} GTG agarose SeaPlaque^{TM} LTG agarose
Gibco BRL Ltd.	Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)
Koch-Light Laboratories Ltd.	DMSO (dimethyl sulphoxide)
May and Baker Ltd.	chloroform, glycerol.
Melford Laboratories Ltd.	caesium chloride
National Diagnostics	Ecoscint A (scintillation solution), Sequagel^{TM}
Prolabo, Rhone-poulenc Ltd.	acetic acid (glacial), hydrochloric acid
TAAB Laboratory Equipment Ltd.	epon 812 resin, lead citrate, osmium tetroxide, phosphotungstic acid, sodium silicotungstate uranyl acetate

1.2. Radiochemicals

All radioisotopes were supplied by Amersham International plc. They had the following activities:-

[^{35}S]-L-methionine	>1000Ci/mmol
5' [α-^{32}P]dNTPs	~3000Ci/mmol
[^{14}C] methylated proteins	5uCi/ml

1.3. Miscellaneous materials

Bacterial and tissue culture plates	Gibco BRL Ltd.
BEEM capsules	Agar Aids
Belco spinner flasks	Scientific Laboratory Supplies Ltd.
Cellulose nitrate (E)	Schleicher and Schuell.
DNA ladders; 100bp and 1Kb	Gibco BRL Ltd.
Hybond N nylon membrane	Amersham International plc.
Nick™ prepacked column (sephadex G-50)	Pharmacia
Rainbow™ protein molecular weight markers	Amersham International plc.
RNasin recombinant ribonuclease inhibitor	Promega
Sephaglass™ bandprep kit	Pharmacia
Sequenase™ version 2.0 DNA sequencing kit	Amersham International plc.
Slot-blot hybridisation kit; S+S Minifold II	Schleicher and Schuell.
TNT™ lysate system	Promega
T7Sequencing™ kit	Pharmacia
Ultrapure dNTP set (2'-deoxynucleoside 5'-triphosphate)	Pharmacia
Universal primer (Sequenase Version 2.0; DNA Sequencing kit)	Pharmacia
X-Omat-S film	Kodak Ltd.

1.4. Enzymes

Restriction Enzymes were obtained from either Bethesda Research Laboratories (BRL) or New England Biolabs (NEB). The following were supplied by:

Agarase (from *Pseudomonas atlantica*)	Boehringer Mannheim
Calf intestinal phosphatase (CIP)	Boehringer Mannheim
T4 polynucleotide DNA ligase and buffer	Gibco BRL Ltd.
T4 polynucleotide kinase	New England Biolabs
T7 DNA polymerase	Pharmacia
Proteinase K (from *Tritirachium album*)	Sigma
Ribonuclease A (RNaseA)	Sigma

Deoxyribonuclease I (DNase 1)	Sigma
Klenow large fragment polymerase	Made by Dr. A. Davison and M. Watson
Taq polymerase	Life Technologies Ltd.

1.5. Oligonucleotides

Synthetic restriction enzyme linker oligonucleotides were obtained from New England Biolabs.

Oligonucleotides 1 and 2 used in the cloning of UL35 were synthesised by Dr. John McLauchlan using a Biosearch 8600 DNA synthesiser.

1.6. Bacterial Growth Media

L-broth:- 10g/l NaCl (170mM), 10g/l bactopeptone, 5g/l yeast extract.

L-broth agar:- L-broth containing 1.5% (w/v) agar.

Ampicillin, where appropriate was added to L-broth or L-broth agar at 50ug/ml.

1.7. Bacterial strains

The strain used for cloning was the *E. coli* K 12 derivative, JM101 [F'traD36, supE, thi, lacproAB, proAB, lac192 M15] as described by Yanisch-Perron *et al.* (1985).

Bacteria were stored as overnight cultures in 40% (v/v) glycerol at -70°C.

1.8. Bacterial Plasmids

pUC18 and **pUC19** are the cloning vectors described by Vieira and Messing (1982) and Norrander *et al.* (1983) and were supplied by Bethseda Research Laboratories.

PT3T7 18U is a cloning vector which consists of the pUC18/19 polylinker region flanked by T3 and T7 promoters and was obtained from Pharmacia.

pGEM-2 plasmid contains SP6 and T7 RNA polymerase promoters either side of a multiple cloning region to allow for transcription of genes cloned downstream of the promoters. This plasmid was obtained from Promega.

pAcYM1 is the baculovirus transfer vector described by Matsuura *et al.* (1987). This vector contains a section of the *Autographa californica* nuclear polyhedrosis virus EcoR1 restriction fragment I with a BamH1 cloning site 3' of the polyhedrin gene promoter.

pAcCL29.1 is a baculovirus transfer vector derived from pAcYM1 and is described by Livingston and Jones (1989). It contains a polylinker region downstream of the polyhedrin promoter and has single strand capability.

pGX122 contains the HSV-1 DNA restriction fragment Kpn *c* in the vector pAT153, and was constructed by Davison and Wilkie (1983). Kpn *c* was inserted into the Pst1 site of pAT153 following addition of homopolymer tracts of deoxycytidine residues to the Kpn1 fragment and deoxyguanosine residues to the Pst1 site.

pMJ521 contains the UL18 ORF cloned as a Nar1 restriction fragment into the Nar1 site of the vaccinia transfer vector pMJ601 as described by Nicholson *et al.*, 1994. Nar1 cuts 2 residues upstream from the UL18 initiation codon within the sequences encoding the untranslated leader of UL18 mRNA and 381 residues downstream from the stop codon.

pBJ199 contains the UL19 ORF cloned into the BamH1 site of PAcYM1 as described by Nicholson (1992) and Tatman *et al.* (1994).

pBJ182 contains the UL38 ORF cloned into the HincII site of pUC18; the construction of which is described by Nicholson (1992) and Tatman *et al.* (1994).

pCMV10 is a eukaryotic expression vector containing the major immediate-early (IE) promoter of HCMV and RNA processing signals of SV40 and is described by Stow *et al.* (1993).

1.9. Cell Lines

The insect cells used were the *Spodoptera frugiperda* cell line, **Sf21** which are derived from ovarian tissue (IPLB-Sf21; Vaughn *et al.*, 1977).

The BHK cells used were the **BHK-21 clone 13** fibroblastic cell line derived from baby hamster kidney cells (MacPherson and Stoker, 1962).

1.10. Tissue culture media

Sf21 cells were grown in **TC100/5** which consisted of TC100 medium (Gibco/BRL) supplemented with 5% foetal calf serum (FCS); supplied by Gibco/BRL and 1% PS (1000units/ml penicillin/10mg/ml streptomycin; supplied by Gibco/BRL).

BHK21/C13 cells were grown in **ETC_{10}**, consisting of Glasgow-modified Eagle's medium (GMEM; Busby *et al.*, 1964) supplied by Gibco/BRL and supplemented with 10% (v/v) tryptose phosphate broth, 10% newborn calf serum (NCS); supplied by Gibco/BRL and 1% PS.

Variations on the basic growth media were:

TC100/10	TC100 medium containing 10% FCS and 1% PS
$TC100_{-met}$	TC100 medium minus methionine (Gibco/BRL) used in protein labelling with [^{35}S]methionine .
$ETC_{1/5met}$	ETC_{10} containing 1/5 the normal concentration methionine and 2% NCS; used for labelling with [^{35}S]methionine.
EMC_{10}	Eagles medium containing 1% carboxymethyl cellulose and 10% newborn calf serum.
Opti-MEM	Supplied by Gibco/BRL; used in transfections.
BHF	Brain heart infusion (37g/l) used in sterility checks.
SAB	Sabouraud medium (30g/l) used in sterility checks.

1.11. Viruses

Two parental baculoviruses were used: the wild type *Autographica californica* nuclear polyhedrosis virus **AcNPV** and **AcPAK6**, a polyhedrin-negative derivative which has the *lacZ* gene under control of the polyhedrin promoter (Bishop, 1992). Both were gifts from R. Possee.

The wild-type **HSV-1** virus was strain 17 syn$^+$ which has been described by Brown *et al.* (1973).

The temperature sensitive mutant ***ts*1201** which contains a mutation mapping to the UL26 ORF and which displays a distinctive capsid morphology has been described by Preston *et al.* (1983).

AcUL26 and **AcUL26.5** are baculoviruses recombinant for the HSV-1 capsid genes UL26 and UL26.5 which express the proteins VP21/VP24 and the unprocessed form of VP22a (pre-VP22a) respectively and are described by Preston *et al.* (1994).

1.12. Antibodies

MCA 406 is a mouse monoclonal antibody against the HSV-1 capsid protein VP22a, the processed product of the UL26.5 gene. It is commercially avaliable from Serotec and was used in Western blot analysis of VP22a.

TrpE-UL35 is a rabbit antibody raised against a trpE-UL35 chimeric protein and recognises the HSV-1 capsid protein VP26, the product of the UL35 gene. Its

construction and characterisation is described by McNabb and Courtney (1992a). It was a gift from Dr. R. Courtney.

GARTRITC (tetramethylrhodamine isothiocyanate) is a goat anti-rabbit IgG (whole molecule) antibody conjugated to texas red and is used in immunofluorescence experiments. It was obtained from Sigma.

1.13. Commonly used buffers and solutions

Cell lysis buffer	0.6% SDS, 10mM Tris-HCl (pH7.5), 1mM EDTA
Gel soak 1	600mm NaCl, 200mM NaOH
Gel soak 2	600mm NaCl, 1M Tris-HCl, adjusted to pH8.0 with HCl
HBS	20mM HEPES, 150mM NaCl; pH7.4
Hybridisation buffer	7% SDS, 0.5M $NaPO_4$ (1M NaH_2PO_4:1M Na_2HPO_4, 19:81)
Loading buffer (5x)	5x TBE, 50% glycerol, bromophenol blue, xylene cyanol
Membrane wash buffer	1x SSC, 0.25% SDS
Phenol/chloroform (1:1)	This is a 1:1 mixture of phenol and chloroform and has been saturated with 10mM Tris-HCl, pH8.0.
PBS A	170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH7.2
PBS complete	PBS A plus 6.8mM $CaCl_2$ and 4.9mM $MgCl_2$
PBS/CT	PBS complete containing 5% NCS and 0.05% Tween 20
SDS-PAGE buffers:	
dissociation mix (3x)	30% SGB, 30% glycerol, 15% 2-mercaptoethanol, 6% SDS, 0.03% bromophenol blue
4x RGB	1.5M Tris pH8.9, 0.4% SDS
4x SGB	0.488M Tris pH6.7, 0.4% SDS
Tank buffer	52mM Tris, 53mM glycine, 0.1% SDS
Commassie blue stain	Fix containing 0.2% Coomassie Brilliant Blue R250
Destain	methanol:H_2O:acetic acid; 50:880:70
Fix	methanol:H_2O:acetic acid; 50:50:7
Solution 1	25mM Tris pH8.0, 10mM EDTA, 50mM glucose
Solution 2	0.2M NaOH, 1% w/v SDS
Solution 3	3M KAc, 2M acetic acid
SSC	150mM NaCl, 15mM trisodium citrate, pH7.5
TBE	2mM EDTA, 89mM boric acid, 89mM Tris-HCl pH8.0
TE	10mM tris-HCl, 1mM EDTA, pH8.0
Trypsin	0.25% (w/v) trypsin dissolved in tris-saline
Versene	0.6mM EDTA dissolved in PBS containing 0.002% (w/v) phenol red

2. METHODS

2.1 RECOMBINANT DNA TECHNOLOGY

2.1.1. Transformation of *E. coli.*

i) Preparation of competent cells

10ml of L-broth was inoculated with 10ul of a glycerol stock of JM101 bacteria and incubated in an orbital shaker at 37°C overnight to produce a starter culture. 1.6ml of this culture was used to inoculate 90ml of L-broth which was shaken for approximately 1 1/4 h, or until the culture had reached a density of OD_{600nm} 0.2-0.3. The bacterial cells were transferred to two 50ml Falcon tubes and centrifuged at 3,000 r.p.m. for 10 minutes in a Sorvall RT6000B centrifuge. Each cell pellet was resuspended in 40ml of ice-cold, sterile $CaCl_2$ (0.1M) and incubated on ice for 2 h. The cells were then pelleted, resuspended in a total of 2ml of the $CaCl_2$ and incubated on ice for a further 2 h after which they were ready to use for transformation. The low temperature and presence of calcium ions provide optimal conditions for the interaction of plasmid DNA and E. coli cells (Cohen *et al*, 1972; Hanahan, 1983).

ii)Transformation

Typically, 1ul and 5ul of a ligation mix or plasmid preparation were incubated on ice with 100ul of competent JM101 cells. After 30 minutes the cells were subjected to a 40 second heat shock at 42°C, which promotes the uptake of plasmid DNA, and immediately placed on ice. 100ul of the transformed bacteria were plated onto L-broth agar plates containing 50ug/ml ampicillin. If *B*-galactosidase selection was used, 20ul of X-gal (50mg/ml) and 100ul of IPTG (100mM) were first plated onto the agar. Plates were allowed to dry at room temperature, before incubation in an inverted position at 37°C overnight.

2.1.2. Small scale plasmid preparation

DNA was prepared by the alkaline lysis method as described by Maniatis *et al.* (1982). Single, transformed bacterial colonies were inoculated into 5ml of L-broth containing 50ug/ml ampicillin, and shaken overnight at 37°C. 1.5ml of each culture was transferred into a 1ml reaction vial and the cells pelleted at low speed (6,500 r.p.m.) in a microfuge. The supernatant was discarded and the cell pellet resuspended in 100ul of solution 1. Following 5 minutes incubation at RT, 200ul of freshly made solution 2 was added and the preparation vortexed. 150ul of solution 3 was added and after 5 minutes incubation on ice, the resulting white precipitate consisting of the cell debris was

pelleted at high speed (13,000 r.p.m.) in a microfuge. The supernatant was transferred to a fresh 1.5ml reaction vial, the plasmid DNA extracted and precipitated as described in section 2.1.3 before resuspension at 37°C in 50ul dH_2O containing RnaseA at a final concentration of 10ug/ml. Typically, 7ul of DNA was used for restriction enzyme analysis (section 2.1.7).

2.1.3. Phenol/chloroform extraction and ethanol precipitation of DNA

If the DNA was in a volume of less than 200ul, it was increased to this volume by the addition of dH_2O. An equal volume of phenol/chloroform (1:1) was added, the mixture shaken, centrifuged for 5 minutes, either at 13,000 r.p.m. in a microfuge or at 3,000 r.p.m. in a Sorvall RT600B centrifuge, depending on the volume. The top aqueous layer was removed into a separate tube and re-extracted. To precipitate the DNA, 1/10 volume of 3M sodium acetate (pH 5.4) together with 2.5 volumes of ethanol were added, mixed and incubated for 20 minutes at -20°C. The DNA was pelleted at 13,000 r.p.m. (or 3,000 r.p.m.) for 5 minutes, washed in 70% ethanol to remove any salts, dried in a rotary evaporator and resuspended in an appropriate volume of dH_2O. The DNA was then incubated at 37°C in the presence of RNaseA (10ug/ml final concentration) and subsequently stored at -20°C.

2.1.4. Large scale plasmid preparation

Either single, transformed colonies from a L-broth agar plate or 10ul of a glycerol stock, were inoculated into 10ml L-broth containing 50ug/ml ampicillin and shaken for 4-8 h to produce a starter culture. This was then transferred into 900ml L-broth containing 100ug/ml ampicillin in a 2 litre dimpled flask and shaken overnight.

The bacteria were pelleted by centrifugation at 5,000 r.p.m. for 10 minutes in a Sorvall GSA rotor. After discarding the supernatant, the pellets from the 900ml culture volume were resuspended in 14ml of solution 1, transferred to a Sorvall SS34 tube and incubated at RT for 10 minutes. 28ml of freshly made solution 2 was added and the preparation vortexed. 30ml of ice-cold solution 3 was then added and incubated on ice for 10min. The bacterial debris and cellular DNA were pelleted by centrifugation at 12,000 r.p.m. for 30 minutes. The supernatant, containing the plasmid DNA was transferred to a 50ml Falcon tube and was extracted three times with an equal volume of phenol/chloroform (1:1) and ethanol precipitated (section 2.1.3). The DNA was resuspended at 37°C in 500ul dH_2O containing RnaseA at a final concentration of 10ug/ml.

2.1.5. CsCl banding of plasmid DNA

To remove residual host DNA and RNA, plasmid DNA was further purified by isopycnic banding on caesium chloride (CsCl) density gradients. The DNA solution was prepared for caesium banding by dissolving 1.15g of CsCl in 1ml of DNA solution and adding 50ul of 10mg/ml EtBr, resulting in a final CsCl density of 155g/ml and a final EtBr concentration of 500ug/ml. This mixture was incubated on ice for 5 minutes and then centrifuged in a microfuge at 13,000 r.p.m. for 3 minutes to pellet any precipitate. The supernatant containing the DNA was transferred to a Beckman TLV-100 ultracentrifuge tube and topped up with stock solution containing similar amounts of CsCl and EtBr. The tube was heat-sealed and centrifuged at 20°C, either for 4 1/2 h at 100,000 r.p.m., or alternatively, for 16 h at 80,000 r.p.m.

DNA was visualised either by daylight or long-wave U.V. transillumination and the lower band containing supercoiled plasmid DNA was recovered with a large bore needle and syringe. EtBr was extracted by shaking with equal volumes of isopropanol equibrilated with saturated CsCl solution. The plasmid DNA was diluted three fold with dH_2O and precipitated with ethanol as described in section 2.1.3. The DNA was dissolved in dH_2O and its concentration was determined by spectrophotometry ($O.D._{260nm}$ 1.0 = 50mg DNA/ml) and by running a small quantity on an agarose gel.

2.1.6. Preparation of glycerol stocks

To preserve the viability of plasmid DNA, 1.5ml of the starter culture (section 2.1.1) was transferred to a 1.5ml reaction vial and the cells were pelleted at a low speed in a microfuge for 1 minute. The cell pellet was resuspended in 1ml of sterile mixture consisting of 40% glycerol and 1% bactopeptone. The suspension was then frozen at -70°C until required.

2.1.7. Restriction endonuclease digestion of DNA

Restriction enzyme digests were carried out using the buffer and incubation temperature specified by the manufacturers. The number of units of enzyme added was dependent on the activity of the enzyme and the amount of DNA being digested; typically 10 units of enzyme was added per ug of DNA. If the digested DNA was to be dephosphorylated, 1 unit of calf intestinal phosphatase (CIP) per ug of DNA was added to the reaction mixture which was incubated at 37°C for a further 30 minutes.

In situations where the DNA required to be digested with two enzymes which utilised incompatable restriction buffers the first digestion was carried out and the DNA was extracted and precipitated as described in section 2.1.3 prior to digestion with the second enzyme.

2.1.8. Analytical agarose gel electrophoresis of DNA

The restriction enzyme digested or extracted DNA was mixed with 1/5 volume of loading buffer and loaded into wells on a horizontal 1% (w/v) agarose gel. Either 1kb or 100bp size markers of known concentration were run alongside fragments to enable confirmation of the vector/fragment size. The gels consisted of 1% (w/v) agarose dissolved in 1 x TBE and contained 0.5ug EtBr per ml of gel. Electrophoresis was carried out with the gel submerged in 1 x TBE at 75V for approximately 40 minutes. DNA was examined and photographed by U.V. transillumination.

2.1.9. Purification of DNA from agarose gels

5-20ug of digested plasmid DNA was mixed with an appropriate amount of loading buffer onto a 0.8-1% (w/v) agarose gel and electrophoresed in 1 x TBE at 75V. The DNA was visualised using a long-wave length ($300\text{-}600_{nm}$) transilluminator, a gel slice containing the desired fragment was excised using a sterile scalpel and transferred to a 1.5ml reaction vial.

Two different methods have been employed to extract the DNA.

i) Agarase

For this method of purification the DNA was required to be electrophoresed on a GTG agarose (NuSieve^{TM}) gel. An equal volume of 1 x TBE buffer was added to the excised gel slice which was incubated at 65^{o}C for 10-15 minutes or until the agarose was completely molten. After the reaction mixture had cooled to 45^{o}C, 2 units of agarase per 100ul of molten agarose was added and this was incubated at 45^{o}C for 1 h. The DNA was then phenol/chloroform extracted and ethanol precipitated as previously described (section 2.1.3) and the DNA resuspended in a suitable volume of dH_2O, usually 20ul.

ii)Sephaglass

The DNA was purified using the materials from a Sephaglass^{TM} BandPrep kit (Pharmacia) according to the manufacturer's instructions.

2.1.10. DNA ligation

Both vector and insert were cut with the appropriate restriction enzymes. To prevent self-annealing of the vector, it was usually treated with 1 unit CIP per ug DNA following restriction enzyme digestion (section 2.2.7). The DNA was either phenol/chloroform extracted or was purified from an agarose gel. DNA fragments were mixed in the ratio of 3 parts insert to one part vector in a total volume of 20ul containing 1 x BRL ligation buffer and 1 unit of T4 DNA ligase and incubated for a minimum of 3

h at 15°C. The ligated DNA was either used immediately in a transformation reaction or was stored at -20°C until required.

i) Ligation of a linker:

Phosphorylated oligonucleotide linkers were inserted into blunt ended restriction sites using the same procedure as for vector/fragment ligations. A 50 fold molar excess of linker over plasmid DNA was used.

Certain restriction digests produced ends that were incompatible for ligation and so had to be treated to generate the correct type of fragment ends.

ii) 5' overhangs:

For 5' overhangs purified digested DNA (section 2.1.9) was resuspended in 50ul dH_2O. dNTPs each to a final concentration of 100uM, nick translation buffer (10x:- 1M Tris pH7.5, 1M $MgCl_2$, 1M DTT, 10mg/ml BSA), 1 unit of Klenow large fragment polymerase and dH_2O was added. The reaction mixture was incubated at 15°C for 2 h before being phenol:chloroform extracted, precipitated (section 2.1.3) and ligated as described above.

iii) 3' overhangs:

For 3' overhangs purified digested DNA (section 2.1.9) was resuspended in 50ul dH_2O and T_4 polymerase buffer (10x:- 1M Tris-HCl, pH7.9, 1M $MgCl_2$, 1M DTT), T_4 chase (20x:- 4 dNTPs each at 2mM) and 12 units of T_4 polymerase, which has a strong 3'-5' exonuclease activity were added. The reaction mixture was incubated at 15°C for 2 h before being extracted with phenol:chloroform, precipitated (section 2.1.3) and ligated as described above.

2.1.11. Purification of synthetic oligonucleotides

Oligonucleotides were synthesised by Dr. J. McLauchlan on a 8600 Applied Biosystems synthesiser. The oligonucleotide was eluted from the preparation column with ammonia solution (Fisons) and incubated at 55°C for 5 h. The deprotected oligonucleotide was transferred to a 1.5ml reaction vial and the ammonia was removed by evaporation in a rotary evaporator overnight. The oligonucleotides were resuspended in 50ul of dH_2O, divided into 2 aliquots and stored at -20°C until required.

2.1.12. PAGE of synthetic oligonucleotides

Oligonucleotides were purified on a 1.5mm thick 15% acrylamide gel containing 4% bis-acrylamide and 7M urea. The acrylamide:bisacrylamide ratio of 24:1 was suitable for the purification of 15-100mer oligonucleotides. One of the DNA aliquots (section 2.1.11) was mixed with an equal volume of 90% (w/v) deionised formamide in 1 x TBE and heated at 100°C for 5 minutes prior to loading onto the gel. 10ul of the above

formamide mix containing 0.5% (w/v) bromophenol blue and 0.5% (w/v) xylene cyanol was added to the wells adjacent to the sample well to act as a migration marker. The gel was electrophoresed overnight at 60V until the bromophenol blue had reached the bottom of the tank.

To visualise the DNA, the gel was removed, wrapped in clingfilm and viewed over a silica gel thin layer chromatography plate with a short-wave U.V. lamp. The DNA in the gel absorbed the U.V. light thus casting a shadow on the fluorescent plate. If the synthesis had been successful then a predominant band, with possibly a few lower molecular weight bands, was observed. The higher band was excised with a sterile scalpel, added to a 15ml Falcon tube together with 500ul of dH_2O and shaken overnight at 37°C. The oligonucleotide solution was then transferred to a 1.5ml reaction vial, phenol/chloroform extracted and ethanol precipitated (section 2.1.3) and the resulting oligonucleotide was resuspended in 100ul dH_2O. The concentration of the oligonucleotide was estimated by spectrophotometry (OD_{260nm} 1.0 = 20ug single stranded DNA.).

2.1.13. Polymerase chain reaction (PCR)

The PCR reaction was carried out in a volume of 50ul using a Biometra Trio-Thermoblock machine. Purified oligonucleotides were diluted in dH_2O to a concentration of 3 OD/ml and 2.5ul of each were used together with 0.1ug of pGX122. dNTPs (final concentration of 200uM), PCR buffer (10x:- 500mM KCl, 100mM Tris-HCl pH8.3, 50mM $MgCl_2$) and 5 units of *Taq* polymerase were added. The reaction mixture was gently mixed, centrifuged briefly and a few drops of mineral oil was added to the surface to prevent evaporation.

The amplification reactions were carried out by the following steps:

Step 1:- 95°C for 4 min
Step 2 :- 95°C for 1 min, 10 s
55°C for 1 min 30 s
73°C for 2 min
This step was cycled 30 times
Step 3:- 73°C for 5 min
Step 4:- held at 10°C

PCR products were checked by agarose gel electrophoresis alongside the appropriate DNA size marker before gel purification and subsequent cloning.

2.1.14. Sequencing analysis of DNA

The method of sequencing used was the chain-termination method of sequencing developed by Sanger *et al.* (1977). In the original procedure, the primer extension was catalysed by the Klenow fragment of *E. coli* DNA polymerase 1. However in the method of Kristensen *et al.* (1988), T7 DNA polymerase is used. A Pharmacia T7 sequencing kit was used and the procedure was carried out according to the manufacturer's instructions. As the DNA to be sequenced had been cloned into the pUC19 plasmid, the primer used was a universal primer (-40'); 17mer, sequence 5'GTTTTCCCAGTCACGAC, concentration 0.5pmol/ul.

2.1.15. Sequencing gels

Two glass plates (44.5 x 33cm), one notched, were treated with Repelcote (BDH), a water repellent which produces a silicone surface on glass, and a 0.22mm cast prepared. The products of the sequencing reaction were resolved on an 8% denaturing polyacrylamide gel prepared from Sequagel™ (National Diagnostics). 75ml of gel mix was prepared as per manufacturer's instructions. After pre-electrophoresis in tank buffer (0.5 x TBE) at 40W for 30 minutes, the wells were washed and 1.5ul aliquots of the denatured sequencing reactions (section 2.1.14) were loaded into the wells. Electrophoresis was carried out at 40W for approximately 2 h, or until the bromophenol blue dye front reached the bottom of the gel. The gel was then removed from the apparatus, the plates separated and the gel plate supporting the gel immersed for 10 minutes in a solution containing 10% acetic acid and 10% methanol. This fixed the DNA into the gel and prevented diffusion of the bands The gel was transferred to Whatman paper, covered in clingfilm, dried down under vacuum and set up for autoradiography against Kodak XS-1 film.

2.1.16. *In Vitro* Transcription-translation

The method used was the TNT™ Coupled Reticulate Lysate system (Promega) which provides one step translation in rabbit reticulocyte lysate of DNA sequences cloned downstream of an RNA polymerase promoter. The gene to be expressed (UL35) was cloned into pGEM-2 downstream of the SP6 polymerase promoter. 1ug plasmid DNA was mixed with the reagents specified in the TNT lysate system and the reaction continued as per manufacturer's instructions. Luciferase control DNA provided by Promega was used as a control. Proteins were labelled with [^{35}S]-methionine and at the end of the translation assay, a fraction of each sample was analysed by SDS-PAGE and visualised by fluorography as described in section 2.2.10.

2.1.17. Transfection of plasmid DNA and immunofluorescence

The UL35 ORF was placed under the control of the CMV I.E. promoter in the plasmid pCMV10. The procedure was carried out essentially as described by Nicholson *et al.* (1994).

i) Transfection of plasmid DNA

13mm coverslips were placed in 24-well culture dishes and seeded with 2 x 10^5 BHK21 cells in 1ml of ETC_{10}. After overnight incubation at 37°C the cells, which formed a 50% confluent monolayer, were washed in PBS complete and the plasmid DNA transfected by the lipofectin method (Rose *et al.*, 1991). 1ug of plasmid DNA was made up to a total volume of 20ul in HBS and mixed with 6ul of the synthetic liposomes and 14ul Opti-MEM. Following incubation for 10 minutes at RT, an further 500ul Opti-MEM was added and the transfection mix added onto the cells. After 4 h incubation at 37°C, 500ul of ETC_{10} was added. After a further 24 h incubation, the cells were washed in PBS complete and fixed in chilled methanol (-20°C) for a minimum of 30 minutes. The cells could be stored in methanol at -20°C if required.

ii) Immunofluorescence

Subsequent steps were performed at RT in a humidified chamber. Each coverslip was incubated for 10 minutes in PBS/CT, drained and incubated for 45 minutes in 50ul of the primary antibody; TrypE-UL35 (McNabb and Courtney; 1993) diluted 1:300 in PBS/CT. After 3 x 10 second washes in PBS/CT the coverslips were incubated in 50ul of a 1:100 dilution (in PBS/CT) of the secondary antibody; GARTRITC (Sigma). After 45 minutes, the coverslips were washed 3 x in PBS/CT, 2 x in dH_2O, drained, mounted upside down on glass microscope slides in Citifluor and sealed using clear nail polish. The slides were stored in the dark prior to being viewed using a Nikon microphot-SA fluorescence microscope. Photographs were taken using Kodak Ektachrome 400 film.

2.1.18. *In vitro* labelling of DNA

Plasmid or gel purified fragments of DNA were labelled with [^{32}P] using the Nick Translation method. The reaction was carried out in a 50ul volume consisting of DNA, nick translation buffer (10x:- 1M Tris pH7.5, 1M $MgCl_2$, 1M DTT, 10mg/ml BSA), 1.5ul of each [^{32}P]-labelled dNTP, 4 units DNA Polymerase 1 and 2ul DNase 1 (10^{-4} mg/ml). The reaction mix was incubated at RT for 2-4 h. A nick prepacked column (Pharmacia) was equilibrated with TE. The [^{32}P]-labelled probe was loaded onto the column and eluted with TE; the presence of the probe in the 2nd or 3rd 5 drop fractions could be confirmed by determination of the peak counts of radioactivity. The volume of the probe was made up to 1ml with TE prior to denaturing with the addition of 200ul

1M NaOH for 10 minutes followed by neutralisation with 200ul 1M HCl. The [^{32}P]-labelled probe was then ready for hybridisation.

2.1.19. Immobilisation of DNA to a membrane and hybridisation of a labelled probe

i) Slot blot analysis of DNA

To identify viruses recombinant for a specific sequence of bases, DNA prepared from infected cell extracts as described in section 2.2.6 was subjected to slot blot analysis using the S+S minifold^{R} II apparatus (Schleicher and Schuell).

The method used was essentially that of Ausubel *et al.*, (1990). Basically ~5ug of DNA was diluted to 440ul in TE and denatured by the addition of 49ul 1M NaOH for 30 minutes at RT. The DNA was neutralised on ice by the addition of 4.9ml of 6 x SSC. The DNA sample was immobilised onto the S+S nitrocellulose membrane (which had been pre-soaked in 6 x SSC buffer) as described by the manufacturer. The slots were washed with 2 x 500ul aliquots of 6 x SSC buffer and the DNA cross-linked onto the membrane using a U.V. stratalinker 1800 (Stratagene).

ii) Southern blot analysis of DNA

Infected cell viral DNA or plasmid DNA was digested with the appropriate restriction enzyme(s) and the fragments separated by electrophoresis on agarose gels as described in section 2.1.8. The DNA in the gel was denatured by washing in Gel Soak 1 for 1 h, rinsed with dH_2O and transferred to Gel Soak 2 for a further 1 h. The DNA was transferred to a sheet of Hybond N nylon membrane (Amersham) using the capillary transfer method as described by Maniatis *et al.* (1982). The DNA was cross-linked to the nylon membrane as decscribed above.

iii) Hybridisation of [^{32}P]-labelled probe

Hybridision was carried out in a sealed bag. Prior to hybridisation, the nylon membrane containing the DNA was pre-treated by soaking in 10ml of hybridisation buffer for 30 minutes at 70^{o}C. The denatured, radiolabelled DNA probe was injected into the bag which was resealed and the incubation continued overnight at 70^{o}C. The membrane was washed three times with pre-warmed (70^{o}C) membrane wash buffer before being set up for autoradiography against Kodak XS-1 film.

2.2. VIRUS PREPARATION AND POLYPEPTIDE EXPRESSION

2.2.1. Tissue culture

i) Spodoptera frugiperda 21 (Sf21) cells

Sf21 cells were routinely passaged every 3-4 days in 150cm^2 tissue culture flasks in 75ml of TC100/5 medium at 28^{o}C. Confluent monolayers were dislodged into 10ml of TC100/5 by banging the flask against a bench. The yield from a confluent flask was ~4-6 x 10^7 cells and the cells remained viable for 24 h when stored at 4^{o}C. In some instances, for example, large scale production of virus and capsid purification, suspension cultures of Sf21 cells were required. Cells harvested from a confluent 150cm^2 flask were added to a 850cm^2 roller bottle containing 300ml TC100/5 medium which was then turned at a speed (~4 r.p.m.) sufficient to prevent the cells growing on the surface of the bottle. Alternatively, the cells from the flask were added to a 1000ml spinner culture bottle containing 300ml TC100/5. After 5 days, when the cells had reached a density of 2-3 x 10^6 cells/ml, fresh cultures were seeded at 1-2 x 10^5 cells/ml (1:10 split). These were passaged every 4-5 days when the cell density had again reached 2-3 x 10^6 cells/ml. Cell densities were determined by counting a sample of the cells in a Neubauer counting chamber.

ii) BHK21 C13 cells

These were routinely passaged in 850cm^2 plastic roller bottles seeded with approximately 2 x 10^7 cells at 37^{o}C in 100ml ETC_{10} and an atmosphere of 5% CO_2 in air. Confluent monolayers were resuspended in 20ml ETC_{10} after 2 versene washes and brief trypsinisation with trypsin/versene (1:4). A confluent roller bottle yielded approximately 1 x 10^8 cells which remained viable for at least 5 days when stored at 4^{o}C.

2.2.2. Freezing, storage and recovery of *S. frugiperda* cells

Protocols followed were based on those described by King and Possee (1992).

i) Freezing and storage

A confluent flask of healthy Sf21 cells was harvested in 10ml TC100/5 medium. The cell suspension was transferred to a 15ml falcon tube and the cells pelleted at 1,500 r.p.m. for 5 minutes in a Sorvall RT6000B centrifuge. Cells were resuspended in 2ml TC100/10 and an equal volume of ice-cold cell freezing mix (80% (v/v) TC100/10, 20% (v/v) DMSO) was added. 2ml aliquots were placed into cryogenic vials which had

been chilled on ice. The cells were transferred to -20°C for 2-4 h prior to long-term storage in liquid nitrogen.

ii) Recovery of cells

Cells were thawed rapidly at 37°C and each 1ml of cells was transferred to a 25cm^2 tissue culture flask containing 10ml TC100/5. The flasks were incubated at 28°C for 4-5 days or until the cells had formed a confluent monolayer after which they were transferred into a 150cm^2 flask containing 75ml TC100/5 and passaged as normal.

2.2.3. Preparation of high titre virus stocks

i) Baculovirus

Parental, AcPAK6, and recombinant baculoviruses were propagated in 300ml suspension cultures of Sf21 cells. Cultures which had obtained a density of approximately 5 x 10^5 cells/ml were inoculated with virus at a m.o.i. of 0.1 p.f.u./cell and incubated at 28°C for 4 to 5 days. The cells were pelleted in 250ml Falcon tubes at 2,000 r.p.m. for 10 minutes at 4°C in a Sorvall RT6000B benchtop centrifuge and the virus was then pelleted from the supernatant at 12,000 r.p.m. for 2 h at 4°C using a Sorvall RC-5B centrifuge (GS-A rotor). The supernatant was discarded and the pellet was allowed to resuspend overnight at 4°C in TC100/5 (4ml/300ml culture). The resuspended virus was transferred to a glass universal and sonicated in a QH Kerry sonibath. The virus was stored at -70°C until required and was subsequently stored at 4°C. This method of storage prevented possible contamination in the first instance and a reduction of the titre arising from continued freeze-thawing.

ii) HSV-1

HSV-1 strain 17 and *ts*1201 were grown on confluent monolayers of BHK21 C13 cells. Each roller bottle was inoculated with virus at a m.o.i. of 0.001 p.f.u./cell in 40ml ETC_{10} medium and incubated for 4-5 days at 31°C (31°C rather than 37°C was found to be better for growth of high titre virus stocks). The cells were shaken into the medium, transferred to a 250ml Falcon bottle and pelleted. Cell-associated virus (CAV) was prepared by sonicating the pellet in 2ml ETC_{10} followed by centrifugation at 3,000 r.p.m. in a Sorvall RT6000B centrifuge for 5 minutes to pellet cell debris. Cell-released virus (CRV) in the supernatant was transferred to Sorvall GSA bottles and the virus pelleted as described above for baculovirus. The virus was resuspended by sonication in 2ml ETC_{10}. Stocks were then frozen at -70°C.

2.2.4. Sterility checks on virus stocks

To check for bacterial contamination, 10ul of the viral stock was added to each of two 20ml volumes of BHI which were then incubated for 7 days; one at 37°C and the other at 28°C. To identify the presence of fungal contamination 20ml SAB was treated as above but was incubated at 31°C.

2.2.5. Titration of virus stocks

i) Baculovirus

Baculovirus stocks were titrated by the method described by Brown and Faulkner (1977). 35mm dishes were seeded with 1 x 10^6 cells in 1.5ml of TC100/5 medium and the cells were incubated overnight at 28°C. Virus titration is best carried out on 70-80% confluent cell monolayers as cells which are too dense will either produce very small plaques or none at all. Serial 10-fold dilutions of the virus stock were prepared in TC100/5 medium and the virus was titrated from 10^{-5} to 10^{-8} for high titre stocks and 10^{-2} to 10^{-6} for low titre stocks. Basically, the medium was removed from the plate and 100ul of each dilution was gently inoculated onto the centre of the dish. The virus was left to absorb to the cells at RT with occasional rocking of the plates. After 1 h, the inoculum was removed and 1.5ml overlay medium was added. This consisted of 50% TC100/5 and 50% 3% LTG agarose (SeaPlaque™) which had been incubated at 37°C to prevent it setting. Once this had set, 2ml of TC100/5 was added as a liquid overlay and the titration incubated at 28°C. After 4-5 days the liquid overlay was removed and 1.5 ml of neutral red stain (0.4% neutral chloride mixed 1:50 with TC100/5) was added and the cells incubated at 28°C for 4-8 h before the stain solution was removed. The plates were inverted and the plaques left to clarify overnight at 28°C. Plaques could be easily observed against the red background of the stain and the virus titre was calculated as p.f.u./ml. If the titration was required for the identification of recombinant virus, either resulting from a transfection or as part of a plaque purification assay, then 250ug X-gal/ml of stain was added to pre-warmed (37°C) neutral red stain. Recombinant virus plaques were distinguished from parental plaques by the absence of the blue colour produced by the parental *B*-galactosidase activity.

ii) HSV-1

HSV-1 virus stocks were titrated on 80% confluent BHK21 cell monolayers in 50mm plates. Cells were inoculated with serial 10-fold dilutions of virus in 0.1ml of PBS complete containing 5% NCS. After adsorption for 1 h at 37°C, the inoculum was removed and the plates were overlaid with 4ml of EMC_{10} and incubated for 3-4 days at 37°C before being fixed and stained by the addition of 4ml Giemsa.

2.2.6. Preparation of Baculovirus DNA

i) DNA for analytical purposes.

DNA required for slot blot or southern blot analysis was prepared as follows. 35mm plates were seeded with 6 x 10^5 cells in 1.5 ml of TC100/5. After overnight incubation (cell monolayer 50-60% confluent) the medium was removed and the cells inoculated with 200ul of each plate harvest stock. The virus was allowed to absorb for 1 h at RT before the addition of 1.5ml of TC100/5 and incubation at 28°C. After 4 days, or if there was extensive CPE, the cells were scrapped into the medium with the rubber plunger of a 1ml syringe and transferred to a 1.5ml reaction vial. The cells were pelleted at 6,500 r.p.m. for 5 minutes and the supernatant discarded. Cells were resuspended in 1ml of cell lysis buffer (CLB) and incubated at 37°C for 4 h. Following the addition of NaCl to a final concentration of 375mM the DNA was extracted with an equal volume of phenol:chloroform (1:1), followed by a single chloroform extraction and ethanol precipitation. The DNA was ethanol precipitated as previously described (section 2.1.3) and was resuspended in 50ul TE (pH7.5).

ii) AcPAK6 DNA for transfections

Infectious viral DNA was prepared as follows. 2 roller bottles of Sf21 cells at a density of 2 x 10^5 cells/ml were infected with AcPAK6 virus at a m.o.i of 1 p.f.u./cell. After incubation at 28°C for 5 days, the virus was harvested as described in section 2.2.3. The virus pellet was removed fom the centrifuge bottle by resuspension in 2ml TC100/5 medium. It was sonicated in a sonibath and the virus transferred to a SS34 tube and reconcentrated by centrifugation at 18,000 r.p.m. for 90 minutes at 4°C in a Sorvall RC-5B centrifuge. The virus was resuspended in 2ml of TE (pH7.5) and SDS and proteinase K added to final concentrations of 0.25% and 500ug/ml respectively After overnight incubation at 31°C, the DNA was prepared by 2 x phenol/chloroform (1:1) and 1 x chloroform extraction followed by ethanol precipitation. The DNA was resuspended by overnight incubation at 37°C in 200ul of dH_2O.

To be used in a transfection reaction the DNA required to be digested with Bsu361 and phosphatased. 50ug AcPAK6 DNA was digested in a total volume of 200ul. The digestion mix; consisting of 1 x NEB buffer 3 supplemented with 100ug/ml acetylated BSA and 100 units of Bsu361, was incubated at 37°C for 4-5 h. 100 units of CIP was added during the final 30 minutes of the incubation. The DNA was phenol:chloroform (1:1) extracted, ethanol precipitated and resuspended in 100ul of dH_2O. To check that the digestion was complete, 0.5ug Bsu361 digested DNA was digested with EcoR1 and the DNA profiles compared to AcPAK6 DNA digested solely with EcoR1. If the DNA had been correctly digested there was a noticeable shift in one of the larger fragments and the appearance of a smaller band.

2.2.7. Construction of recombinant viruses

i) Transfection of viral DNA

The transfection procedure used a synthetic lipid reagent (lipofectin) prepared according to the method of Rose *et al.* (1991). Transfections were performed on 60-70% confluent monolayers (35mm dishes) of Sf21 cells. 0.5ug AcPAK6 DNA which had been digested with Bsu361 and phosphatased as described above, was mixed with 2 to 5ug of transfer vector DNA and 500ul TC100 in a 15ml polystyrene Falcon tube. 15ul lipofectin was added, the mixture vortexed gently and incubated at RT for 10 minutes. The medium was removed from the monolayers of Sf21 cells which were then rinsed with 2ml of TC100. The transfection mixture was added to the cells together with a further 500ul of TC100. After incubation at 28°C for 4 h, 1ml of TC100/10 was added. The incubation was continued at 28°C for a further 65 h after which the cells were removed by centrifugation at 13,000 r.p.m. in a microfuge for 2 minutes. The supernatant was transferred to a sterile 1.5ml screw cap tube and stored at -70°C.

ii) Isolation of recombinant viruses

Putative recombinants were selected on the basis of their *lac*Z-minus phenotype. 100ul of the transfection harvest was assayed for plaques from neat to a dilution of 10^{-3} as described in section 2.2.5. Plaques were picked into 1ml of TC100/5, freeze-thawed three times on dry ice to release the virus from the agar and stored at -70°C. Viruses obtained from picked plaques were grown up on 35mm plates. 50% confluent monolayers (1 x 10^6 cells) were inoculated with 500ul of the picked plaque virus suspension which was allowed to adsorb for 1 h at RT when a further 1ml of TC100/5 medium was added. Following incubation at 28°C for 4-5 days, the supernatant from this plate harvest (PH1) was harvested as described in i) above and used for a further round of plaque purification. Virus from the second round of plaque purification was grown up on 35mm plates as described above to produce plate harvest 2 (PH2) virus stocks which were then used to infect for DNA for analysis of protein profiles and for the production of high titre virus stocks.

2.2.8. Production of low titre seed stock of virus

Tissue culture flasks ($25cm^2$) were seeded with 1 x 10^6 cells in 10ml of TC100/5 medium and incubated at 28°C for 2-3 days until the cells formed a 50% confluent monolayer. The medium was removed from the flask, 500ul of the virus supernatant PH2 (above) was added to the flask and the virus allowed to adsorb for 1 h at RT. 10ml of fresh TC100/5 was added and the cells incubated at 28°C for 4-5 days or until extensive CPE was observed. The medium was harvested into a 15ml Falcon tube, the cells spun out at 2,000 r.p.m. for 5 minutes and the supernatant frozen at -70°C. 5ml of

this supernatant was used to infect a large tissue culture flask ($150cm^2$) seeded with $1x\ 10^7$ Sf21 cells in the manner described. 50ml of TC100/5 was added and the cells incubated for 4-5 days. The harvested supernatant was used in the production of high titre virus and was stored at -70^o.

2.2.9. Expression of viral polypeptides

i) Baculovirus

35mm dishes were seeded with 6×10^5 Sf21 cells in 1.5 ml of TC100/5 and incubated overnight to a final density of $1x10^6$ (cell monolayer 50% confluent). The medium was removed and the virus was added at a m.o.i. of 5 p.f.u./cell, except in the initial identification of viral phenotype when 200ul of the PH2 virus (section 2.2.7) was added. The virus was allowed to adsorb for 1 h at RT after which 1.5ml of TC100/5 was added and the cells incubated at 28^oC. At 24-30 h p.i., the medium was replaced with 1.5ml of $TC100_{-met}$ for 1 h prior to incubation in TC100/5:$TC100_{-met}$ (1:4) containing 30uCi/ml of [^{35}S]-methionine. Incubation was continued a further 18 h unless stated otherwise. The cells were transferred to a 1.5ml reaction vial, pelleted at 6,500 r.p.m. for 3 minutes, washed in 1ml of PBS complete, pelleted and resuspended in 100-150ul dissociation mix. Cell lysates were stored at -20^oC prior to analysis by SDS-PAGE.

ii) HSV-1/*ts*1201

35mm dishes were seeded with 1×10^6 BHK21 cells in 2ml of ETC_{10}, incubated overnight at 37^oC and the confluent monolayers were infected with 5 p.f.u./cell of virus in 100ul of ETC_{10}. After 1 h adsorption at 37^oC (31^oC for *ts*1201) 2ml ETC_{10} was added. Following a further 2 h incubation, the medium was removed and replaced with 2ml of $ETC_{1/5met}$ containing 30uCi/ml [^{35}S]-methionine. The cells were incubated for a further 21 h. At 24 h.p.i., the cells and medium were transferred to a 1.5ml reaction vial and the cells pelleted at 6,500 r.p.m. for 3 minutes after which they were washed in PBS complete and resuspended in 100-150ul dissociation mix.

2.2.10. SDS-PAGE of infected cell extracts

SDS-polyacrylamide gels were run essentially as described by Marsden *et al.* (1976). Single concentration gels were cross-linked with 2.5% (w/w) N,N'-methylenebisacrylamide (acrylamide:bisacrylamide ratio of 39:1) whereas gradient gels used 5% (w/w) crosslinker (acrylamide:bisacrylamide ratio of 19:1). Proteins were resolved on of 9, 10 or 15% final acrylamide concentration gels or 5-15% gradient gels. The samples for electrophoresis were boiled in dissociating mix for 5 minutes prior to loading into the wells. If required, protein standards of known molecular weights

(rainbow markers or [^{14}C]-labelled proteins; Amersham) were run in adjacent wells. Gels were electrophoresed until the bromophenol blue marker had reached the bottom of the gel. The gel was removed from the apparatus and the proteins visualised by shaking in Coomassie Brilliant Blue stain for 15 minutes followed by destaining overnight in destain. Alternatively, if radioactive samples were to be analysed, the proteins were fixed into the gel with 3 changes of Fix solution, treated with EN^{3}HANCE (Du Pont Ltd.) for 1 h followed by 3 x 15 minute washes in dH_2O. The gel was then dried down under vacuum onto Whatman paper and exposed to Kodak XS-1 film at -70^{o}C.

2.3. ANALYSIS OF CAPSID ASSEMBLY

2.3.1. HSV-1 capsid assembly in baculovirus

35mm plates were either mock-infected, infected with AcPAK6, infected singly or with combinations of the recombinant baculoviruses each at 5 p.f.u./cell. Cells were harvested at 48 h p.i. and analysed by electron microscopy for the presence of HSV-1 like capsid structures. To confirm the expression of the recombinant proteins, cell monolayers were infected in duplicate, labelled with [^{35}S]-methionine, harvested at 48 h p.i. and their protein profiles determined by SDS-PAGE. To serve as a control, 35mm dishes of BHK cells were infected with 5 p.f.u./cell of either HSV-1 wt or *ts*1201 viruses as described in section 2.2.9.

2.3.2. Electron microscopy of cells

Electron microscopy of thin sections was performed essentially as described by Preston *et al.* (1983). Cells were harvested in their medium, transferred to a 1.5ml reaction vial and pelleted by centrifugation at 6,500 r.p.m. before being gently resuspended in 800ul PBS complete and transferred to 2 BEEM capsules. The cells were pelleted by centrifugation at 1,000 r.p.m. for 5 minutes in a vertical centrifuge (Beckman microfuge 12) and fixed overnight at 4^{o}C with 2.5% gluteraldehyde diluted 1:10 in PBS complete. Fixed pellets were washed 3 x in PBS complete and care was taken not to disturb the pellet. Pellets were then treated with osmium tetroxide for 1 h at RT followed by 3 washes in PBS complete. The fixed cells were dehydrated through a series of increasing ethanol concentrations: 30%, 50%, 70%, 90% (v/v) followed by 2 changes in 100% ethanol. Each dehydration was for a minimum of 2 h in each concentration. The alcohol was then carefully removed and the pellets were infiltrated with Epon resin for 6 h at RT. The resin was replaced with fresh resin which was left overnight before incubation at 60^{o}C for 2 days to enable polymerisation to take place.

The resin blocks were cut using a diamond knife on an ultra-microtome (Ultracut-E, Reichart-Jung) and the thin sections collected on parlodion coated copper grids before being stained for 1 h with saturated uranyl acetate in 50% (v/v) ethanol. The grids were rinsed in dH_2O and counter-stained with lead citrate for 1 minute and the excess stain removed by rinsing in dH_2O. The cell morphology was examined using a JEOL 100S electron microscope and photographed on Kodak 4489 film.

2.3.3. Purification of capsids

i) Baculovirus system

150-300ml suspension cultures of Sf21 cells which had reached a density of 1 x 10^6 cells/ml were infected with 5p.f.u./cell of each recombinant baculovirus and incubated at 28°C. After a period of 48-65 h p.i., the cell suspension was transferred into 250ml Falcon tubes and the cells harvested by centrifugation at 2,000 r.p.m. for 10 minutes in a Sorvall RT6000B centrifuge. If the capsids were to be labelled with [^{35}S]-methionine then, at 24 h p.i., the cells were harvested by centrifugation, resuspended in $TC100_{met}$/TC100/5 (4:1) containing 20uCi/ml [^{35}S]-methionine and incubated for a further 18 h before being harvested.

ii) HSV-1 and *ts*1201

Confluent monolayers of BHK21 cells grown in 850cm^2 roller bottles were infected with 5 p.f.u./cell of either HSV-1 wt or *ts*1201 in 100ml ETC_{10} and incubated at 37°C (38°C for *ts*1201). The cells were harvested at 24 h p.i., when there was obvious CPE. Alternatively, to produce radiolabelled capsids, the viruses were infected in 40ml $ETC_{1/5met.}$ After 4 h 10uCi/ml of ^{35}S-methionine was added and the incubation continued for a further 44 h when the cells were harvested.

iii) Preparation of capsids

Capsids were isolated using a modification of the method of Booy *et al.* (1991). Cells were resuspended in 30ml ice-cold NTE (0.5M NaCl, 20mM Tris-HCl pH7.4, 1mM EDTA) containing 1% Nonident-P40 (N-P40) and incubated on ice for 10 minutes. The nuculei were disrupted using a Branson soniprobe and the debris was pelleted by centrifugation at 2,000 r.p.m. for 10 minutes at 4°C in a Sorvall RT6000B centrifuge. The supernatant containing the capsids was spun through a 5ml 40% sucrose cushion (w/w in NTE) at 20,000 r.p.m for 1 h at 4°C in a AH629 rotor in a Sorvall OTD ultracentrifuge. This sedimented the capsids which were rinsed once in NTE to remove excess sucrose. Capsids were then resuspended by sonication in 1ml of NTE, layered onto a 12ml gradient of 5-40% sucrose in NTE and centrifuged at 40,000 r.p.m. at 4°C for 20 minutes. The gradients were viewed under a fibre optic light in the dark and if present the capsid band was removed by side puncture. For capsid preparations which

were radioactively labelled, 8-drop fractions were collected by dripping the gradient through an 18-gauge needle. The positions of the capsid bands were determined following TCA precipitation and scintillation counting (section 2.3.5) and by SDS-PAGE analysis of the protein profiles (section 2.2.10).

Gradient fractions containing capsids were pooled and diluted 10-fold with NTE prior to centrifugation at 24,000 r.p.m. at 4^{o}C in a Sorvall TsT41 rotor for 1 h. The capsid pellet was resuspended in 100ul of NTE by sonication in a Q H Kerry sonibath. Protein profiles of the resuspended capsids (5ul) were examined by SDS-PAGE.

2.3.4. Electron microscopy of capsids

Samples (2ul) of the concentrated banded capsids (above) were adsorbed onto parlodion-coated microscope grids and were negatively stained with either 1% sodium silicotungstate or 1% phosphotungstic acid. The grids were rinsed with dH_2O, dried in air and examined in a JEOL 100S electron microscope.

2.3.5. TCA precipitation of radiolabelled proteins

25-50ul of each 8 drop fraction were added to a 1cm diameter grade 1 Whatman disc, which was allowed to dry before being subjected to 2 x 10 minute washes in ice-cold 5% trichloroacetic acid (TCA) followed by 1 x 5 minute wash in ice-cold ethanol (100%) to precipitate the protein. A control disc was included in the treatments to provide a count of the background radioactivity. Once dry the discs were added to 5ml Ecoscint A (National Diagnostics) and the amount of incorporation of [^{35}S]-methionine determined as counts/minute (c.p.m) in a 1600TR liquid scintillation analyser (Packard). The results were plotted as c.p.m against fraction number and the position of the capsids identified as a peak on the graph.

2.3.6 Western blotting

i) Transfer of proteins

To determine the positions of the VP22a on the capsid gradient obtained from the baculovirus system (section 2.3.3) proteins from gradient fractions 6 to 21 (50ul), together with 10ul of AcPAK6 infected cell lysate (2.2.9) and 50ul of a HSV-1 B capsids preparation, were separated by SDS-PAGE on a 9% polyacylamide gel and transferred to a nitrocellulose membrane using a 2117 Multiphor II electrophoresis unit (LKB Bromma) essentially as described by Harlow and Lane (1988).

ii) Detection of proteins

The method used was based on that described by Biocell International who provided the reagents. Basically, the membrane was first washed in PBS containing 5% BSA for 30

minutes at 37°C to block the non-occupied protein binding places on the membrane. This was followed by 3 x 5 minute washes at RT in PBS containing 1% BSA (PBS/1%). The membrane was transferred to a hybridisation bag and 20ml of PSA/1% and 1:200 dilution of the primary antibody MCA 406 was added. After incubation under constant agitation at RT for 2 h, the membrane was subjected to 3 x 5 minute washes in PBS/1% at RT. The membrane was incubated in a hybridisation bag for 2 h at RT in 20ml of a 1:100 dilution of the secondary antibody (protein A gold conjugate) in PBS/1% containing 1:20 v/v gelatin which was added to inhibit non-specific binding of gold particles. The excess unbound gold particles were removed by washing the membrane twice in PBS/1% for 5 minutes followed by 2, 1 minute washes in distilled water. The presence of the bound gold particles was identified by a 1 to 15min incubation in the silver enhancing solutions provided.

CHAPTER 3

RESULTS

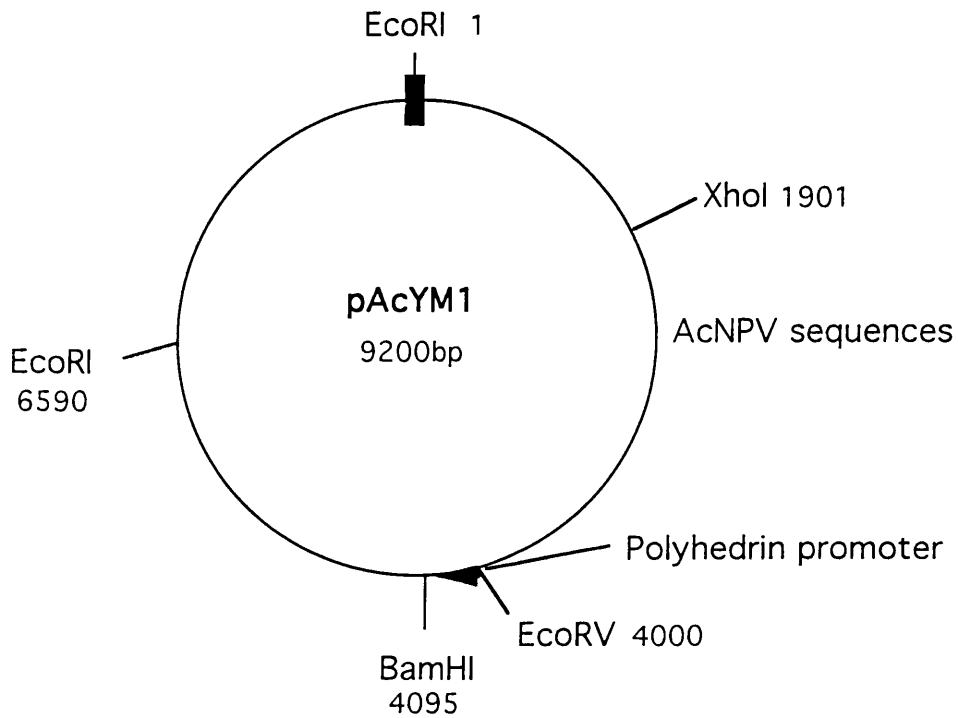


Figure 5:- pAcYM1, the transfer vector used in the construction of the recombinant baculoviruse AcUL19. This figure has been adapted from Matsuura *et al.* (1987)

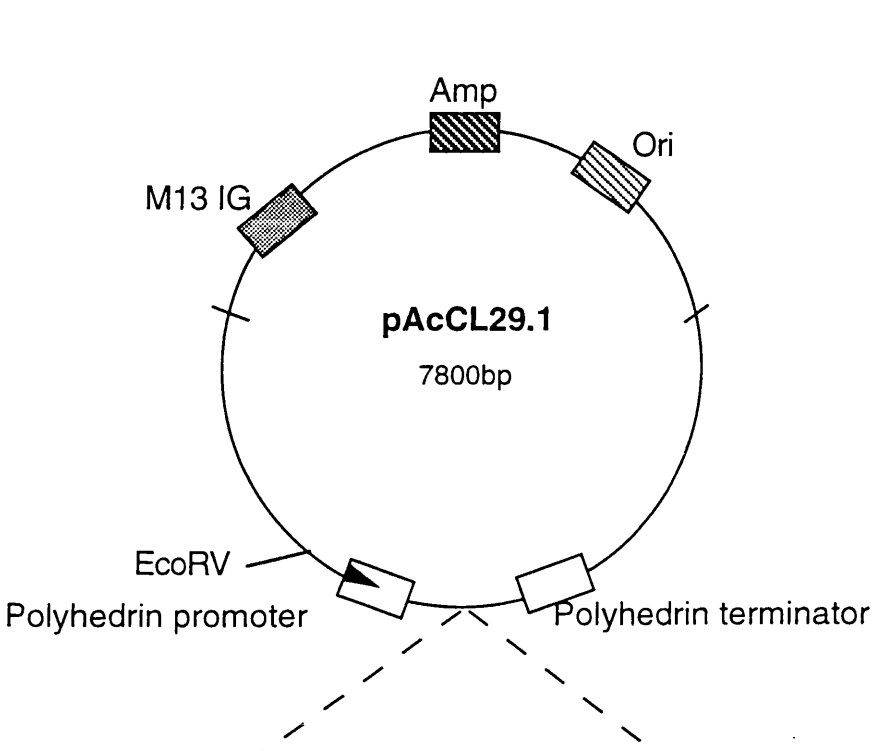


Figure 6:- pAcCL29.1, the transfer vector used in the construction of recombinant baculoviruses AcUL18, AcUL35 and AcUL38. This figure has been adapted from Livingston and Jones (1989).

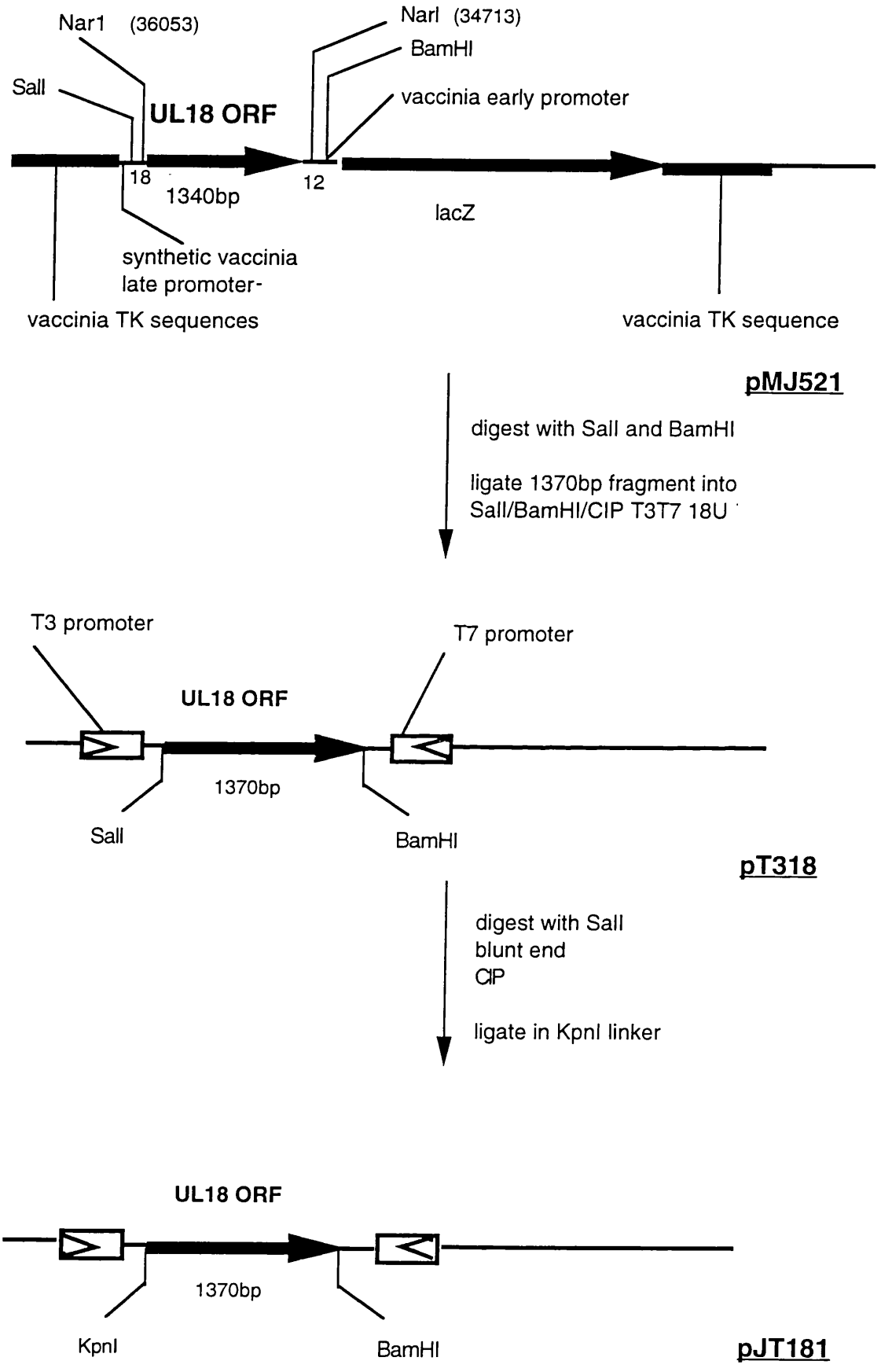


Figure 7:- The Cloning of UL18

cont./

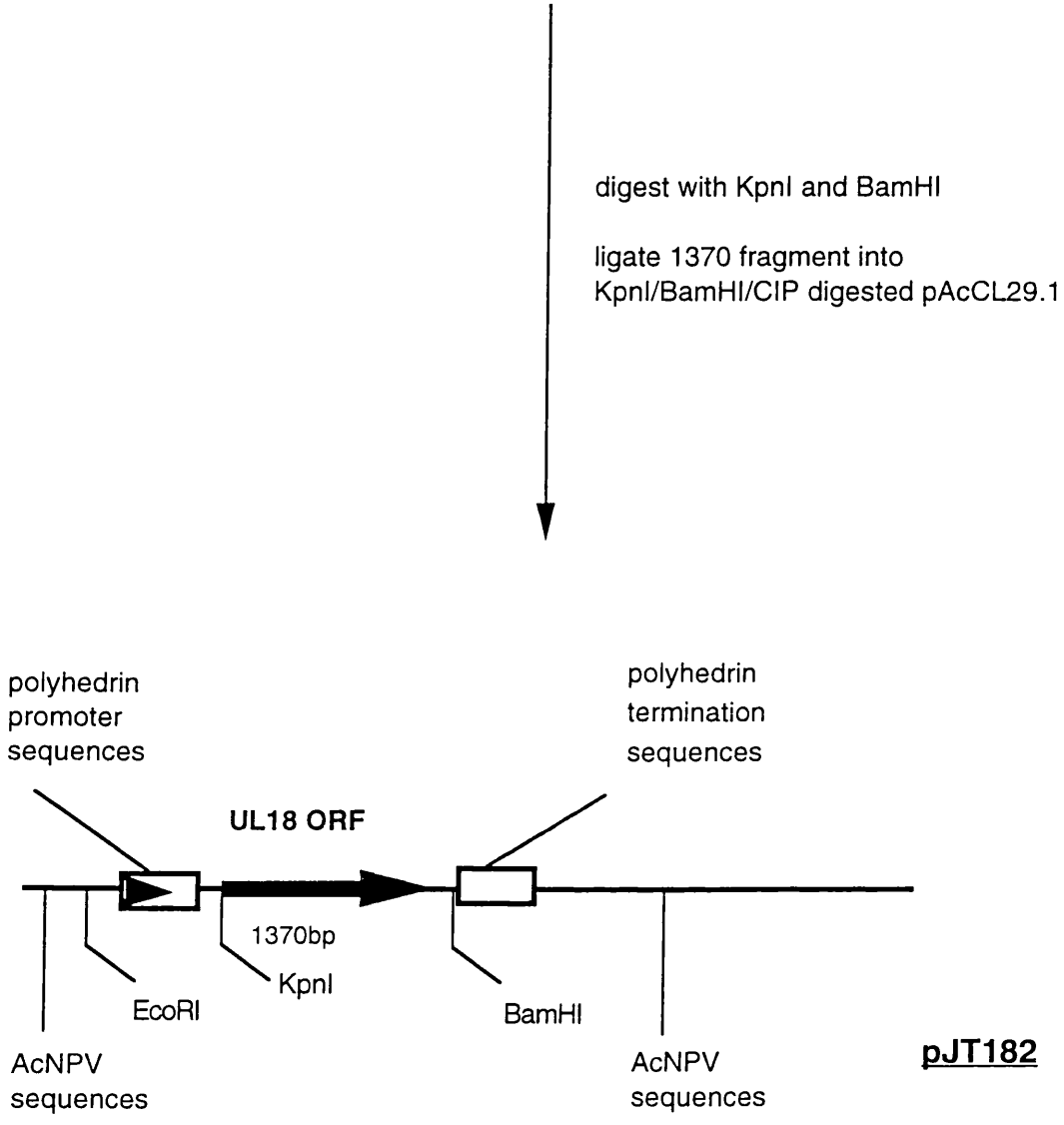


Figure 7 (continued):- The cloning of UL18

1. CLONING OF THE CAPSID GENES

1.1 BACULOVIRUS TRANSFER VECTORS

The transfer vector pAcYM1 described by Matsuura *et al.* (1987) is shown in figure 5. pAcYM1 contains sequences from AcNPV which flank the polyhedrin gene which has had the coding residues +2 to +751 deleted and thus lacks a complete initiation codon. A BamHI restriction site allows insertion of foreign genes between the polyhedrin gene promoter and polyadenylation sequences. This vector was used in the construction of AcUL19 (Nicholson; 1992). pAcCL29.1 is a derivative of pAcYM1 and its construction is described by Livingstone and Jones (1989). Its structure is shown in figure 6. Basically, a 5kb XhoI/EcoRI fragment encoding all the signals necessary for efficient expression and recombination was removed from pAcYM1 and cloned into the plasmid pUC118 which provides the M13 intergenic region (M13 IG; necessary for single strand DNA production), origin of replication (ori) and β-lactamase gene (amp). The pUC polylinker was cloned into the BamHI site to generate a variety of unique restriction sites for the cloning of genes to be expressed. UL18, UL35 and UL38 were cloned into this vector. A modified version of pAcCL29.1 that had the SmaI site converted to a BglII site generating pAcCL29.1B was used in the construction of AcUL26 and AcUL26.5 (Preston *et al.*, 1994).

In the following descriptions of the cloning of the capsid genes, the numbering of the bases corresponds to their position on the prototype arrangement of the HSV-1 DNA as described by McGeoch *et al* (1988).

1.2 THE CLONING OF UL18 INTO PACCL29.1

The UL18 ORF had previously been cloned as a NarI fragment into the plasmid pMJ521 (Nicholson, 1992; Nicholson *et al.*, 1994). NarI cuts two residues upstream from the UL18 ATG initiation codon (position 36051) and 381 residues downstream from the stop codon (35097) at positions 36053 and 34713 respectively. The strategy for cloning UL18 into pAcCL29.1 is shown in figure 7. A 1370bp fragment containing the UL18 ORF was released from pMJ521 by digestion with BamHI and SalI and was ligated into BamHI/SalI digested pT3T7 18U to give pT318. This was then digested with SalI, the site blunt ended with T4 polymerase and the phosphate groups removed by treatment with CIP. A KpnI linker was inserted by ligation to form pJT181. The UL18 ORF was released by digestion with BamHI and KpnI and ligated into BamHI/KpnI digested pAcCL29.1. This plasmid which was to be used in the transfer of the UL18 gene into baculovirus was designated pJT182.

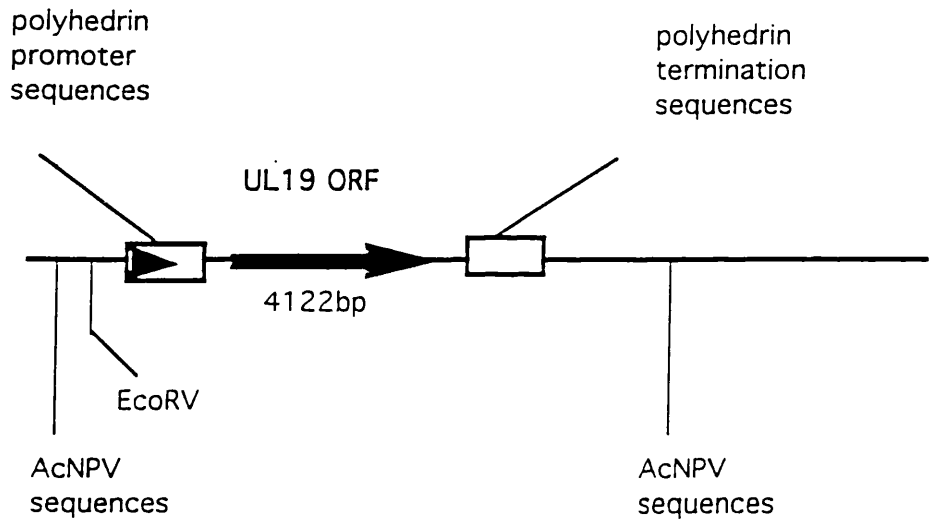


Figure 8:- Structure of pBJ199

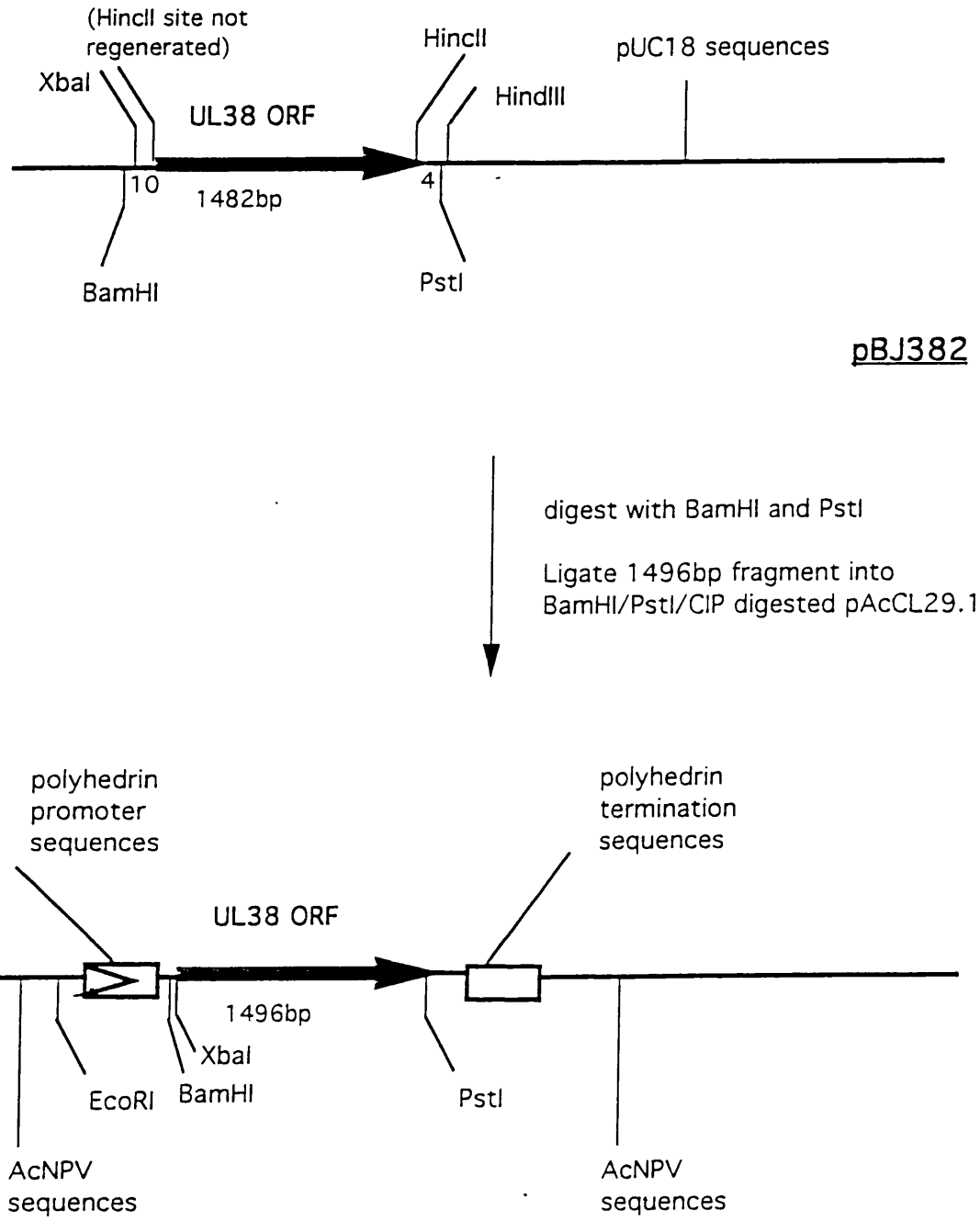


Figure 9:- The Cloning of UL38

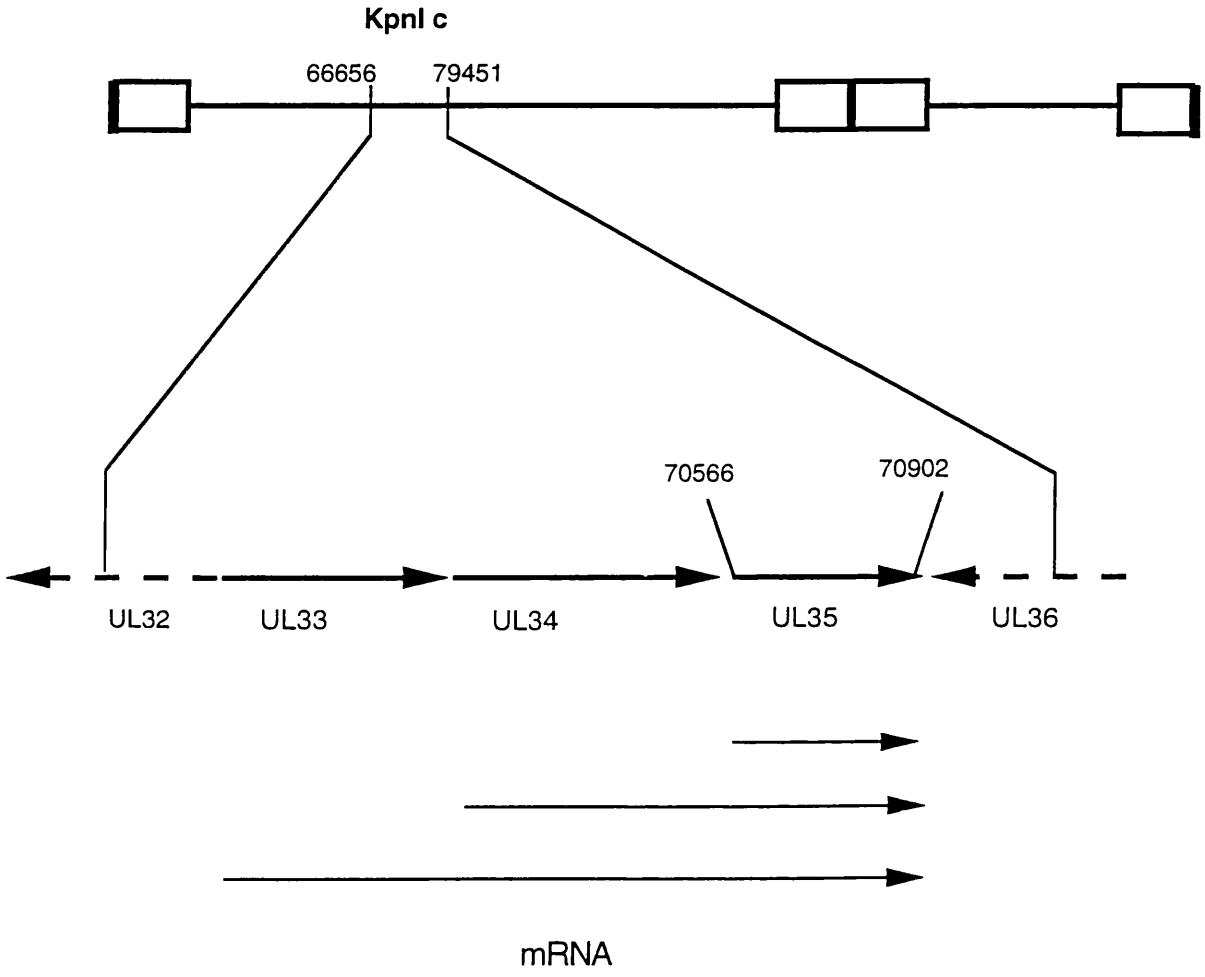


Figure 10:- The locus of UL35

1.3 THE CLONING OF UL19 INTO pAcYM1

The cloning of the UL19 ORF into pAcYM1 was carried out before the work in this thesis began and has been described elsewhere (Nicholson, 1992; Nicholson *et al.*, 1994; Tatman *et al.*, 1994). The final construct, pBJ199 which contains the 4122bp UL19 ORF under the control of the polyhedrin promoter is shown in figure 8.

1.4 THE CLONING OF UL38 INTO pAcCL29.1

The cloning of the UL38 ORF from the HindIII *k* fragment into pUC18 was described by Nicholson (1992). Basically a 1482bp fragment containing the residues from 15bp upstream of the initiation codon to 70 residues downstream from the stop codon (positions 84516 to 85996) was cloned into the HincII site of pUC18 to form pBJ382. The UL38 ORF was then subcloned from pBJ382 as a 1496bp BamHI/PstI fragment into BamHI/PstI digested pAcCL29.1 to give pJT381 as shown in figure 9.

1.5 THE LOCUS OF UL35

The UL35 gene encodes the capsid protein VP26 (Davison *et al.*, 1992; McNabb and Courtney, 1992a). It is located on the KpnI *c* restriction fragment which lies between 66656 and 79451 on the HSV-1 viral genome (Preston *et al.*, 1978; McGeoch *et al*, 1988). Analysis of HSV-1 transcripts located within this region of the viral genome suggest that UL33, UL34 and UL35 are transcribed late in infection as a nested set of 3' co-terminal mRNAs (Wagner, 1985) as shown in figure 10. However precise mRNA mapping studies have not been done. The 336bp UL35 ORF is transcribed from left to right on the prototype arrangement of HSV-1 DNA which encodes a protein with a predicted molecular weight of 12,095Da. The polyadenylation signal, AATAAA, for the three genes is at position 70938. The transcription start site has not yet been identified. The translation initiation codon is at position 70566 and the TGA stop codon is at position 70902 (McGeoch *et al.*, 1988).

1.6 THE CLONING OF UL35 INTO pAcCL29.1

Unlike the other capsid protein genes, no plasmid construct containing the isolated UL35 ORF had previously been made. The KpnI *c* fragment containing the UL35 gene had been cloned into pAT153 to give pGX122 (Davison and Wilkie, 1983). As there were no convenient restriction enzyme sites flanking the UL35 ORF, PCR was employed to amplify the region and so produce a suitable DNA fragment that could be subcloned. pGX122 was used as the template in the PCR. The two oligonucleotides

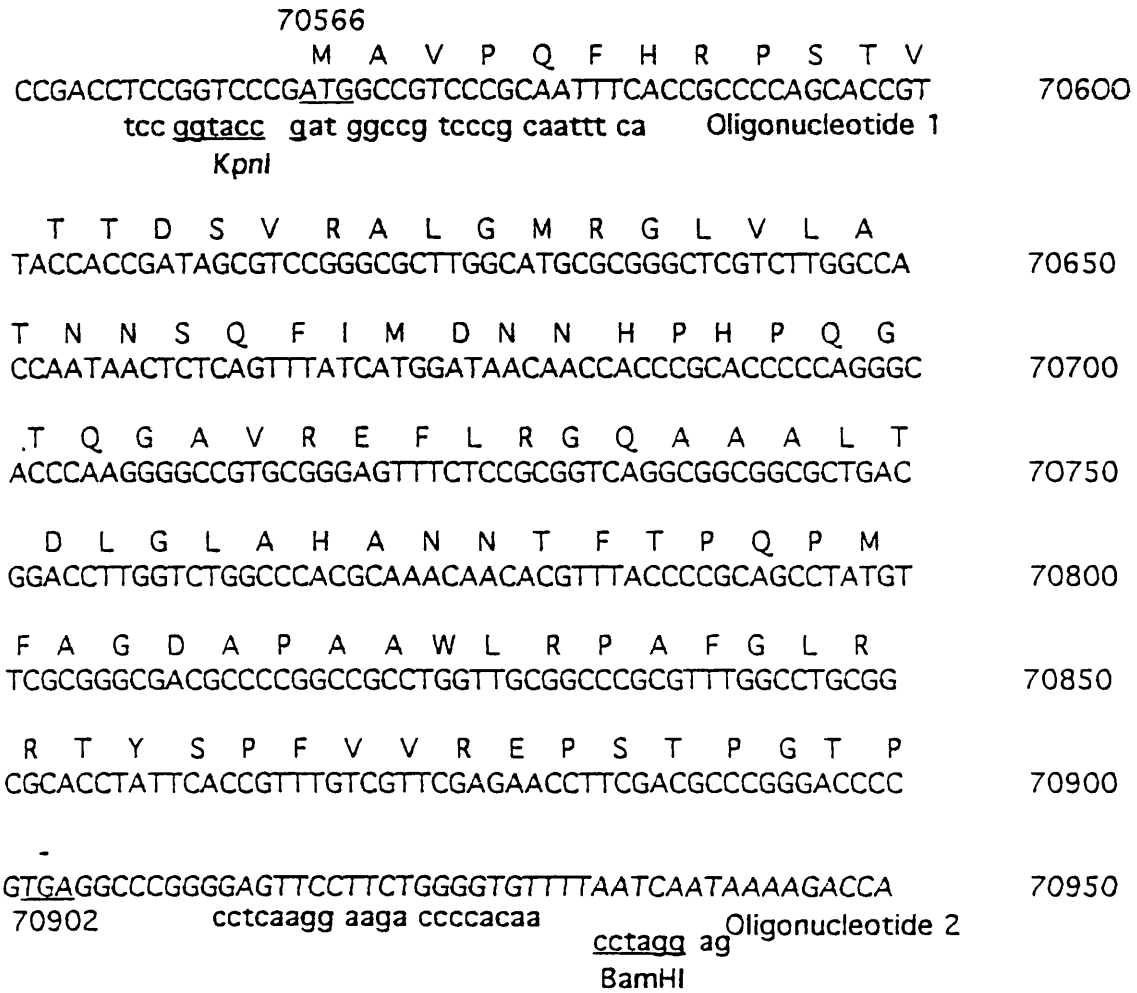


Figure 11:- Synthetic oligonucleotides used in the cloning of UL35

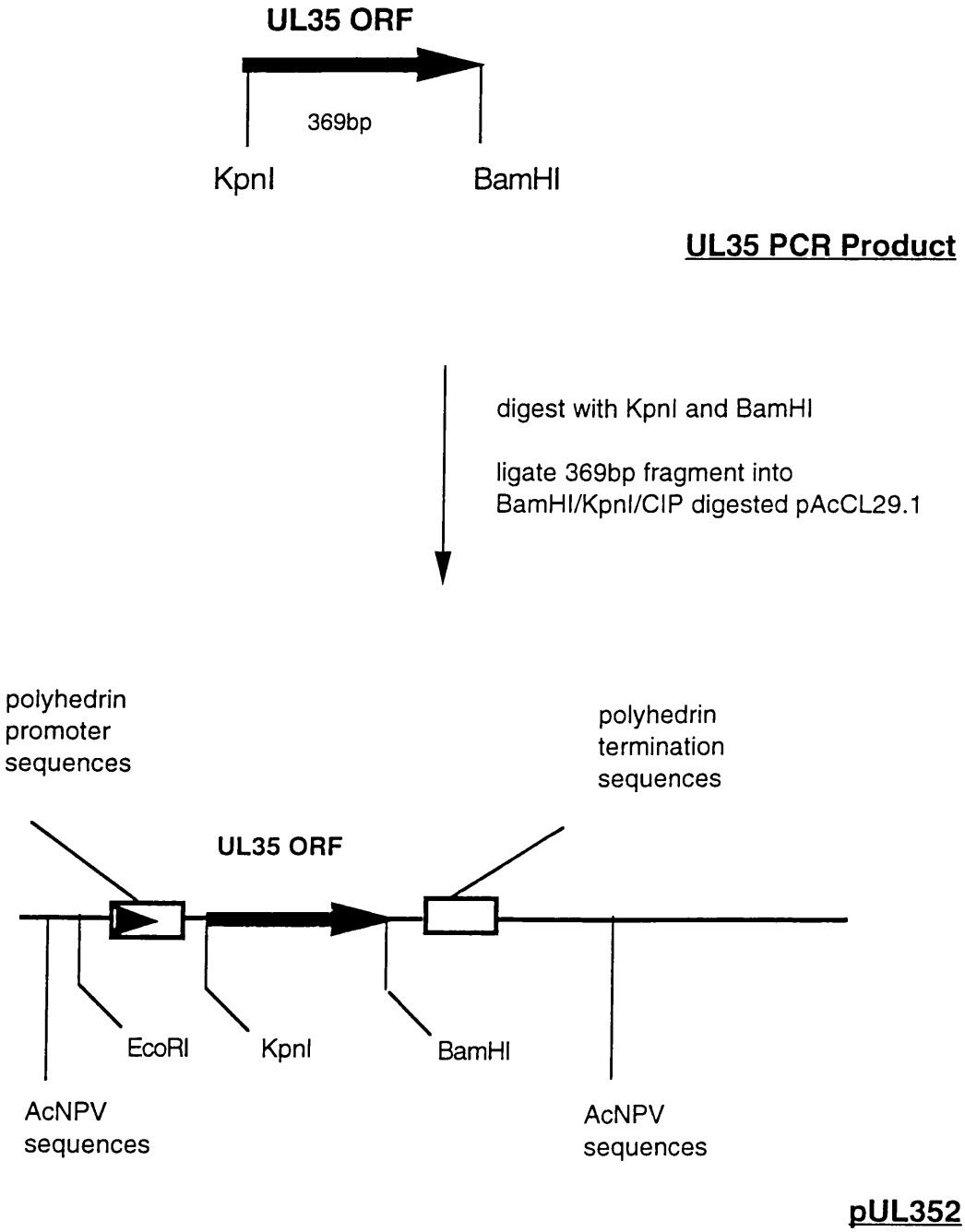


FIGURE 12:- The Cloning of UL35

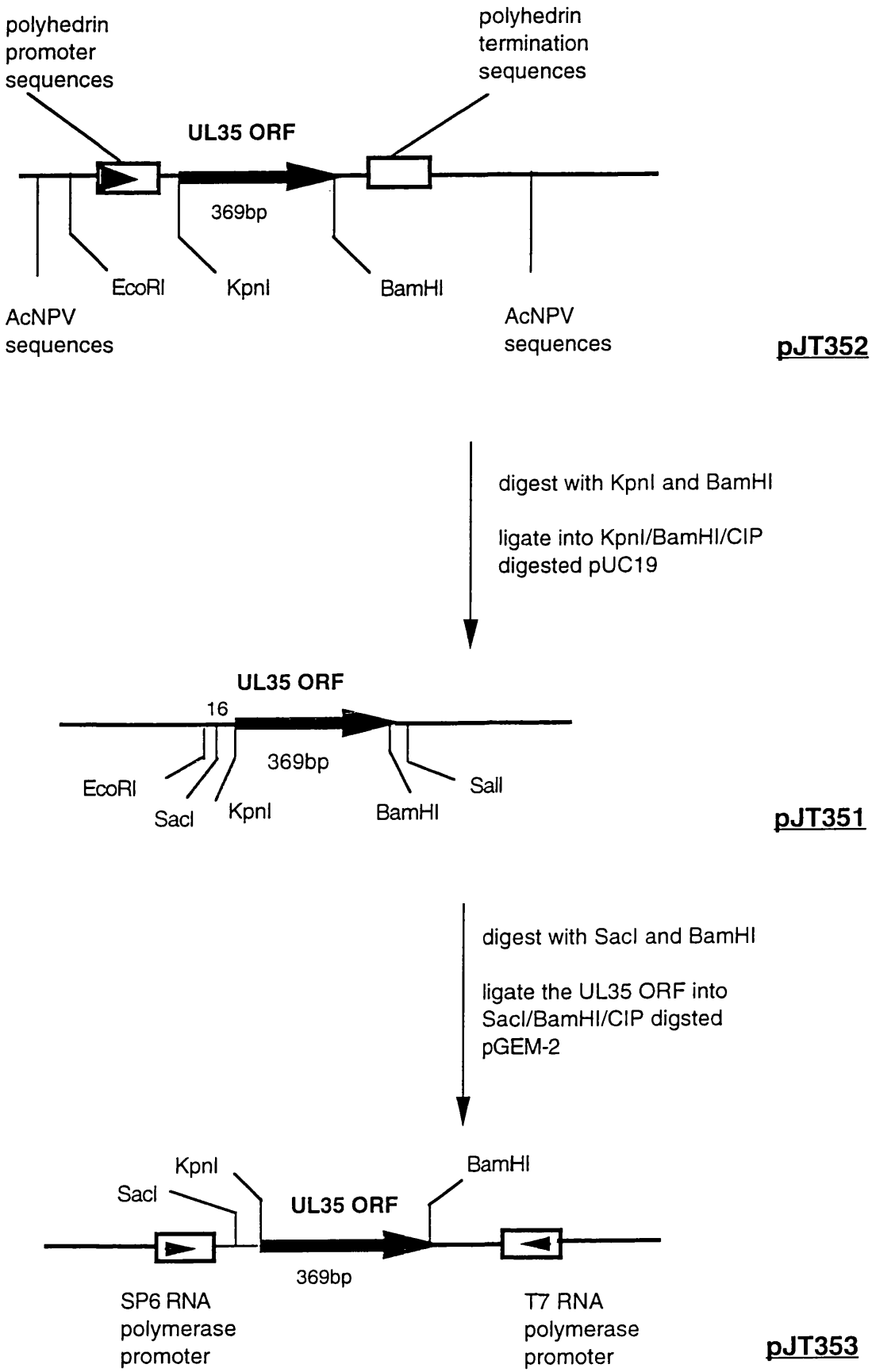


Figure 13: Construction of pJT353

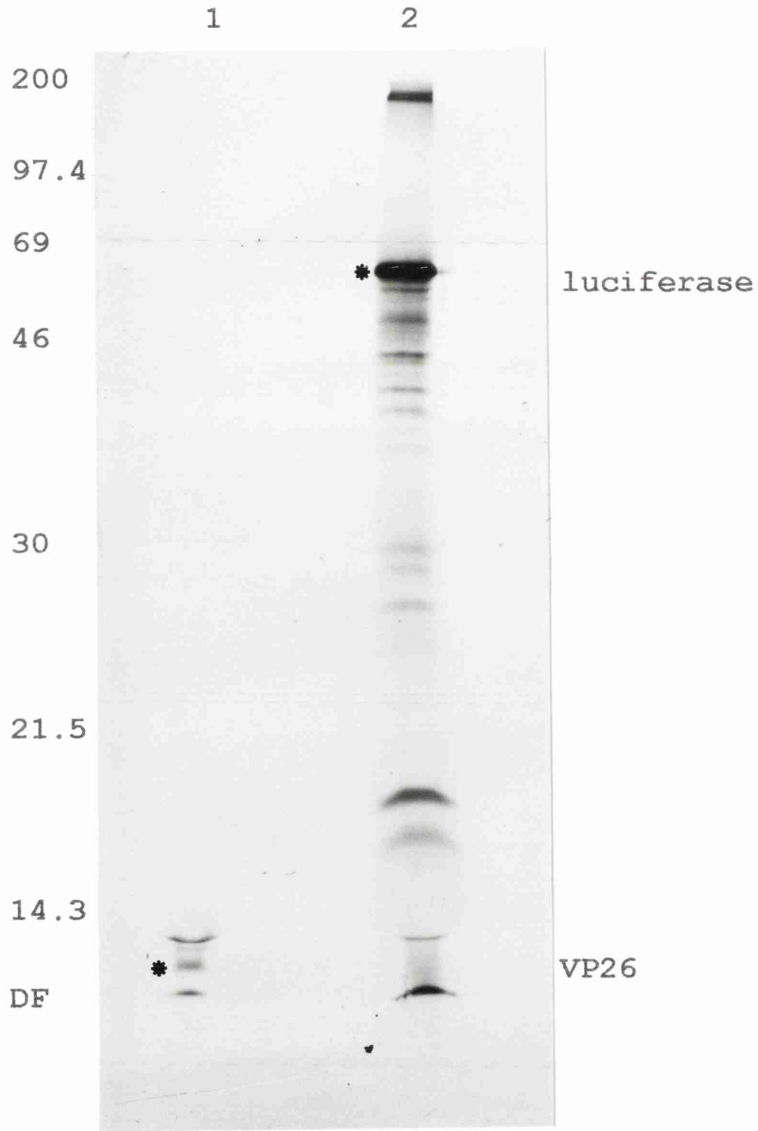

1
2
200
97.4
69
46
30
21.5
14.3
DF
luciferase
VP26

Figure 14:- The protein products of *in vitro* transcription/translation of pJT353

pJT353 (lane1) and a control plasmid consisting of the luciferase gene under the control of the SP6 promoter (lane 2) were used in the *in vitro* linked transcription/translation system as described in Methods. [^{35}S]methionine was included in the reaction so that the protein products could be identified by flurography. The proteins were resolved on a 15% polyacrylamide gel. The major proteins produced (indicated by ✱) are of the same size as VP26 (12,000Da), the product of the UL35 ORF (lane 1) and luciferase; 62,000Da (lane 2). The positions of the marker proteins were observed by staining with Coomassie Brilliant Blue and the sizes are indicated to the left of the gel. The position of the dye front is indicated (DF).

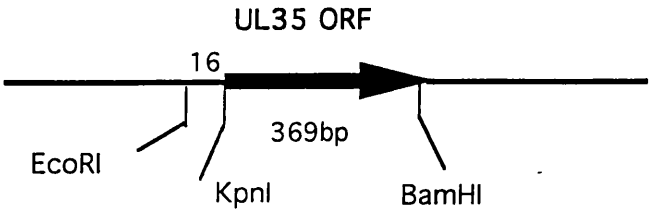
designed as primers are shown in figure 11. Oligonucleotide 1 corresponded to the first 20 bases of the ORF (70566 to 70585) and oligonucleotide 2 to 20 bases downstream from the termination codon (70912 to 70931). To facilitate cloning a KpnI site was introduced at the 5' end of oligonucleotide 1 and a BamHI site at the 5' end of oligonucleotide 2. Neither of these enzymes cut within the UL35 ORF. Since the activity of restriction enzymes may be inefficient when the restriction site is positioned at the end of a sequence, 3 and 2 additional nucleotides were included upstream of oligonucleotides 1 and 2 respectively. The 369bp product was purified from a 1% agarose gel, cleaved with KpnI and BamHI and ligated into KpnI/BamHI digested pAcCL29.1 to generate pJT352 as shown in figure 12. This plasmid was used to make recombinant baculovirus.

To check the fidelity of the PCR product the purified UL35 ORF was sub-cloned from pJT352 as a KpnI/BamHI fragment into pUC19 to generate pJT351, figure 13. This plasmid was then used in the partial sequencing of the UL35 PCR product by the dideoxy chain termination method.

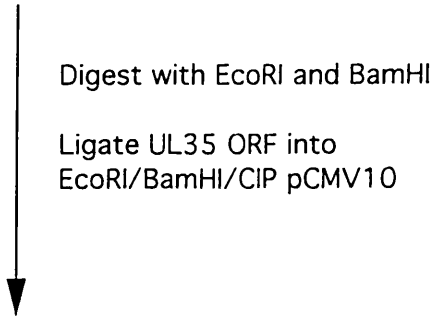
However, since the UL35 gene had not previously been expressed it was necessary to verify that the construct made from the PCR product was correct. Examination of the protein product was carried out by *in vitro* transcription/translation and by immunofluorescence.

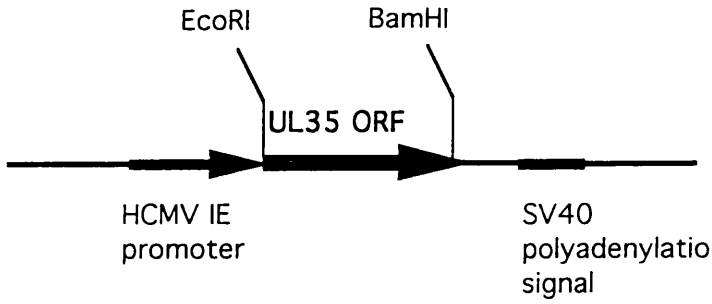
1.7 *IN VITRO* TRANSCRIPTION/TRANSLATION STUDIES OF UL35

The UL35 gene was placed under control of the SP6 RNA polymerase promoter present in the plasmid pGEM-2 as described in figure 13. The UL35 ORF was released from pJT351 by digestion with SacI and BamHI and was ligated into SacI/BamHI digested pGEM-2 to form pJT353. Restriction enzyme analysis was employed to verify the construct. This plasmid was used in conjunction with the linked transcription/translation system as described in Methods. This linked system contains all the components required for the production of protein from protein-encoding DNA sequences cloned downstream of the SP6 RNA promoter. As a control, a construct containing the luciferase gene under the control of the SP6 promoter (provided by the manufacturers) was used (figure 14). The proteins were resolved on a 15% acrylamide gel. Lane 2 shows the proteins produced in the control reaction. The dominant product is the 62,000Da luciferase protein; the bands below presumably represent breakdown products or premature termination products. Lane 1 shows the transcription/translation product of pJT353 and reveals the presence of a novel protein absent from the control. This protein migrated below a 14,300Da marker protein and runs just above the dye



pJT351





pE35

Figure 15:- Construction of pE35

Figure 16:- Immunofluorescence showing the intracellular localisation of VP26

BHK-21 cells were transfected with a plasmid expressing VP26 (pE35) as described in Methods. The distribution of VP26 was examined using the primary rabbit antibody, trypE-UL35 and the immunofluorescence observed was due to a goat anti-rabbit IgG that was conjugated to texas red (GARTRITC).

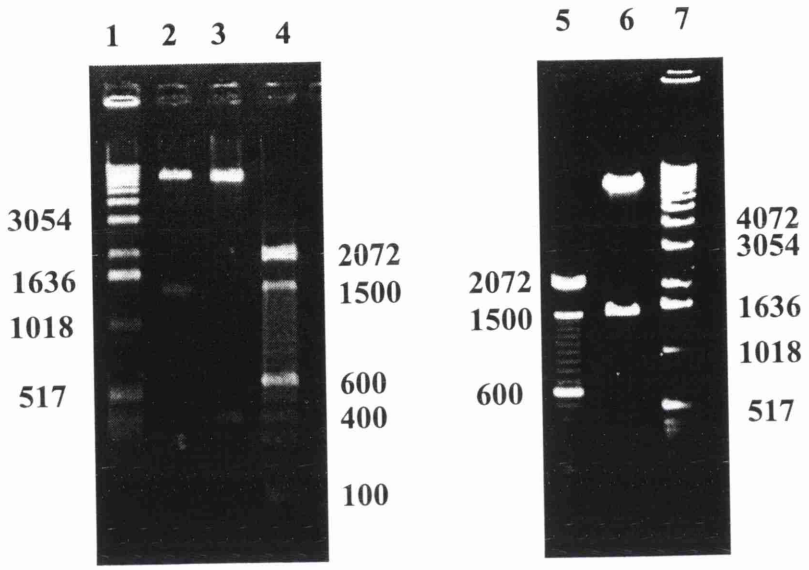

1
2
3
4
5
6
7
3054
1636
1018
517
2072
1500
600
400
100
2072
1500
600
4072
3054
1636
1018
517

Figure 17:- Restriction digest analysis of pAcCL29.1 derived plasmids containing individual HSV-1 capsid genes.

1ug of pJT182 (lane 2) and pJT352 (lane 3) was each digested with both SstI and BamH1 restriction enzymes to release 1376bp and 375bp fragments containing the UL18 and UL35 ORFs respectively. 1ug of pJT381 (lane 6) was digested with XbaI and PstI to release a 1490bp fragment containing the UL38 ORF. The DNA was electrophoresed through a EtBr stained 1% agarose gel and the restriction patterns were visualised by U.V. illumination. The sizes of fragments produced as a result of the restriction digests were estimated from comparison to 1kb (lanes 1 and 7) and 100bp (lanes 4 and 5) DNA ladders.

front. This novel protein corresponds in size to the 12kDa protein, VP26, the product of the UL35 gene.

1.8 IMMUNOFLUORESCENCE STUDIES OF THE UL35 GENE PRODUCT, VP26

The UL35 gene was also cloned under control of the HCMV IE promoter present in the plasmid pCMV10 (Stow *et al.*, 1993). The UL35 ORF was cloned from pJT351 as a EcoRI/BamHI fragment and ligated into EcoRI/BamHI digested pCMV10 to form pE35 (figure 15). The structure of this construct was confirmed by restriction enzyme analysis. On transfection of mammalian cells the intracellular distribution of VP26 was examined with the rabbit antibody trpE-UL35 as described in Methods. Figure 16 shows a cell which is expressing the VP26 protein and demonstrates that epitopes which are recognised by trpE-UL35 are present in the correct configuration in the plasmid expressed protein. The fluorescence is observed throughout the cell indicating that VP26 lacks the nuclear targeting signals necessary to transport the protein to the nucleus.

1.9 RESTRICTION ANALYSIS OF BACULOVIRUS TRANSFER VECTORS

In order to confirm the structures of the plasmids to be used as transfer vectors in the construction of recombinant baculoviruses, each plasmid was analysed by restriction enzyme digestion. The restriction patterns obtained are shown in figure 17. Digestion of pJT182 (lane 2) and pJT352 (lane 3) with SstI and BamHI released 1376bp and 375bp fragments containing the UL18 and the UL35 ORFs respectively. Digestion of pJT381 (lane 6) with XbaI and PstI released a fragment of 1490bp containing the UL38 ORF.

2 CONSTRUCTION AND CHARACTERISATION OF RECOMBINANT BACULOVIRUSES

2.1 INTRODUCTION OF HSV-1 CAPSID PROTEIN GENES INTO BACULOVIRUS

Two parental baculoviruses were used in the construction of recombinant viruses. The wild type *Autographa californica* nuclear polyhedrosis virus (AcNPV) was used in the production of AcUL19 (Nicholson, 1992) whereas AcPAK6, a polyhedrin-negative

derivative of AcNPV which contains the *lacZ* gene under control of the polyhedrin promoter was used in the construction of AcUL18, AcUL35 and AcUL38. This virus has been specifically designed to produce a high percentage (up to 100%) of recombinant viruses which are distinguishable from any parental background due to the absence of the blue colour which is produced on staining with X-gal (Bishop, 1992). It contains two additional Bsu361 sites in addition to the Bsu361 restriction site between the polyhedrin promoter and residual polyhedrin gene sequences in AcNPV; one in an adjacent upstream sequence and one in an essential downstream gene. AcPAK6 and AcNPV were digested with Bsu361 and treated with alkaline phosphatase to reduce the background of non-recombinant virus, before being used in a transfection reaction.

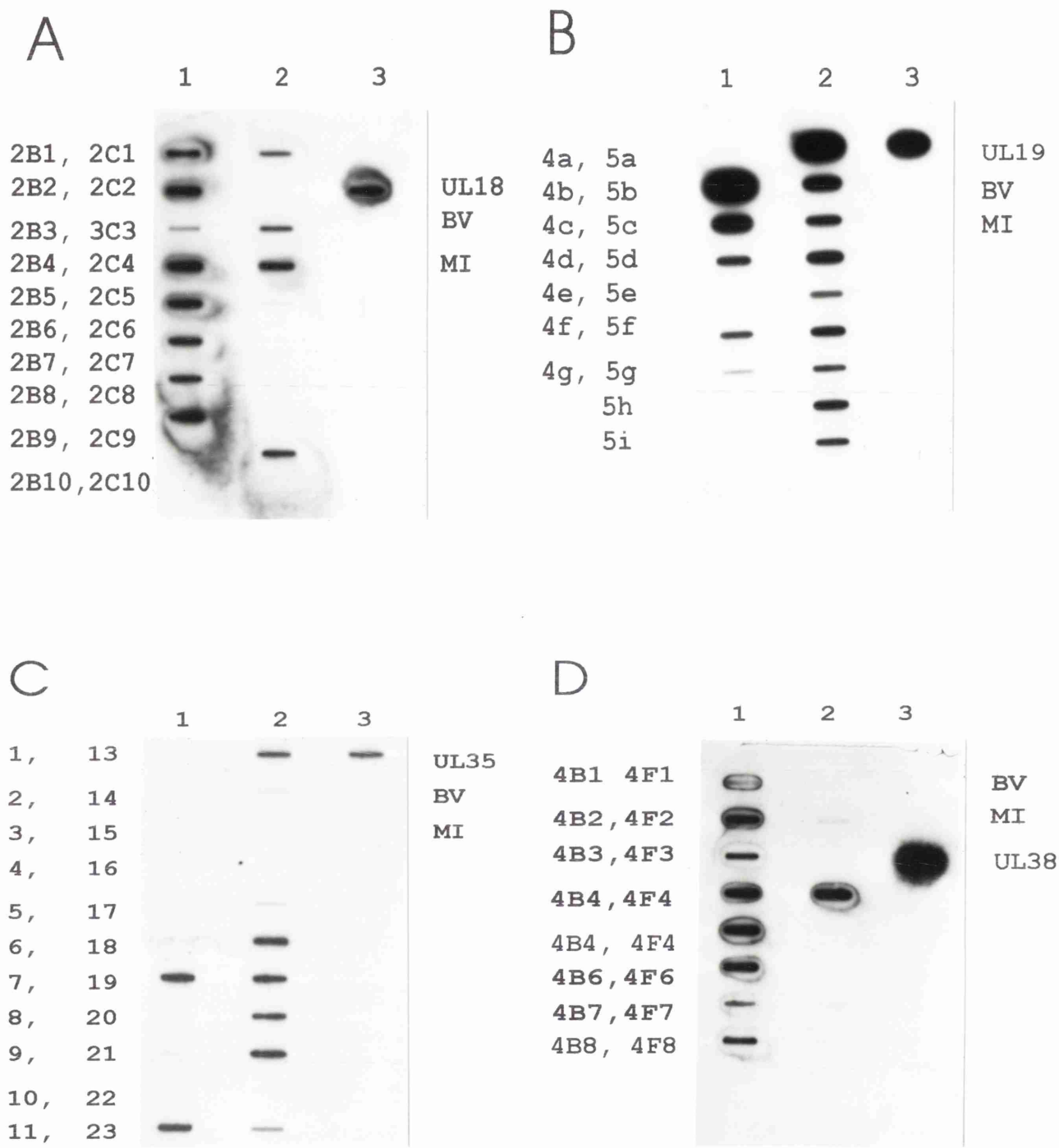
pJT182 (UL18), pJT352 (UL35) and pJT381 (UL38) were cotransfected with parental virus AcPAK6 DNA using the lipofectin method (Rose *et al.*, 1991). Plates were harvested after 3 days and progeny virus titrated. Clear plaques were obtained on the 10^{-3} dilution of the transfection harvest. These were isolated and purified to homogeneity through two cycles of plaque purification as described below.

The UL19 gene was introduced into AcNPV by the calcium phosphate method of transfection essentially as described by Elliott and McGregor (1989). Recombinant viruses partially plaque purified were obtained from P. Nicholson and were subjected to two further rounds of plaque purification.

2.2 PLAQUE PURIFICATION OF RECOMBINANT BACULOVIRUSES

12-20 plaque isolates of each recombinant virus were picked into 1ml of medium which was freeze thawed to release the virus. 200ul of the plaque suspension was used to infect 35mm monolayers of Sf21 cells. The supernatant (1.5ml) containing the virus was harvested after four days incubation at 28°C. 200ul of this plate harvest (plate harvest one) was used to infect cells to produce viral DNA. Slot blot analysis of the DNA was used to identify viruses which contained individual HSV-1 genes.

Isolates which were thought to contain the genes UL18, UL19, UL35 and UL38 were designated with the prefix 'Ac' and were referred to as AcUL18, AcUL19, AcUL35 and AcUL38 respectively. Two isolates which gave positive results with slot blot hybridisation were further purified by a second round of plaque purification, picked plaques were grown up (plate harvest two) and DNA was prepared. Positive isolates were again identified by slot-blot hybridisation.


A
1
2
3
2B1, 2C1
2B2, 2C2
2B3, 3C3
2B4, 2C4
2B5, 2C5
2B6, 2C6
2B7, 2C7
2B8, 2C8
2B9, 2C9
2B10,2C10
UL18
BV
MI
B
1
2
3
4a, 5a
4b, 5b
4c, 5c
4d, 5d
4e, 5e
4f, 5f
4g, 5g
5h
5i
UL19
BV
MI
C
1
2
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1, 13
2, 14
3, 15
4, 16
5, 17
6, 18
7, 19
8, 20
9, 21
10, 22
11, 23
UL35
BV
MI
D
1
2
3
4B1 4F1
4B2,4F2
4B3,4F3
4B4,4F4
4B4, 4F4
4B6,4F6
4B7,4F7
4B8, 4F8
BV
MI
UL38

Figure 18:- Slot blot analysis of recombinant baculoviruses

Recombinant virus DNA was transferred to nylon membranes using the slot blot apparatus. Membranes were probed with nick-translated [^{32}P]-labelled DNA specific for the recombinant gene and positive hybridisation was identified by fluorography. The isolate numbers are indicated on the left hand side of the autoradiograph and correspond to the samples in lanes 1 and 2. Viruses recombinant for UL18 (A) were probed with the UL18 ORF which was released by KpnI/BamHI digestion of pJT181; viruses recombinant for UL19 (B) were probed with the BglII fragment containing the UL19 ORF that was released from pBJ199; viruses recombinant for UL35 (C) were probed with the UL35 ORF PCR product; viruses recombinant for UL38 (D) were probed with the UL38 ORF which was released from pBJ382 by digestion with PstI and BamHI. Lane 3 contains the control DNAs; DNA containing the gene under investigation (UL), DNA from mock-infected Sf21 cells (MI), DNA from Sf21 cells infected with AcPAK6 (BV).

2.3 SLOT BLOT ANALYSIS OF RECOMBINANT BACULOVIRUS DNA

DNAs from each set of recombinant viruses were denatured and hybridised to nylon membranes using the slot blot apparatus as described in Methods. In each case, DNAs from mock-infected and AcPAK6-infected Sf21 cells were included as negative controls. 1ug of the following DNAs: pJT182, pJT352 and pJT381 was used as positive controls in the identification of AcUL18, AcUL35 and AcUL38 viruses respectively. A BglII fragment containing the UL19 ORF was released by digestion from pBJ199 and provided the positive control for AcUL19 viruses. Nick-translation was used to prepare [^{32}]phosphate-labelled DNA probes specific for each virus set under investigation. The autoradiographs obtained from hybridisation of the labelled probe with the viral DNAs are shown in figure 18. It should be noted that the blot of the AcUL35 set of viruses (figure 18C) was obtained using DNA from the first round of plaque purification and those of AcUL18 (figure 18A) and AcUL38 (figure 18D) from the second round of purification. The AcUL19 DNA (figure 18B) was obtained from two rounds of purification subsequent to those carried out by P. Nicholson. DNA from AcUL18 viruses was probed with 1ug of [^{32}P]-UL18 ORF contained on a KpnI/BamHI fragment released from pJT181. 13 out of 18 viruses were identified that contained the UL18 DNA sequence (figure 18A). 14 out of 16 AcUL19 viruses when probed with the [^{32}P]-UL19 BglII fragment gave a positive signal (figure 18B). DNA from AcUL35 viruses was probed with [^{32}P]-UL35 PCR product and identified 7 out of 22 positive viruses (figure 18C). 9 out of 16 AcUL38 viruses were identified as positive when probed with [^{32}P]-labelled UL38 fragment that was released from pBJ381 upon digestion with BamHI and PstI (figure 18D).

From this analysis of recombinant viruses it appeared that the actual efficiency of recombination was lower than expected even though there was a high percentage of clear plaques. It is possible that some of these plaques represented parental virus containing mutations in the *B*-galactosidase gene or that insufficient DNA was transferred to the membranes. It is also possible that the process of picking individual plaques was inefficient and did not result in any virus being collected. The lowest percentage of positive recombinants was observed with the AcUL35 viruses. This was to be expected since this set of viruses represented the first round of plaque purification. The highest percentage of positive plaques was found in the AcUL19 set of isolates which had undergone several additional rounds of plaque purification (Nicholson, 1992).

In each case, an isolate positive for the required gene was grown up to produce a high titre stock of the virus to be used in further characterisation studies. Typically 0.5ml of the final plate harvest was used to infect cells in a small (25cm^2) tissue culture flask and the resultant supernatant was used to infect cells in large flasks to produce low titre

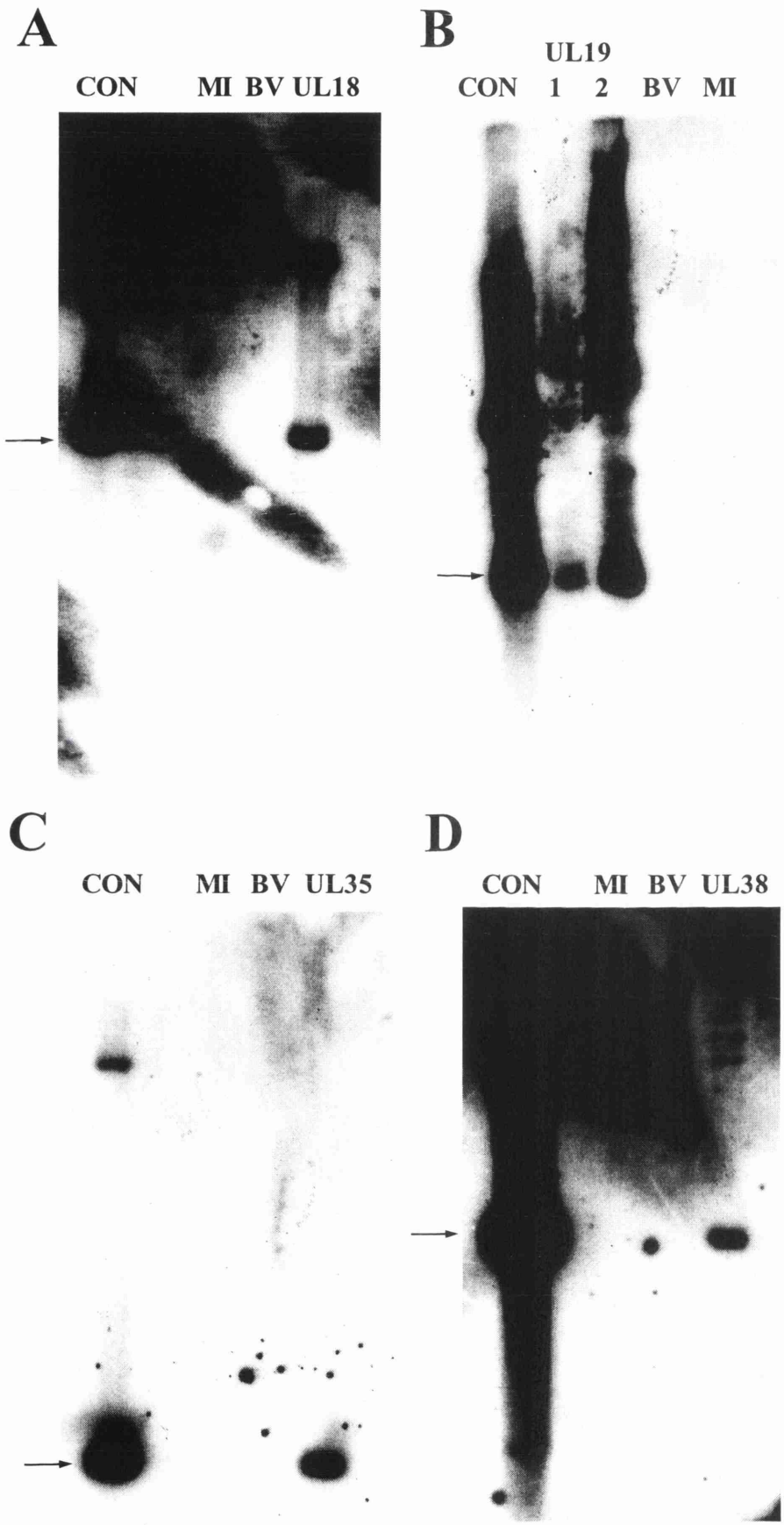

A
CON
MI
BV
UL18
B
UL19
CON
1
2
BV
MI
C
CON
MI
BV
UL35
D
CON
MI
BV
UL38

Figure 19:- Southern hybridisation of recombinant baculoviruses

Recombinant baculovirus DNA was digested with the appropriate restriction enzymes to allow release of the sequences encoding the relevant HSV-1 capsid protein. AcUL18, AcUL35 and the respective control DNAs (pJT182 and pJT352) were digested with SstI and BamHI to release the 1376bp and 375bp UL18 and UL35 ORF containing fragments respectively. Two separate AcUL19 clones (designated 1 and 2) as well as the control DNA (a BglII fragment released from pBJ199) were digested with EcoRV and BamHI to release the 2893 N-terminal base pairs of the UL19 ORF. AcUL38 and pJT381 DNA was digested with XbaI and PstI to release the 1490bp UL38 ORF fragment. DNA from mock-infected and AcPAK6- infected Sf21 cells were digested similarly in each case. DNA fragments were separated by electrophoresis and transferred to nylon membranes as described in Methods. Membranes were probed with nick-translated [^{32}P]-labelled DNA prepared from either plasmids or purified fragments containing the homologous gene. (A) viruses recombinant for UL18 were probed with pJT181. (B) viruses recombinant for UL19 were probed with the UL19 BglII fragment derived from pBJ199. (C) viruses recombinant for UL35 were probed with a KpnI/BamHI UL35 ORF fragment released from pJT351. (D) viruses recombinant for UL38 were probed with pBJ382. Positive hybridisation was identified by fluorography and the position of the capsid gene DNA fragments is indicated by an arrow. In (A) to (D) mock-infected (MI) and AcPAK6 (BV) DNA is indicated and the positive controls are each identified as CON.

virus stock (~$1x10^7$ p.f.u./ml). High titre virus (~$1x10^9$ p.f.u./ml) was subsequently produced from infection of cells in roller bottles. The isolates chosen were 2B4 (AcUL18), 4b (AcUL19), 4B3 (AcUL38) and 18a (AcUL35) which originated from a second round of plaque purification of plaque 18.

2.4 SOUTHERN ANALYSIS OF RECOMBINANT BACULOVIRUSES

35mm plates containing monolayers of Sf21 cells were mock-infected or infected with 5 p.f.u./cell of either AcUL18, AcUL19, AcUL35, AcUL38 or AcPAK6. After four days incubation at 28°C, viral DNA was prepared from the infected cells as described in Methods. 1/10th of this was digested overnight with the appropriate restriction enzymes. Plasmids containing the appropriate HSV-1 capsid protein genes were similarly digested to provide controls. The digested DNAs were separated on a 1% agarose gel and transferred to nylon membranes as described in Methods. [^{32}P]-labelled probes were prepared from plasmids containing the appropriate sequences (figure 19). Digestion of pJT182 (figure 19A, CON) and AcUL18 (figure 19A, UL18) with SstI and BamHI released the 1376bp fragment of DNA containing the UL18 ORF. Digestion of the BglII UL19 fragment from pBJ199 (figure 19B, CON) and 2 individual AcUL19 viruses (figure 19B, UL19 1 and 2) with EcoRV and BamHI each released a 2893bp fragment corresponding to the N-terminus of the UL19 gene. The UL35 ORF was released from both pJT352 (figure 19C, CON) and AcUL35 (figure 19C, UL35) upon digestion with SstI and BamHI. Digestion of pJT381 (figure 19D, CON) and AcUL38 (figure 19D, UL38) with XbaI and PstI released the UL38 ORF. None of the probes hybridised with digested DNA from either mock-infected cells (figure 19, MI) or AcPAK6-infected cells (figure 19, BV). From this analysis it is apparent that each of the recombinant baculoviruses: AcUL18, AcUL19, AcUL35 and AcUL38 contained the appropriate HSV-1 capsid gene sequences.

2.5 EXPRESSION OF HSV-1 CAPSID PROTEINS BY RECOMBINANT BACULOVIRUSES

To examine protein expression by the recombinant baculoviruses, monolayers of Sf21 cells were singly infected with 5 p.f.u./cell of AcUL18, AcUL19, AcUL26, AcUL26.5, AcUL35 or AcUL38. At 30h p.i. [^{35}S]methionine was added and the cells were harvested after a further 2h incubation. Mock-infected and AcPAK6-infected control samples were treated in a similar fashion. Examination of the protein profiles (figure 20) revealed that the Mw. 116,000 *B*-galactosidase protein expressed by the parental virus, AcPAK6, was absent from the recombinant baculovirus-infected cell profiles.

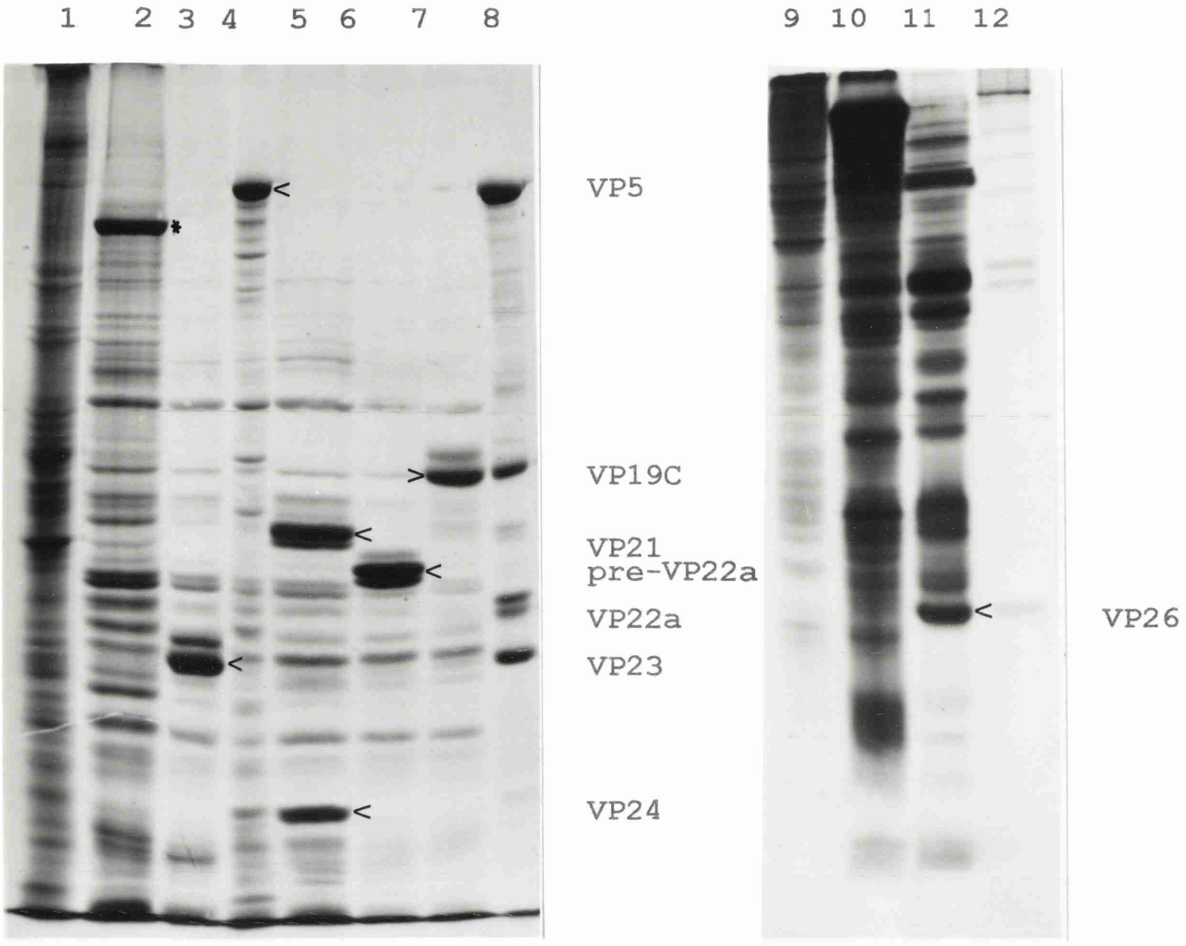

1 2 3 4 5 6 7 8
9 10 11 12
VP5
VP19C
VP21
pre-VP22a
VP22a
VP23
VP24
VP26

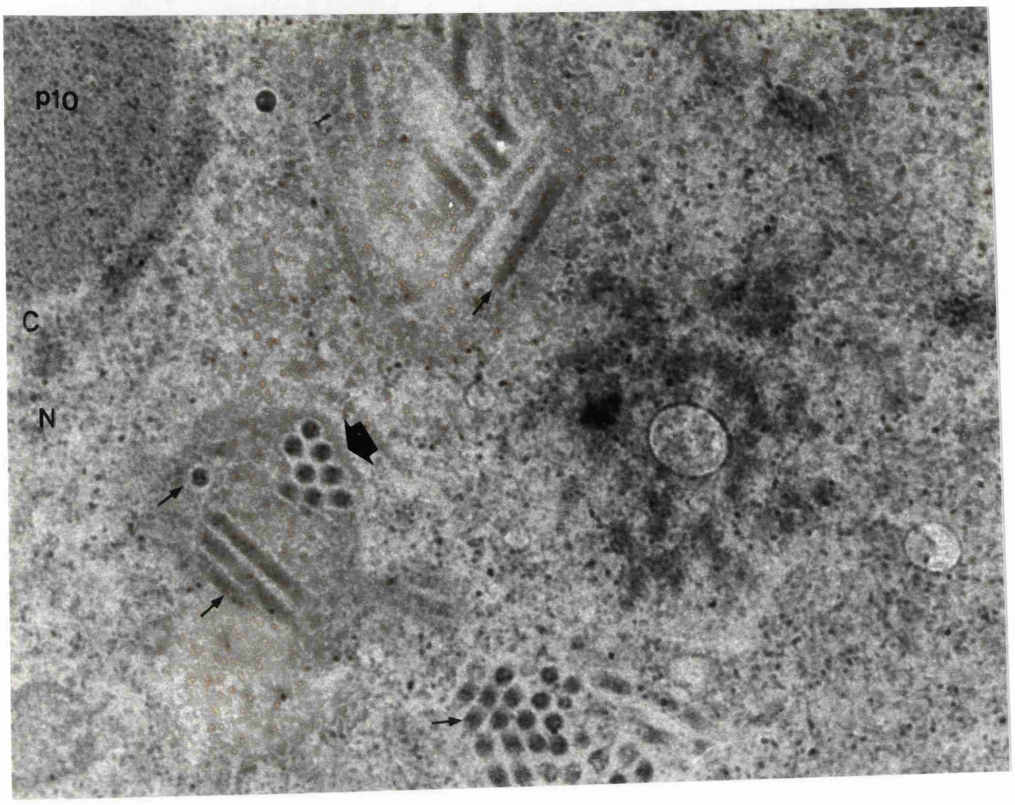
Figure 20:- Expression of HSV-1 capsid proteins by recombinant baculoviruses.

[^{35}S]methionine-labelled polypeptide profiles of mock-infected Sf21 cells (lanes 1 and 9) and cells infected with 5 p.f.u./cell of AcPAK6 (lanes 2 and 10), AcUL18 (lane 3), AcUL19 (lane 4), AcUL26 (lane 5), AcUL26.5 (lane 6), AcUL38 (lane 7) or AcUL35 (lane 11). Purified HSV-1 B capsids are shown in lanes 8 and 12. The capsid proteins VP5, VP19C, VP21, pre-VP22a, VP22a, VP23, VP24 and VP26 are indicated to the right of the purified capsid samples. Novel polypeptides synthesised by the recombinant viruses are marked with arrowheads and the *B*-galactosidase protein synthesised by the parental AcPAK6 virus is indicated (*). Samples shown in lanes 1-8 were resolved on an 11% polyacrylamide gel whereas those in lanes 9 to 12 were resolved on a 15% gel.

Furthermore, each of the recombinant baculoviruses expressed abundant novel proteins which were not present in AcPAK6-infected cells. From examination of Coomassie Brilliant Blue stained gels (not shown) it was apparent that these proteins were expressed to higher levels than any of the other cellular or baculovirus-encoded proteins. The sizes of these novel proteins corresponded to those expected for the appropriate gene products and in most cases they co-migrated with their counterpart proteins in HSV-1 B capsids (figure 20, lanes 8 and 12). Thus AcUL18 expressed a 33,000Da protein corresponding to VP23 (figure 20, lane 3). This protein migrated close to a host cell protein which can be seen just above VP23 in all the lanes. A novel <25,000Da protein (not indicated) was also observed in cells infected with AcUL18 and probably represents a breakdown product of VP23. In cells infected with AcUL19, a 155,000Da protein is produced which corresponds to the major capsid protein, VP5 (figure 20, lane 4). This protein appears to be fairly unstable as is indicated by the presence of a number of breakdown products in this lane. The instability of VP5 has also been observed in capsid preparations produced from HSV-1-infected BHK cells and probably accounts for some of the minor products present in figure 20, lane 8. AcUL38 expressed a 53,000Da protein corresponding to VP19C (figure 20, lane 7). AcUL35 expressed a 12,000Da protein corresponding to VP26 (figure 20, lane 11). This protein migrates very close to the baculovirus encoded 12,500Da basic protein and thus cannot be identified on stained gels. The 12,500Da basic protein is produced in the late phase of baculovirus gene expression (Tweeten *et al.*, 1980; Wilson *et al.*, 1987) and so is not heavily labelled with [^{35}S]methionine under these very late conditions.

As has been shown previously, the product of the UL26 gene is a protease which cleaves itself and the product of the gene UL26.5 to generate a number of protein species. Thus in the single infections described here, the novel proteins present in AcUL26.5-infected cells (figure 20, lane 6) correspond to the unprocessed, primary products designated ICP35 c and d which are here after referred to as pre-VP22a. These proteins are larger than the processed VP22a (Mw. 38,800) which is found in B capsids. Infection with AcUL26 generated novel bands of 42,000Da and 25,000Da corresponding to VP21 and VP24 respectively (figure 20, lane 5) which represent the C- and N- terminal portions of the UL26 gene product (Liu and Roizman, 1991b; Preston *et al.*, 1992).

A



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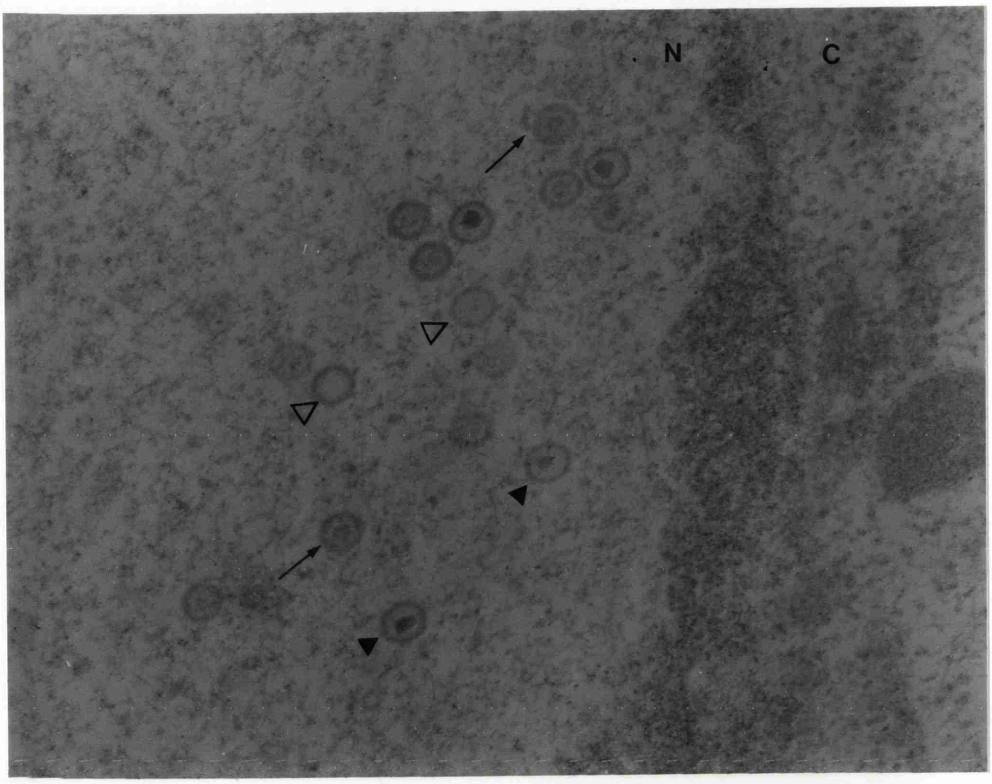

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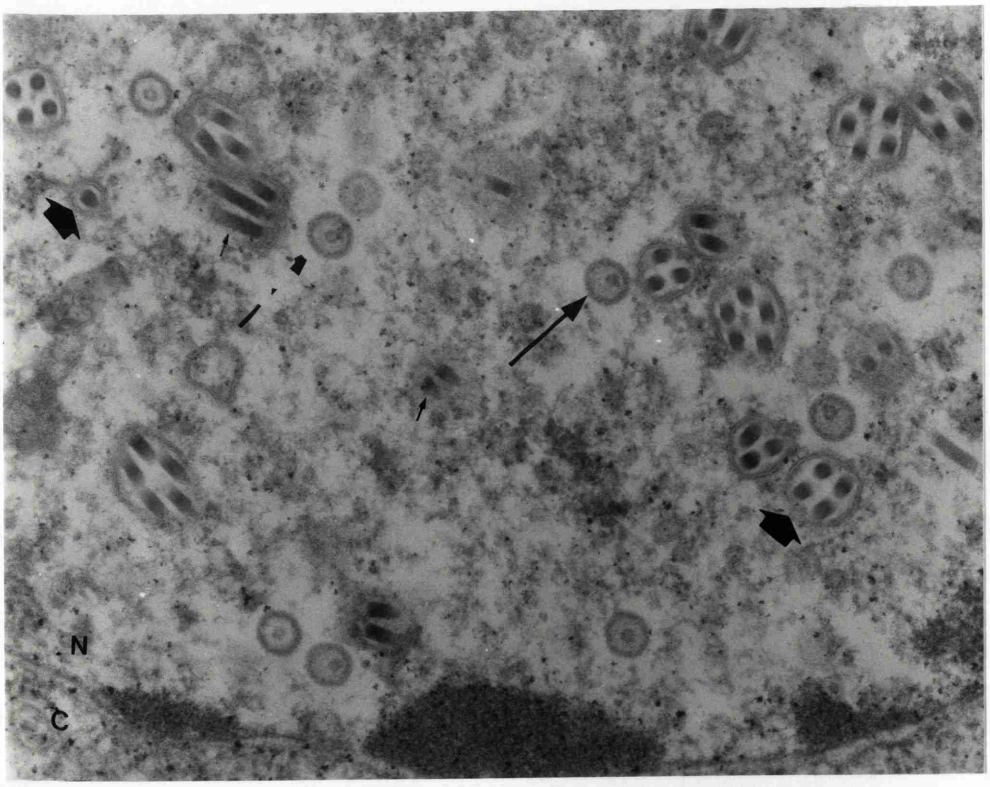


Figure 21:- Capsid assembly in recombinant baculovirus-infected cells.

BHK cells infected with 5 p.f.u./cell of HSV-1 strain 17 were incubated at 37°C for 24h. Sf21 cells infected with 5 p.f.u./cell each of AcPAK6, all 6 recombinant viruses (AcUL18, AcUL19, AcUL26, AcUL26.5, AcUL35 and AcUL38) or mock-infected Sf21 cells were incubated at 28°C for 48h. Panels (A) to (C) show electron micrographs of thin sections of cells infected with the following: (A) AcPAK6, (B) HSV-1 strain 17, (C) all 6 recombinant viruses. N=nucleus, C=cytoplasm. The features characteristic of baculovirus infection: baculovirus nucleocapsid (→), enveloped baculovirus capsids (➧) and p10 protein (p10) are indicated. In panel B: HSV-1 A capsids (▷), B capsids (⟶) and C capsids (▶) are indicated. In panel C: recombinant generated B capsids (⟶) are indicated. The bar marker represents 200nm.

3 CHARACTERISATION AND PURIFICATION OF CAPSID STRUCTURES MADE USING THE BACULOVIRUS EXPRESSION SYSTEM

3.1 ASSEMBLY OF HSV-1 CAPSID STRUCTURES IN RECOMBINANT BACULOVIRUS INFECTED CELLS

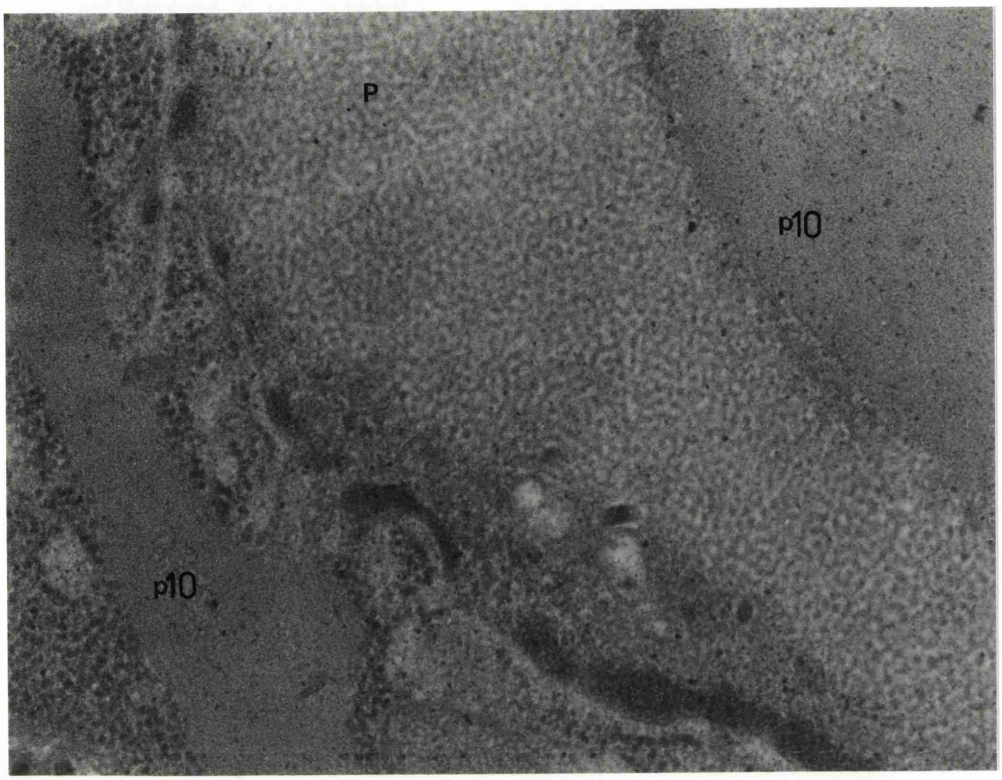
To determine whether expression of the six HSV-1 capsid protein genes would suffice for capsid assembly in insect cells, monolayers of Sf21 cells were co-infected with 5 p.f.u./cell of each recombinant baculovirus. The cells were then prepared for electron microscopy and examined in thin section for the presence of recognisable HSV-1 capsid structures. The internal morphology of these cells was compared to similarly prepared Sf21 cells that were either mock-infected or infected with 5 p.f.u./cell of the parental baculovirus, AcPAK6, or to BHK cells infected with HSV-1.

When Sf21 cells were infected with AcPAK6, baculovirus capsids were observed; often occupying a large volume of the nucleus (figure 21A). Baculovirus capsids are rod shaped and are approximately 210-290nm in length when viewed in longitudinal section. In transverse-section they have a diameter of approximately 40nm. The capsids were observed singly and as enveloped virions; the number of capsids per virion ranged from 1-6. The number of capsids/virions varied between cells and is representative of the degree of infection of individual cells. Other fibrous material resulting from aggregation of the p10 protein of baculovirus was detected in the nuclei and cytoplasm of cells (van der Wilk *et al*, 1987).

As shown in figure 21B, infection of BHK cells with HSV-1 results in three characteristic types of capsids which have been designated type A (or empty) capsids, type B (or intermediate) capsids and type C (or full) capsids (Gibson and Roizman, 1972). The outer shell of each capsid is approximately 100nm in diameter as viewed in transverse section. Type A capsids have no internal structure whereas type B capsids contain a 40-60nm internal scaffold or core. Type C capsids lack the scaffold core but contain the viral DNA which appears as a dark staining mass inside the capsid shell.

When insect cells were infected with all six recombinant baculoviruses, that is; AcUL18, AcUL19, AcUL26, AcUL26.5, AcUL35 and AcUL38 (figure 21C), characteristic HSV-1 capsids were readily observed which appeared indistinguishable from those produced in HSV-1 infected BHK cells (compare figures 21B and 21C). The type of capsids observed (figure 21C) resemble the type B capsids containing the internal proteinaceous core. The lack of type C caspids was to be expected due to absence of HSV-1 viral DNA and packaging proteins in this system. Type A capsids are thought to be the result of abortive packaging and as a result are not observed in this system (Lee *et al.*, 1988; Sherman and Bachenheimer, 1988; Rixon *et al.*, 1988).

A



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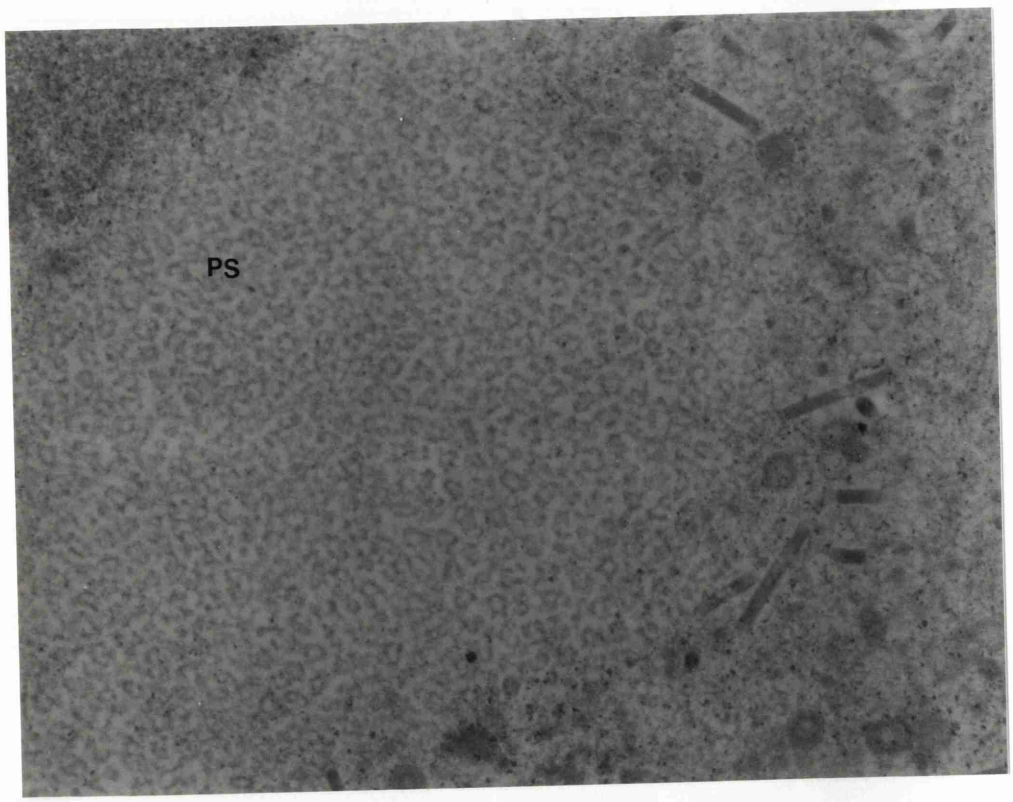

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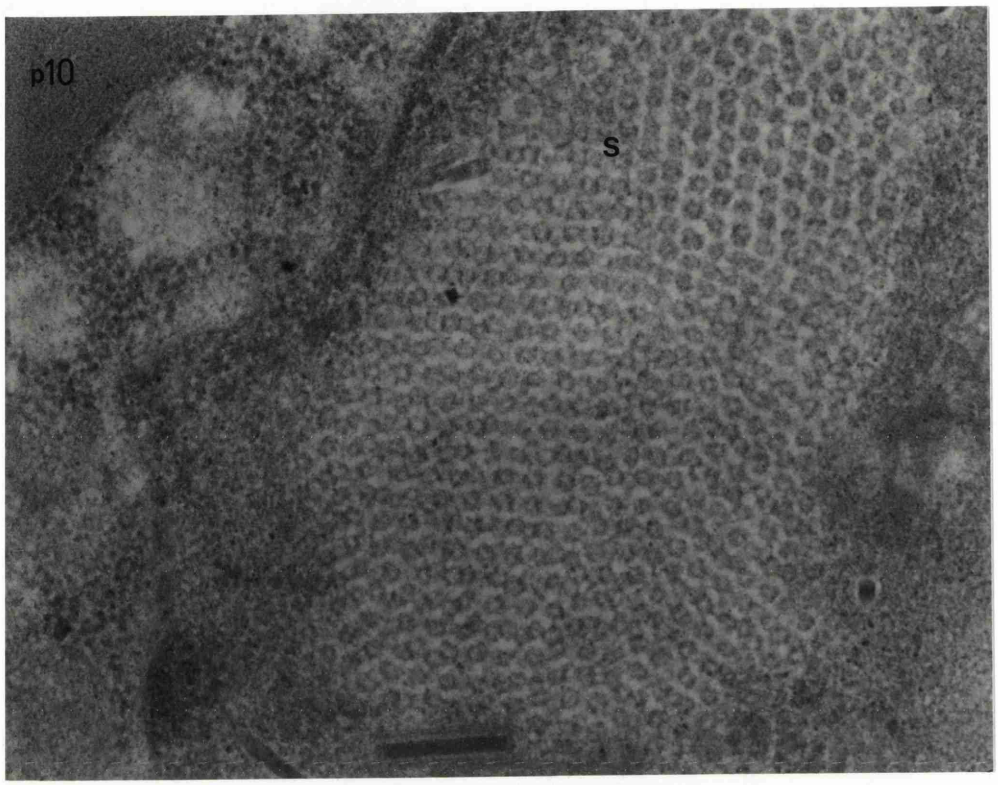

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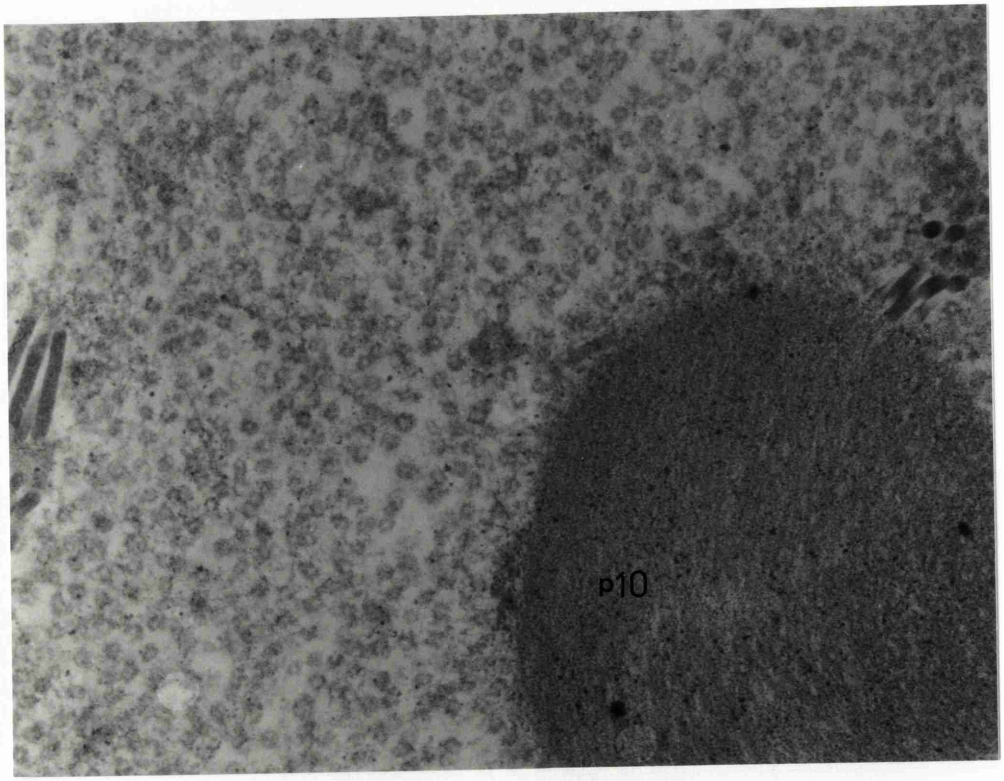


Figure 22:- Structures formed in cells infected with AcUL26 and AcUL26.5

Sf21 cells were infected with 5 p.f.u./cell of each of recombinant baculovirus and were incubated at 28^{o}C for 48h. Electron micrographs of thin sections of cells show the structures formed upon infection with (A) AcUL26, (B) AcUL26.5, (C) and (D) with both AcUL26 and AcUL26.5 In (A) a large aggregate of amorphous protein (P) is indicated whereas in (B) the protein appears to be interspersed with regular circular structures (PS). In (C) the protein has formed semi-crystalline arrays of scaffolds (S) whereas in (D) these scaffold-like particles appear dispersed. The baculovirus-encoded p10 protein (p10) is indicated. The bar represents 200nm.

Baculovirus capsids were also observed in cells infected with the recombinant baculoviruses. Single enveloped baculovirus capsids cut in transverse section could be distinguished from type C capsids by close examination of the baculovirus envelope. This has the appearance of two close thin lines rather than the single shell of the HSV-1 capsid. HSV-1 capsid-like structures were never observed in uninfected cells (not shown) or in cells infected with AcPAK6 (figure 21A).

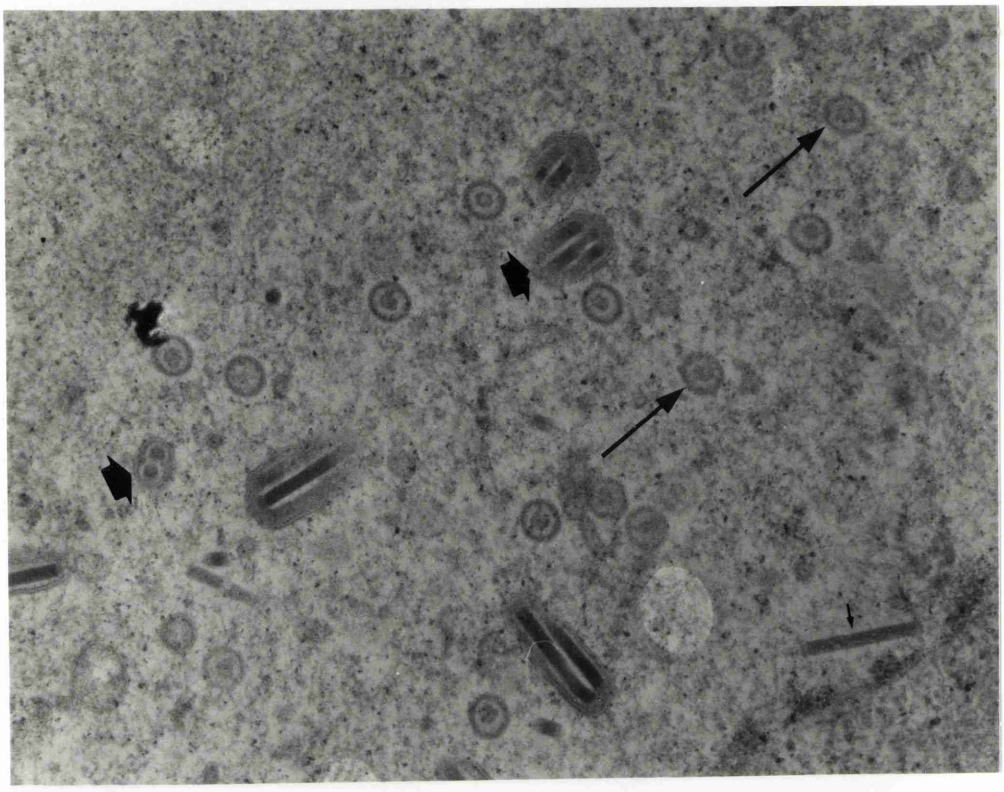
3.2 EM ANALYSIS OF CELLS INFECTED WITH AcUL26 AND AcUL26.5

Monolayers of Sf21 cells were singly infected with each of the recombinant baculoviruses and the cells were prepared for electron microscopy. In cells infected with either AcUL18, AcUL19, AcUL35 or AcUL38, no novel structures could be identified and the cells resembled those infected with AcPAK6. Conversely, in cells infected with either AcUL26 (figure 22A), or AcUL26.5 (figure 22B), large aggregates of fibrous material were observed. In the majority of AcUL26.5 infected cells, the fibrous material was interspersed with particles of similar size and appearance to the internal scaffolds of HSV-1 B capsids (scaffold-like particles) which were 40-60nm in diameter. The accumulations of UL26 and UL26.5 encoded proteins were predominately found in the nucleus although they were also observed in the cytoplasm. When cells were infected with both AcUL26 and AcUL26.5, closely-packed arrays of 40-60nm particles were observed in some cells (figure 22C), while in others similar sized dispersed particles were apparent (figure 22D). Immunoelectron microscopy has confirmed that these particles contain the UL26.5 encoded protein, VP22a (Preston *et al.*, 1994). Neither the 40-60nm particles nor the fibrous material were detected in mock-infected or AcPAK6-infected cells and both were readily distinguishable from aggregations of the baculovirus encoded p10 protein. The similarity between these particles and the cores of B capsids is demonstrated in figure 23B (described below).

3.3 THE EFFECT ON CAPSID FORMATION OF OMITTING INDIVIDUAL RECOMBINANT BACULOVIRUSES

To identify the contribution of each of the capsid proteins to the capsid structure, cells were infected with various combinations of the recombinant baculoviruses. In an initial experiment where AcUL35 was omitted, apparently normal HSV-1 B capsid structures were produced indicating that VP26 is not essential for capsid assembly (figure 23A). Figure 23B shows a cell containing HSV-1 capsids as well as additional smaller structures which resemble the 40-60nm particles formed from co-infection of AcUL26 and AcUL26.5 (figure 22C and D). These can clearly be seen to resemble the internal

A



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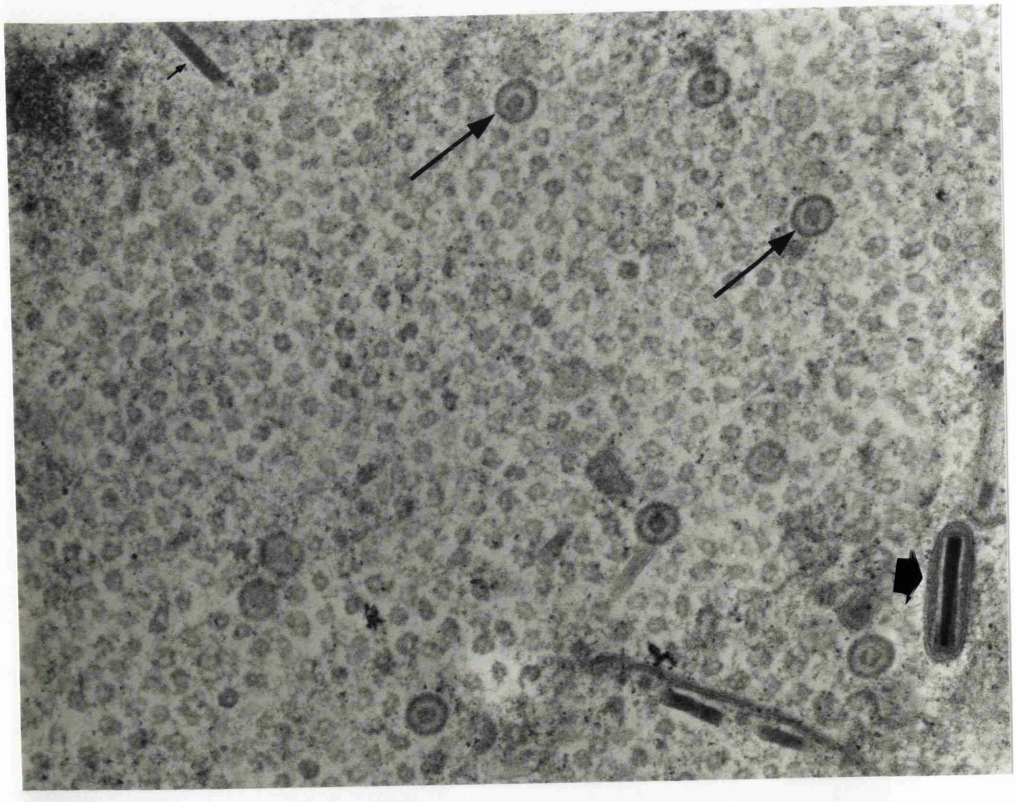

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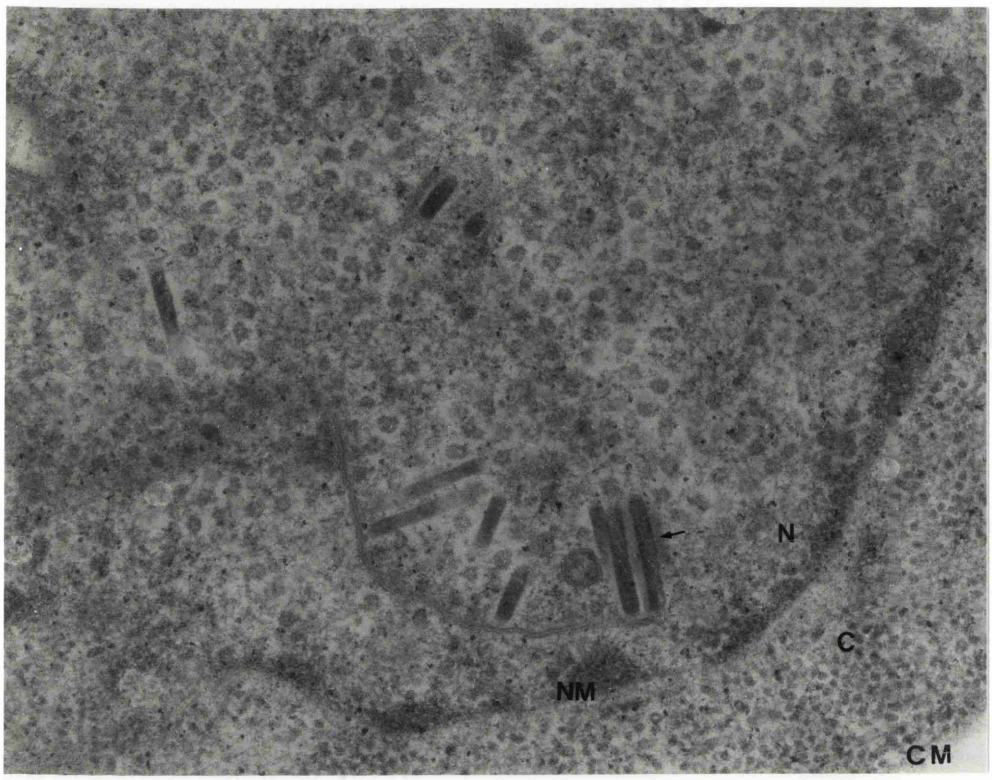

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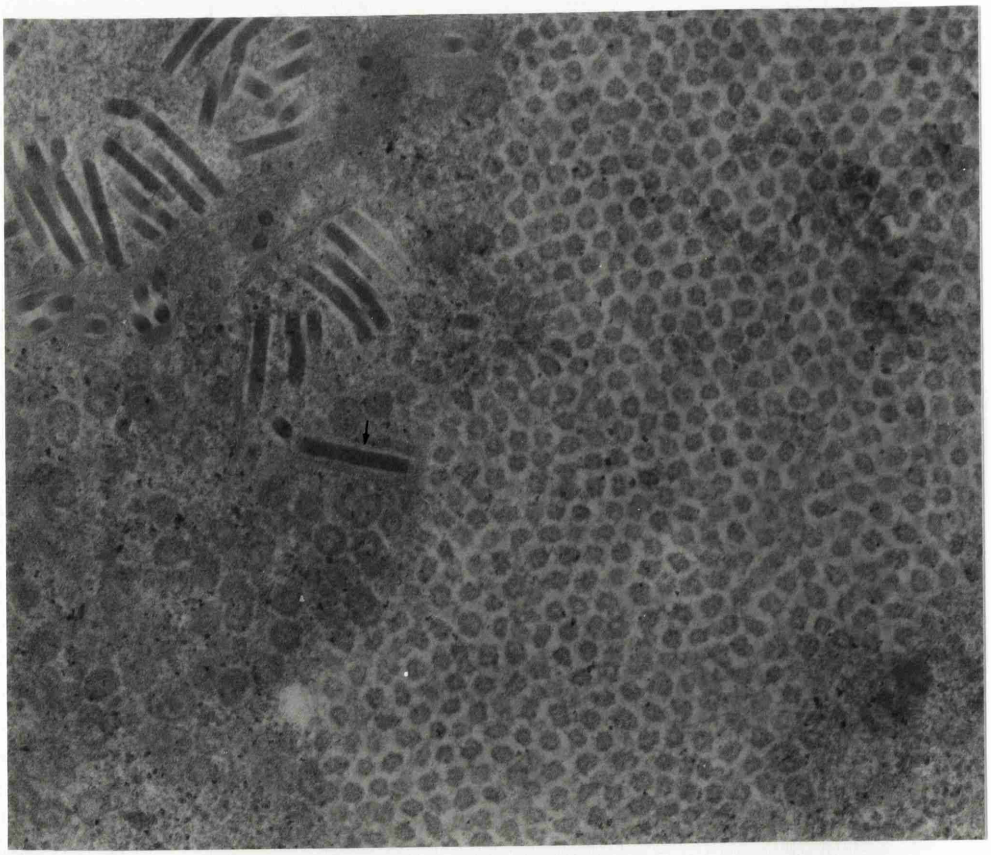

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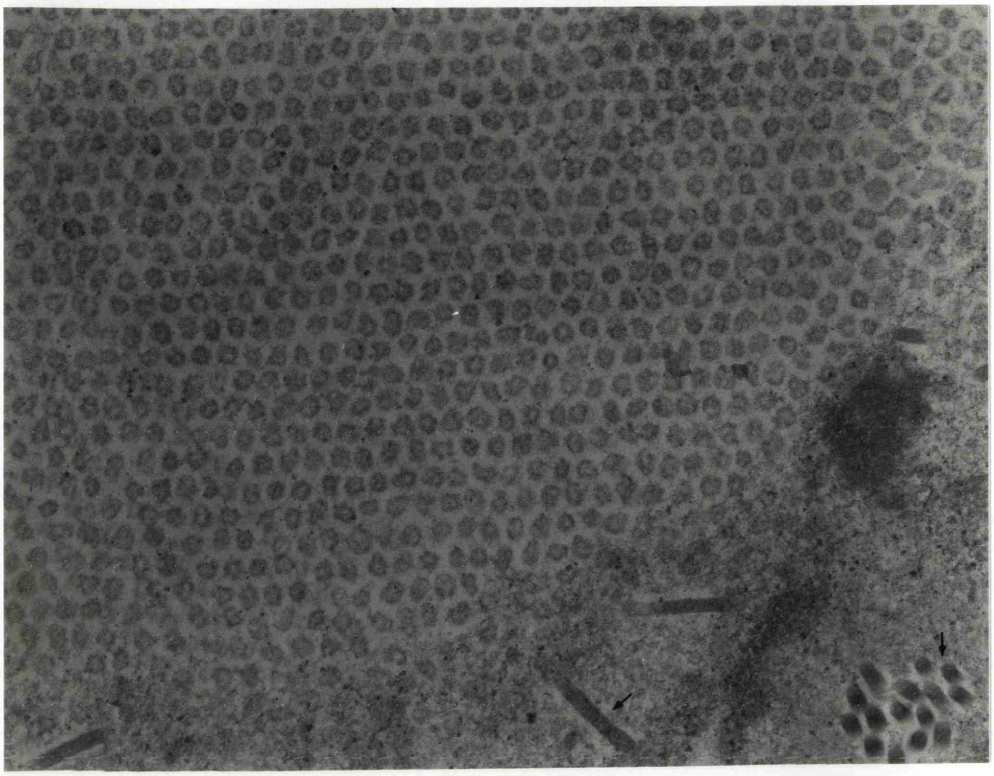

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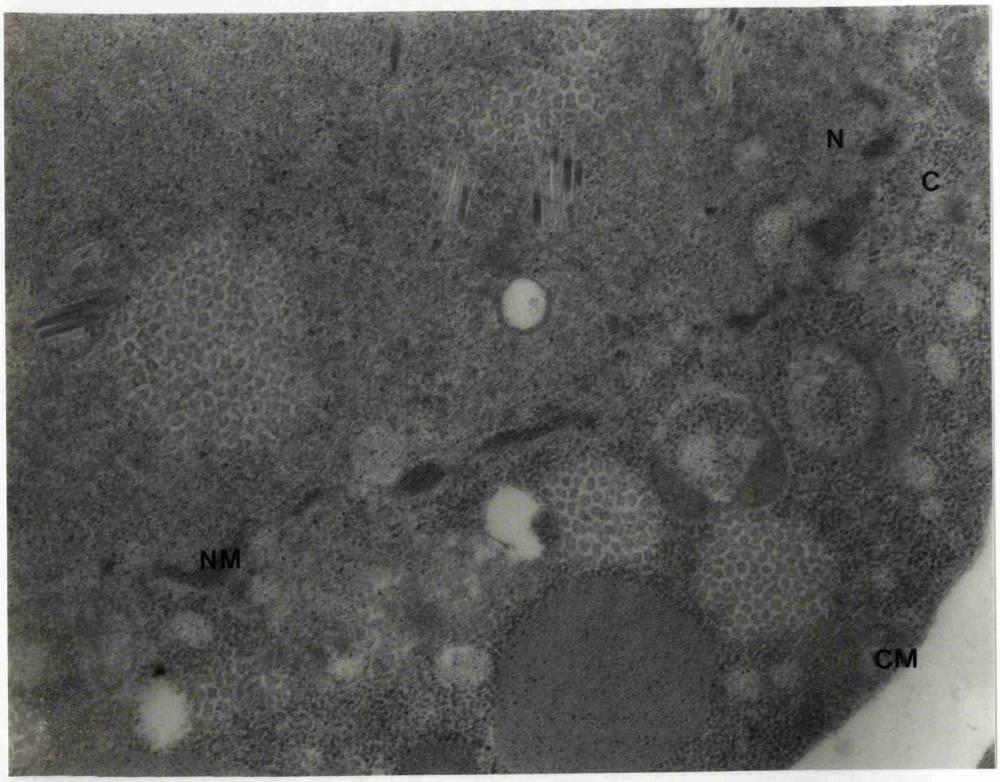

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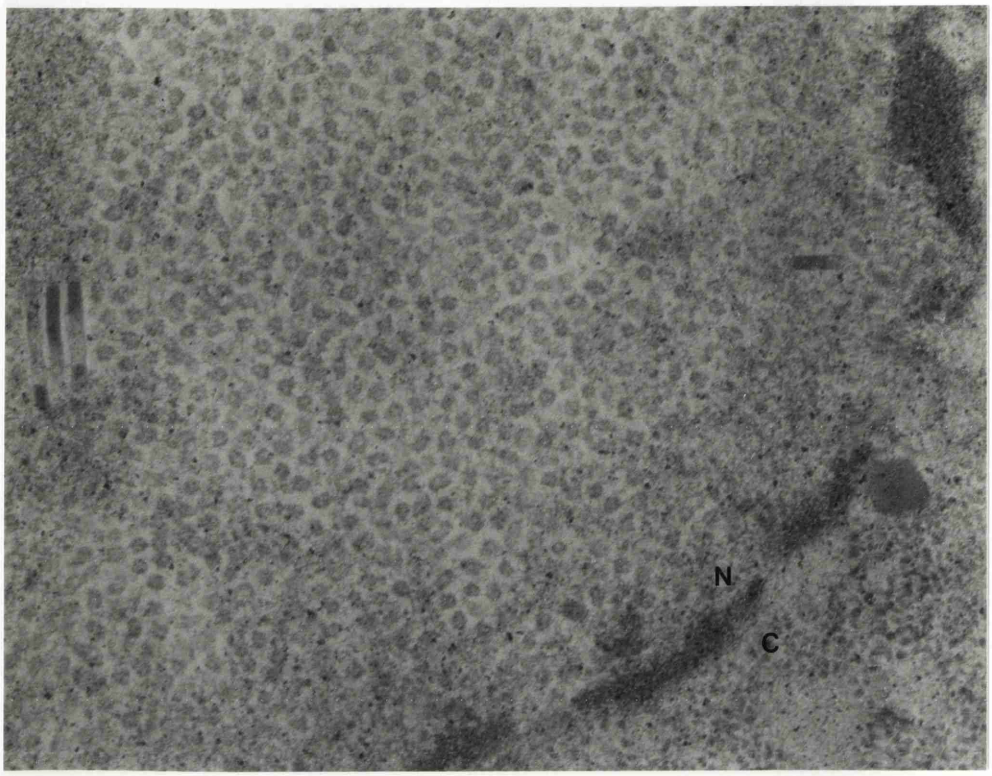

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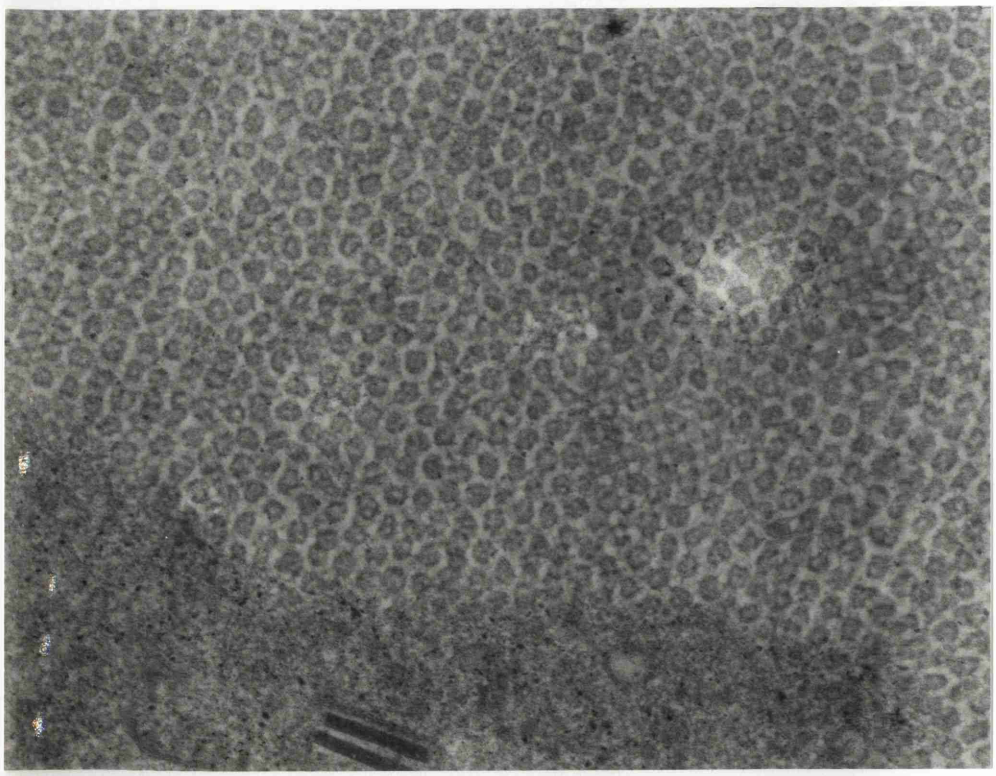

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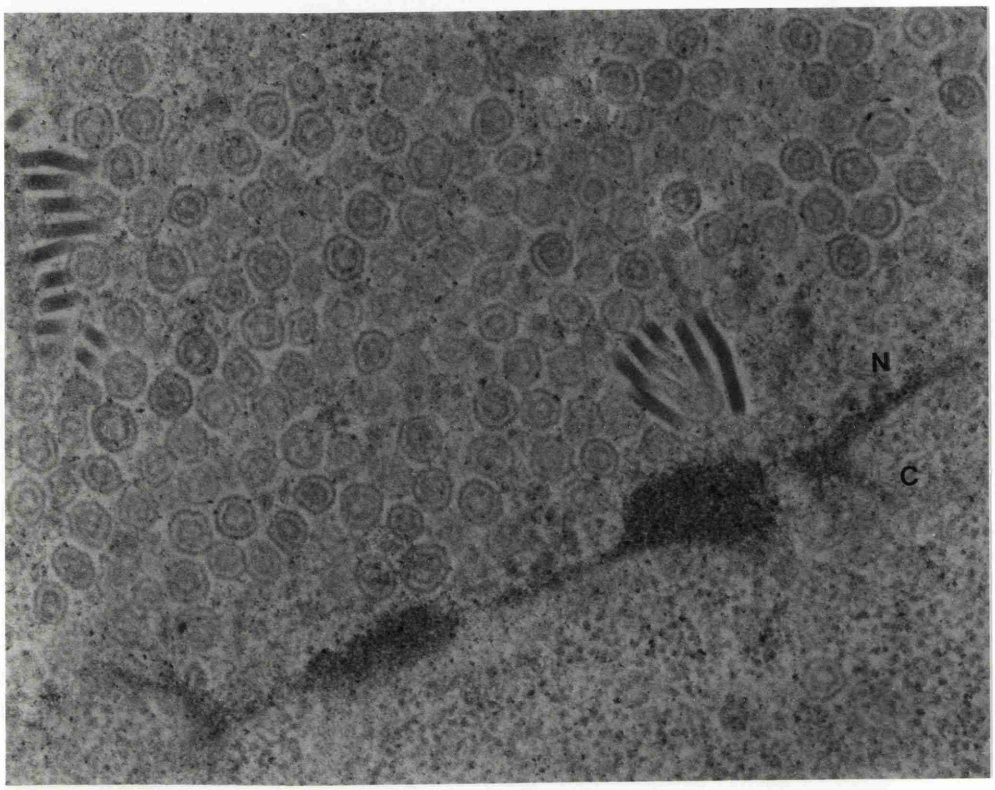

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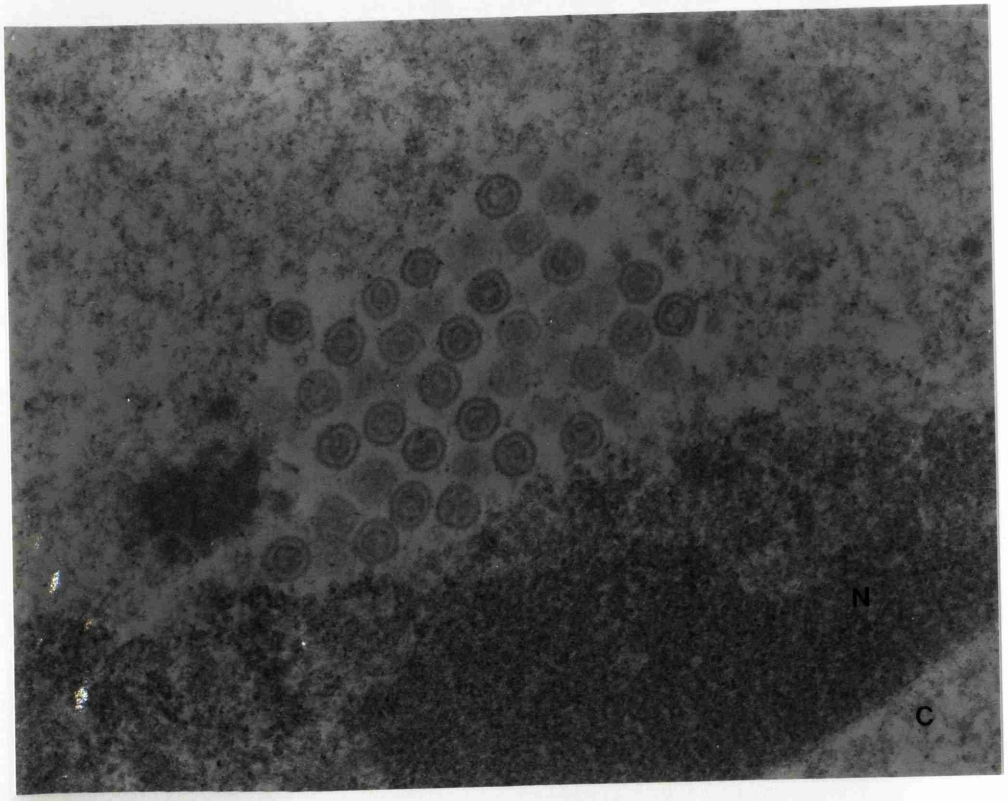

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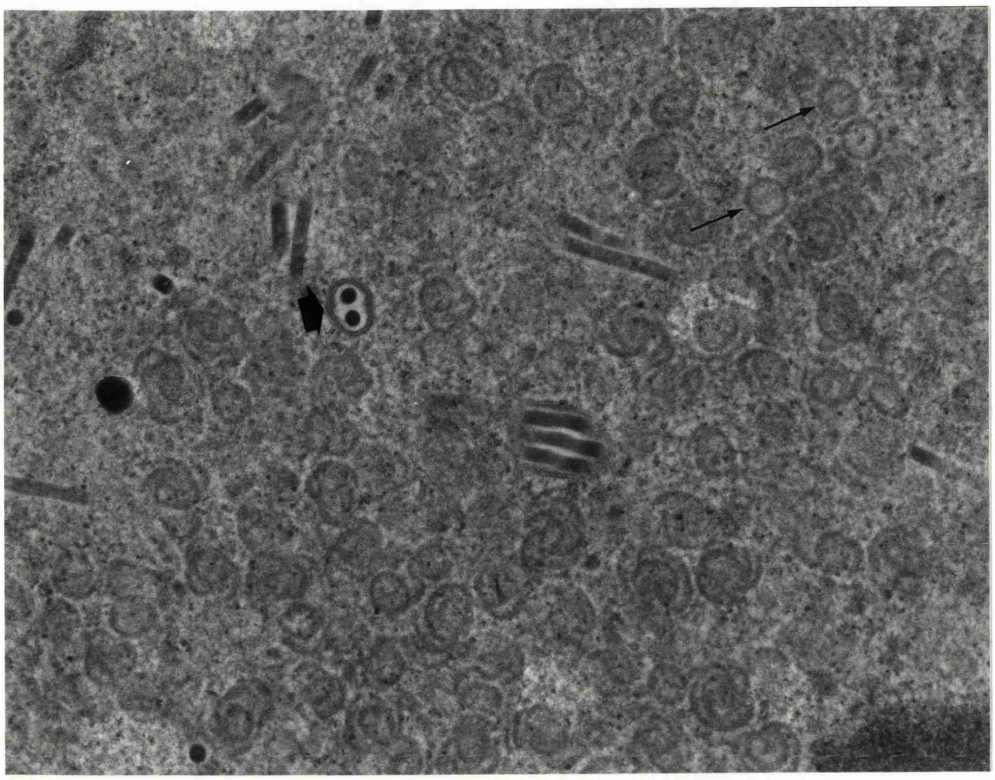
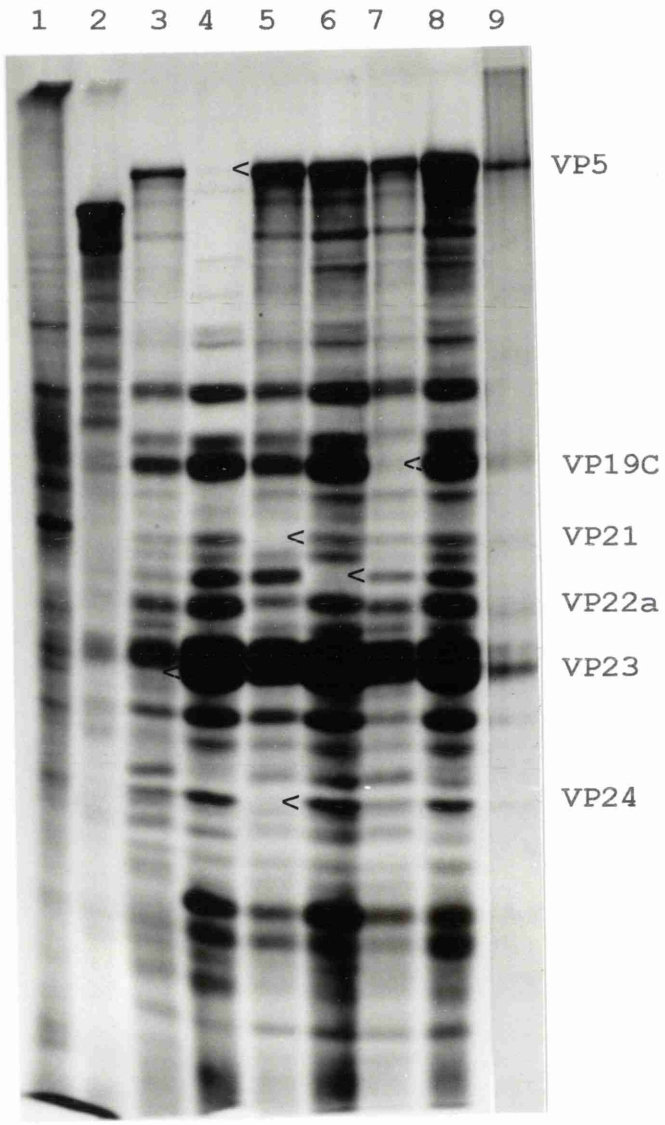


Figure 23:- Capsid assembly in recombinant baculovirus-infected cells

BHK cells infected with 5 p.f.u./cell of *ts*1201 were incubated at 38.5°C for 24h. Sf21 cells infected with different combinations of recombinant baculoviruses (each at 5 p.f.u./cell) were incubated at 28°C for 48h. The six recombinant baculoviruses used were AcUL18, AcUL19, AcUL26, AcUL26.5, AcUL35 and AcUL38 and the prefix ^ is used here to indicate which viruses were ommited from the mixture of recombinant baculoviruses used to infect each of the following samples. Panels (A) to (K) show electron micrographs of thin sections of the following samples: (A) and (B) ^AcUL35, (C) and (D) ^AcUL18/^AcUL35, (E) and (F) ^AcUL19/^AcUL35, (G) and (H) ^AcUL38/^AcUL35, (I) ^ AcUL26/^AcUL35, (J) *ts*1201, (K) ^AcUL26.5/^AcUL35. Where indicated; N=nucleus, C=cytoplasm, NM=nuclear membrane, CM=cytoplasmic membrane. B capsids (⟶), baculovirus capsids (→) and baculovirus virions (➧) are indicated. In panel K, apparently intact capsid shells are indicated (⟶). The bar markers represent 200nm except in panel F when it represents 330nm - this is presented at lower magnification to allow more of the cell to be shown.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VP5
VP19C
VP21
VP22a
VP23
VP24

Figure 24:- Expression of HSV-1 capsid proteins from co-infecting recombinant baculoviruses.

An 11% polyacrylamide gel showing [^{35}S]methionine-labelled polypeptide profiles of mock-infected Sf21 cells (lane 1) and cells infected with 5 p.f.u./cell of AcPAK6 (lane 2). Lane 8 shows the profile from cells infected with 5 p.f.u./cell of each of AcUL18, AcUL19, AcUL26, AcUL26.5 and AcUL38. In the remaining samples the co-infections were carried out as for lane 8 with one virus being left out in each case. The viruses omitted were AcUL18 (lane 3), AcUL19 (lane 4), AcUL26 (lane 5), AcUL26.5 (lane 6), AcUL38 (lane 7). Purified HSV-1 B capsids are shown in lane 9. The capsid proteins are indicated to the right of the gel. The positions of capsid protein bands which are absent from the co-infected samples are marked with arrowheads.

cores of the neighbouring HSV-1 capsids and I interpret them to represent free B capsid scaffolds/cores. Free scaffolds were sometimes seen alongside capsids when all the capsid proteins were present. Thus their presence here does not appear to be related to the absence of VP26 and presumably reflects variations in the extent of infection of each cell with each of the six recombinant viruses.

Since the absence of VP26 had no affect on capsid assembly, AcUL35 was excluded from the following analyses. The protein profiles of duplicate samples infected at the same time were determined by SDS-PAGE to confirm that the capsid proteins were being made (figure 24). In the following descriptions the prefix ^ is used to indicate which of the recombinant baculoviruses was omitted in addition to AcUL35.

3.3.1 ^AcUL18, ^AcUL19 and ^UL38

In cells lacking either VP23 (figure 23C and D; figure 24, lane 3) or VP5 (figure 23E and F; figure 24, lane 4) or VP19C (figure 23G and H; figure 24, lane 7) no HSV-1 capsids were observed. However, in each case, regular structures which appeared as 40-60nm particles, were present. These structures resembled the scaffold-like particles formed by UL26 and UL26.5-encoded proteins (figure 22C) and like them were often present in great abundance and were either found dispersed throughout the nucleus (figures 23C and G) or forming closely-packed arrays (figure 23D, E, F and H). This was true for each of the three combinations mentioned. Whereas in most cells they were confined to the nucleus, they were found in the cytoplasm in a proportion of cells in all three cases. The example of this (figure 23F) was obtained with ^AcUL19. In a minority of cells infected with ^AcUL18, additional novel structures were observed (figure 23D), the implications of which will be discussed in the next section (section 3.4).

3.3.2 ^AcUL26 and ^ AcUL26.5

When AcUL26, which encodes the protease was omitted, VP21 and VP24 were absent from the protein profile and unprocessed forms of VP22a (pre-VP22a) were made (figure 24, lane 5). Large numbers of recognisable capsid structures continued to be made (figure 23I). However, the appearance of these capsids differed from those made in the presence of the protease by virtue of the larger diameter of their cores (compare figures 23A and 23I). This resembles the situation found in BHK cells infected at non-permissive temperatures with the HSV-1 mutant *ts*1201 (figure 23J). The lesion in *ts*1201 maps to UL26 and results in an inactive protease which fails to process pre-VP22a at the NPT. *Ts*1201 capsids also have large cores which, upon downshift to the PT, are converted to the small-cored phenotype as a result of the processing of pre-VP22a to VP22a.

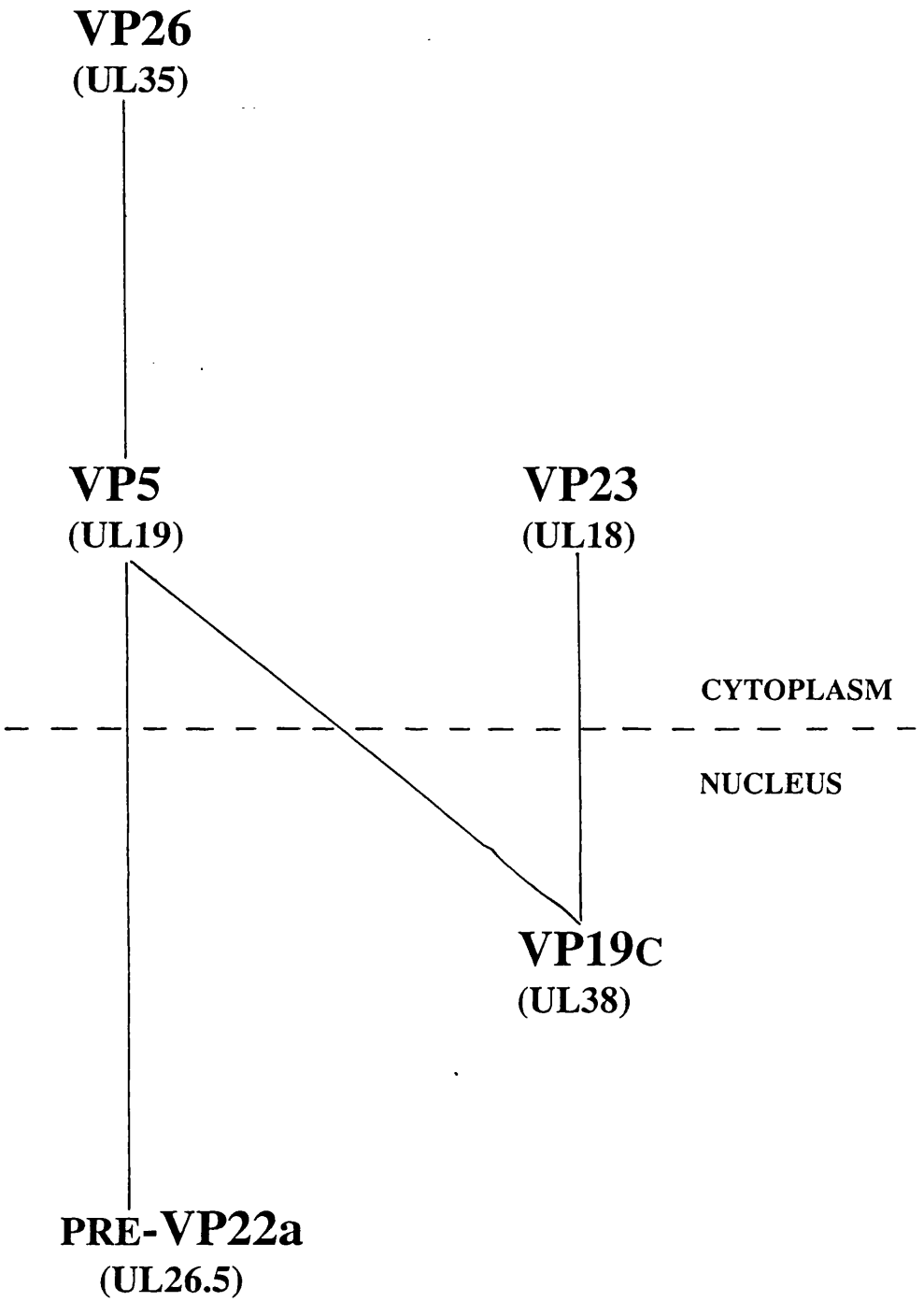

VP26
(UL35)
VP5
(UL19)
VP23
(UL18)
CYTOPLASM
NUCLEUS
VP19C
(UL38)
PRE-VP22a
(UL26.5)

Figure 25:- Interactions between HSV-1 capsid proteins

Interactions between HSV-1 capsid proteins were determined from fluorescent localisation experiments in which the capsid proteins were expressed either singly or in combination from plasmid or vaccinia vectors. The diagram summarises the results of Nicholson *et al.* (1994) and F. Rixon (personal communication).

In experiments in which AcUL26.5 was omitted, (figure 23K, figure 24 lane 6) the majority of the structures observed had the appearance of incomplete or disrupted shells. However some apparently intact capsid shells were also present. These resembled A type capsids (figure 21B) which are thought to be formed as part of an abortive process in DNA packaging. The small numbers of these complete shell structures observed suggest that capsid assembly can take place in the absence of pre-VP22a but does so inefficiently.

3.4 ASSEMBLY OF SUB-CAPSID STRUCTURES IN RECOMBINANT BACULOVIRUS INFECTED CELLS

It has previously been shown by immunofluorescence experiments (Nicholson *et al.*, 1994; F. Rixon, personal communication) that several of the capsid proteins when expressed either by a recombinant vaccinia virus or from a plasmid vector, interact with each other giving rise to altered distributions within cells (summarised in figure 25). Thus the presence of pre-VP22a converts VP5 from a cytoplasmic to a nuclear form while changing the distribution of pre-VP22a from a punctate appearance in the nucleus to a more even distribution throughout the nucleus. Similarly VP19C interacts with both VP5 and VP23 and changes their distributions from predominately cytoplasmic to predominately nuclear. To determine whether these interactions resulted in the formation of any distinct structures, insect cells were infected with different combinations of the recombinant baculoviruses and the cells prepared for electron microscopy as described in Methods.

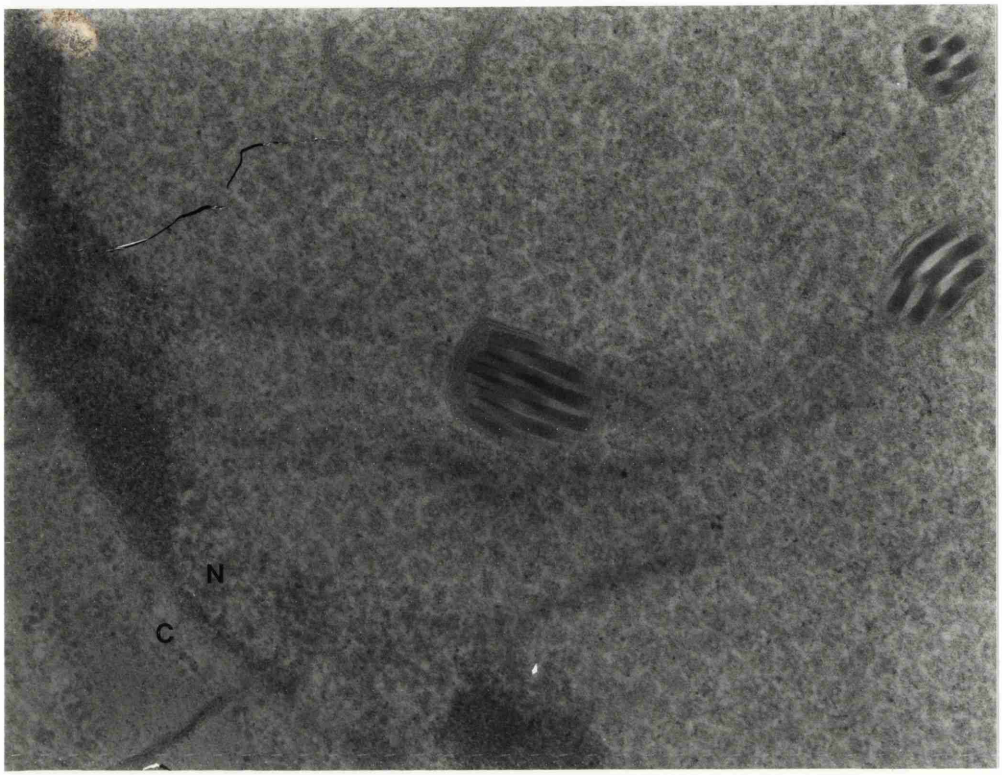
3.4.1 AcUL18 and AcUL38

In cells infected with these two recombinant viruses (not shown), no novel structures were observed and the cell morphology resembled that of cells infected with AcPAK6. Although the two proteins expressed by these viruses (VP23 and VP19C) have been shown to interact by immunofluorecence (figure 25), any complexes formed between these proteins were either too small or indistinct to be observed, or else did not form in the baculovirus system.

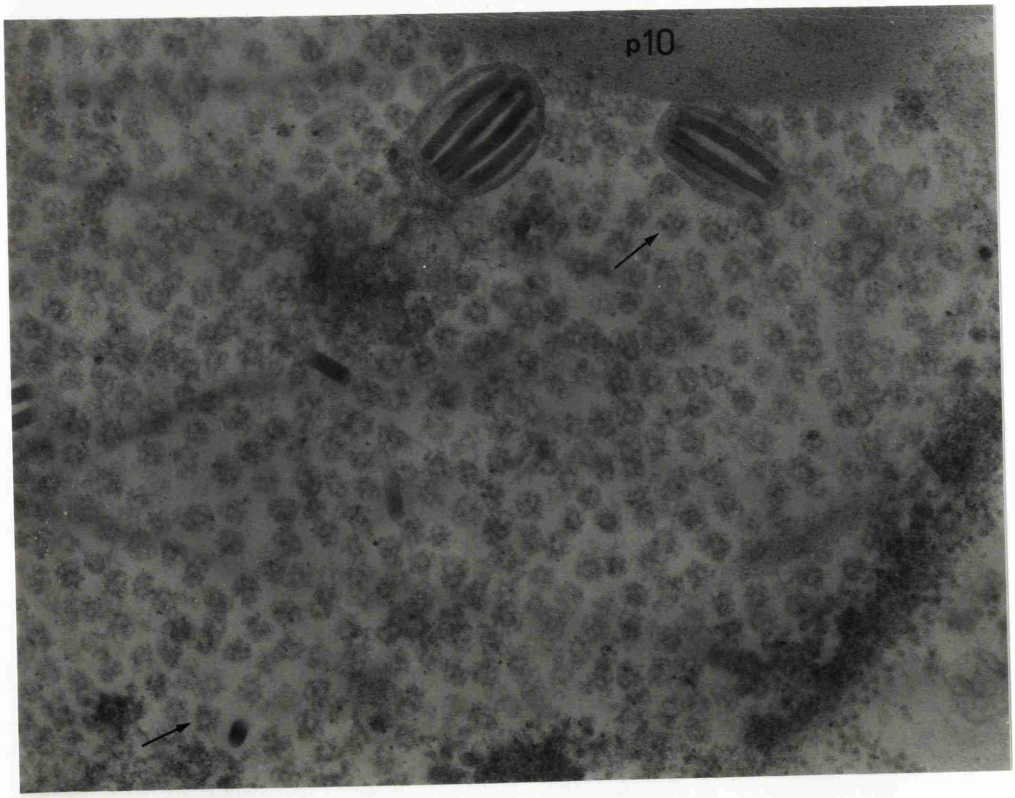
3.4.2 AcUL19 and AcUL26.5

In cells infected with both these recombinant baculoviruses, large numbers of structures approximately 40nm in diameter, with an ill-defined, almost fuzzy appearance were observed uniformly distributed throughout the nucleus. In some cells they appeared densely packed together (figure 26A) whereas in others they were more sparsely dispersed (figure 26B). Some of the individual structures have the appearance of an

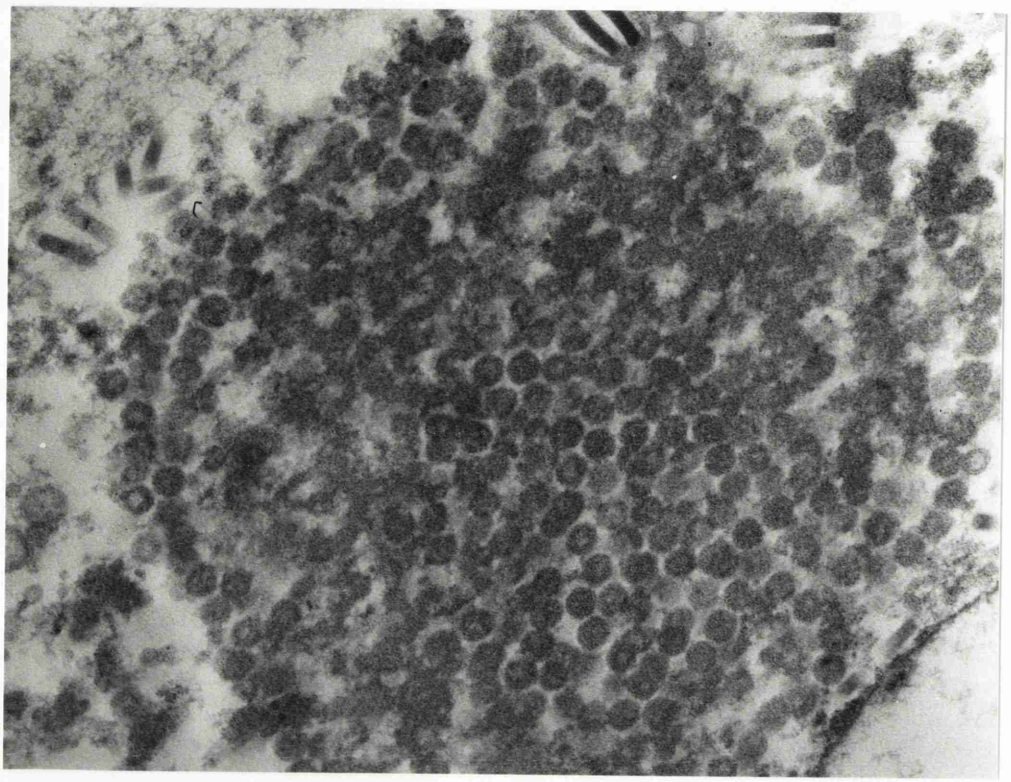
A



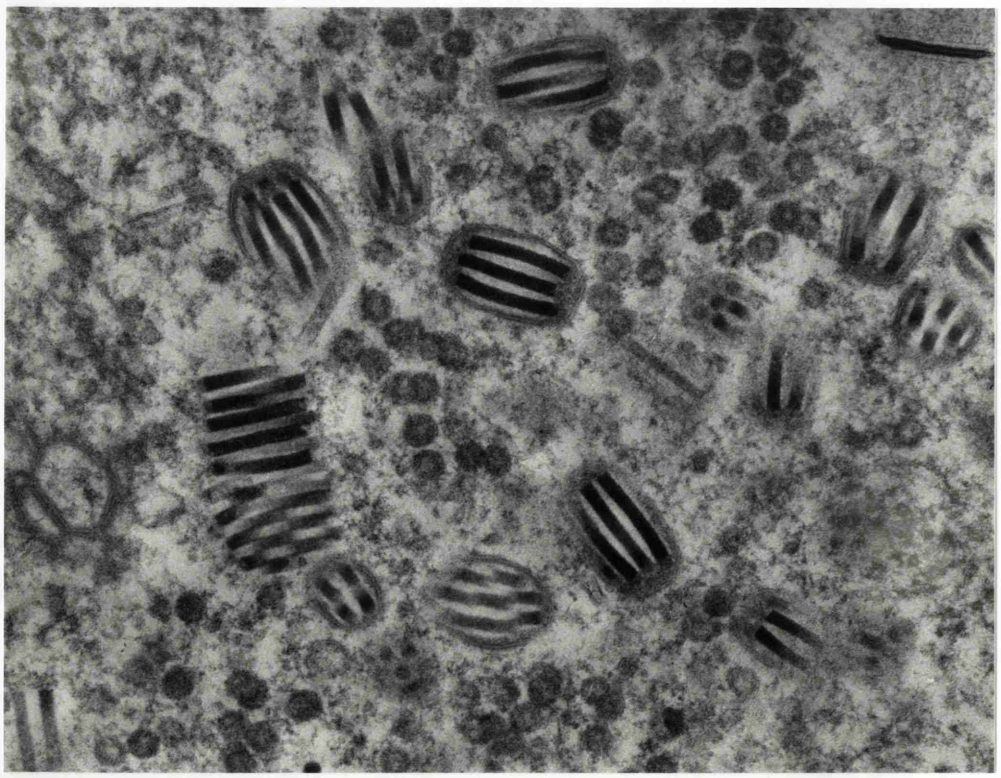
B



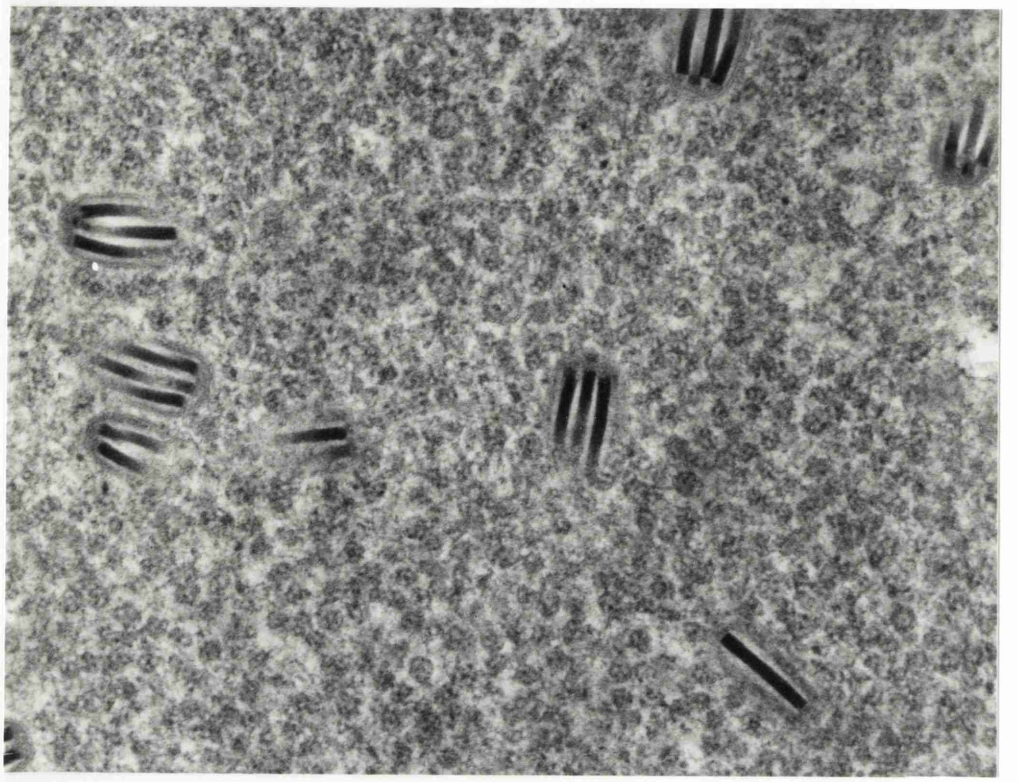
C



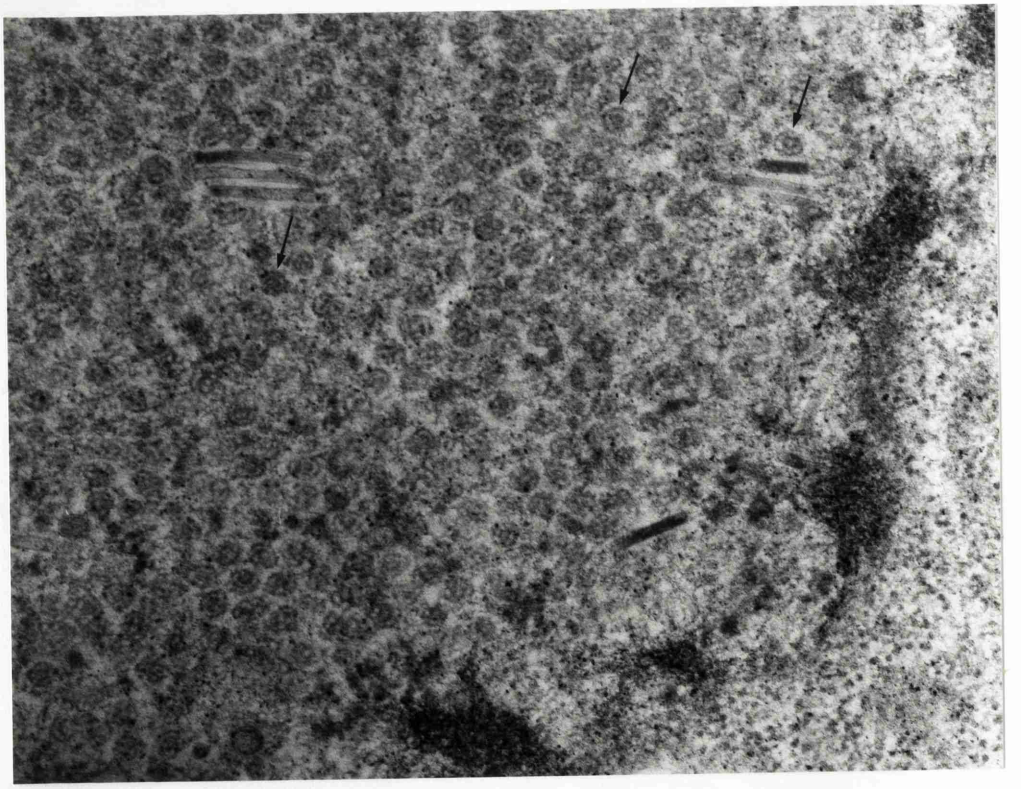
D



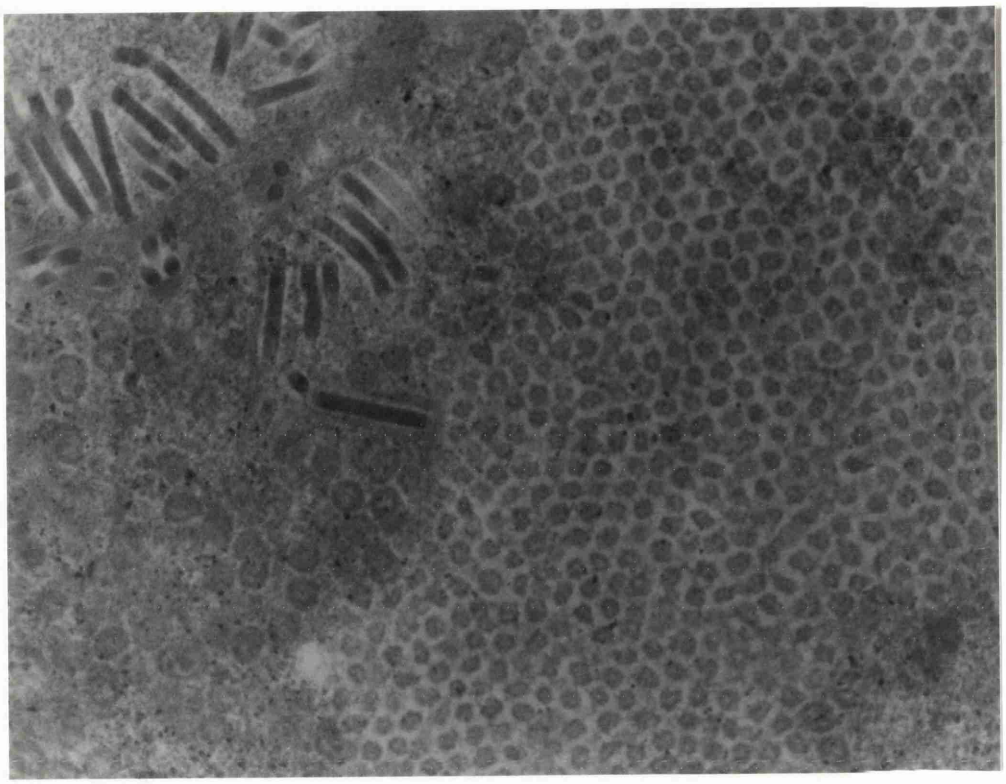
E



F



G



H

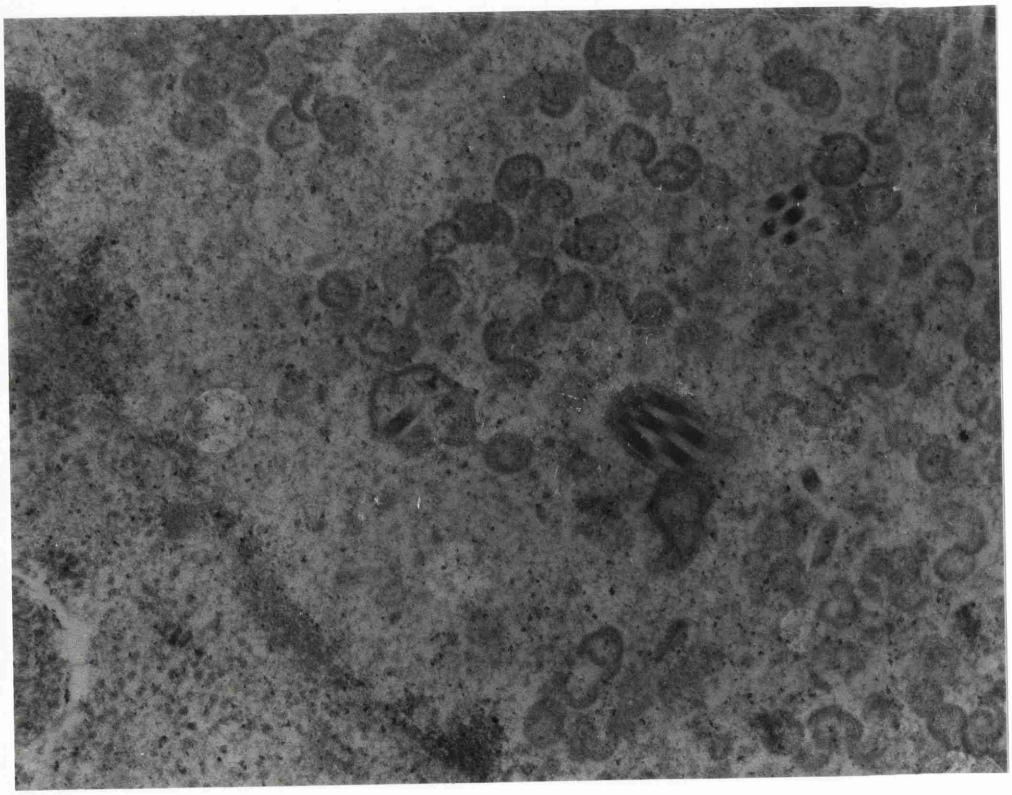


Figure 26:- Sub-capsid structures formed in recombinant baculovirus-infected cells.

Sf21 cells infected with different combinations of recombinant baculoviruses (each at 5 p.f.u./cell) were incubated at 28°C for 48h. The recombinant baculoviruses used were AcUL18, AcUL19, AcUL26, AcUL26.5 and AcUL38. Panels (A) to (H) show electron micrographs of thin sections of cells infected with the following viruses: (A) and (B) AcUL19 and AcUL26.5, (C) and (D) AcUL19 and AcUL38, (E) and (F) AcUL19, AcUL26.5 and AcUL38, (G) AcUL19, AcUL26, AcUL26.5 and AcUL38, (H) AcUL18, AcUL19 and AcUL38. (G) is the same image as that shown in figure 23D.
N=nucleus, C=cytoplasm, p10=baculovirus-encoded p10 protein. In panels B and F the best examples of structures with the appearance of an inner ring with distinct projections are indicated (⟶). The bar markers represents 200nm

inner ring with indistinct projections which may consist of VP5 adhering to the outer surface of pre-VP22a scaffolds. It is not clear whether VP5 was present as capsomeric assemblies or in some other form. Interestingly, the aggregates of fibrous protein observed in AcUL26.5-infected cells are absent (figure 22B) suggesting that VP5 was preventing aggregation of the UL26.5 product, pre-VP22a. This observation supports the idea that these structures represent VP5 bound to pre-VP22a. The absence of this protein mass and the concurrent appearance of these structures explains the changes in fluorescence patterns for VP5 and pre-VP22a described above.

3.4.3 AcUL19 and AcUL38

When baculoviruses expressing the protein products of these two genes, VP5 and VP19C were co-infected, novel structures were seen (figure 26C and D). These densely staining spheres which appeared to be larger than scaffold cores but smaller than capsids have an estimated diameter of 70nm. They did not appear to have any internal structure. Their precise nature is unclear but they do demonstrate that VP5 and VP19C on their own can form relatively uniform self-limiting assemblies. They were often observed in large aggregations within the nucleus (figure 26C) which may account for the nuclear aggregations of VP5 observed by immunofluorescence in cells expressing these two proteins (F. Rixon, personal communication). The nature of these particles is described in further detail in figure 34.

3.4.4 AcUL19, AcUL38 and AcUL26.5

Both the above 40nm fuzzy and 70nm dense structures involve interactions of VP5 with other proteins. When all three proteins (VP5, VP19C and pre-VP22a) were present it was the interaction between VP5 and pre-VP22a that appeared to prevail giving rise to the fuzzy structures shown in figures 26E and 26F. These resembled the structures formed from coinfection of these two baculoviruses alone (figure 26A and B) rather than the densely staining particles observed with co-infection of AcUL19 and AcUL38 (figure 26C and D). Structures formed in the cell section shown in figure 26F are particularly clear in demonstrating that VP5 and pre-VP22a form core-like assemblies with an exterior structure. These interactions suggest that VP5 has a greater affinity for pre-VP22a than it does for VP19C and this may have an important bearing on the sequence of events occurring during the assembly process.

3.4.5 AcUL19, AcUL38, AcUL26.5 and AcUL26 (^AcUL18/^AcUL35)

This combination of viruses is equivalent to the ^AcUL18 set described previously; figure 26G is the same as figure 23D. The presence of the protease produced by AcUL26 resulted in the formation of a different pattern of structures from those seen with AcUL19, AcUL38 and AcUL26.5. Scaffold arrays of the type found earlier

Virus[a] Infection	Proteins present[b] (VP)	Description[c]	Size[d] (nm)	Appearance[e]	EM[f]
HSV-1	5, 23, 19C, 26 22a, 21, 24	small-cored capsids	100		
All	5, 23, 19C, 26 22a, 21, 24	small-cored capsids	100		
^AcUL35	5, 23, 19C 22a, 21, 24	small-cored capsids	100		
*ts*1201	5, 23, 19C, 26 pre-22a, 21+24[g]	large-cored capsids	100		
^AcUL35/ ^AcUL26	5, 23, 19C pre-22a	large-cored capsids	100		
AcUL26 AcUL26.5	22a, 21, 24	cores	40-60		
^AcUL35/ ^AcUL19	23, 19C 22a, 21, 24	cores	40-60		
^AcUL35/ ^AcUL38	5, 23 22a, 21, 24	cores	40-60		
^AcUL35/ ^AcUL18	5, 19C 22a, 21, 24	cores densely staining[h]	40-60 70	[h]	
^AcUL35/ ^AcUL26.5	5, 23, 19C 21, 24	partial shells complete shells[h]	- 100	[h]	
AcUL19 AcUL18, AcUL38	5, 23, 19C	partial shells	-		
AcUL19 AcUL26.5	5 pre-22a	indistinct	~40		
AcUL19 AcUL38	5, 19C	densely staining	70		
AcUL19 AcUL38, AcUL26.5	5, 19C, pre-22a	indistinct	~40		

Figure 27 Summary of novel structures formed following mixed infections with recombinant baculoviruses expressing HSV-1 capsid genes

[a] Sf21 cells were infected with 5 p.f.u./cell of different combinations of the recombinant baculoviruses which each expressed one of the HSV-1 capsid genes: AcUL18, AcUL19, AcUL26, AcUL26.5, AcUL35 and AcUL38. Cells were harvested after 48h incubation at 28°C. 'All' represents infection with all six recombinant baculoviruses and the prefix ^ is used to designate the viruses omitted from infections where only one or two viruses were left out. BHK cells were infected with either HSV-1 strain 17 or *ts*1201 virus and were harvested after 24h incubation at 38.5 °C.

[b] Where particles were purified from infected cells, the protein compositions were determined by SDS-PAGE. Otherwise, the proteins present were assumed from the infecting viruses. The HSV-1 capsid proteins present are listed without the VP prefix.

[c e] The different capsid structures are described in more detail in the text. 'Core' also refers to the scaffold-like structures described in the text.

[d] Estimation of the diameter (nm) of the capsid structures observed by electron microscopy of thin sections.

[f] Infected cells were prepared for analysis in the elecron microscope and thin sections were stained with uranyl acetate and lead citrate. The bar represents 200nm.

[g] Indicates the presence of the inactive protease which has failed process itself into the capsid proteins VP21 and VP24.

[h] Particles present in a lesser amount.

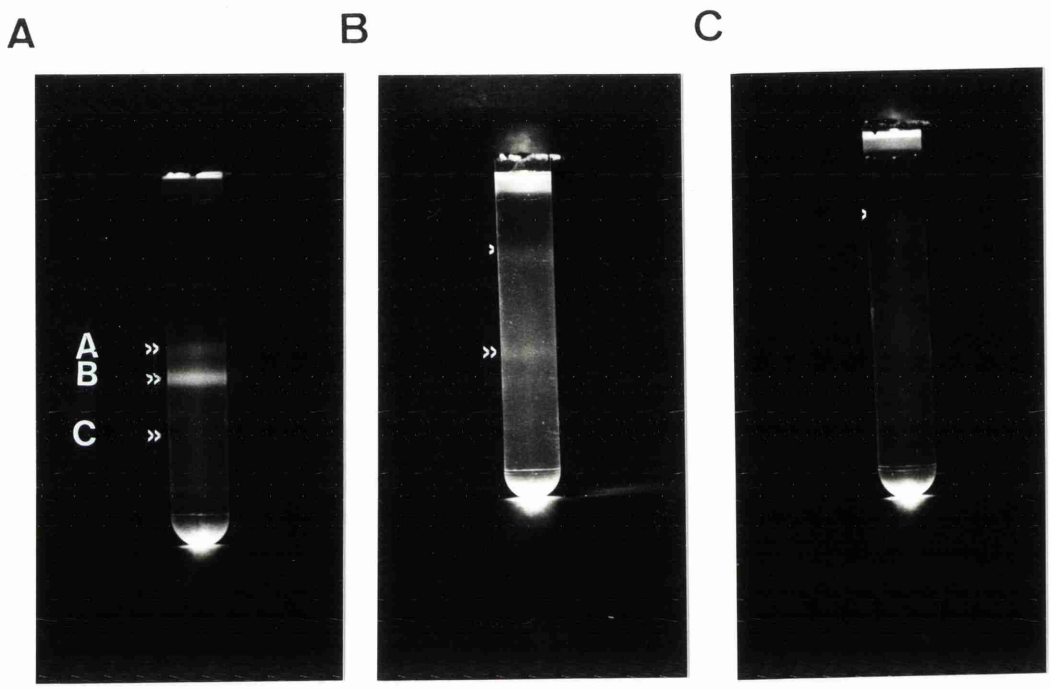

A
B
C
A
B
C

Figure 28:- Velocity gradient centrifugation of intranuclear capsids

Nuclear extracts of BHK cells infected with 5 p.f.u/cell of (A) HSV-1 strain 17, and (B) Sf21 cells co-infected with 5 p.f.u/cell of recombinant baculoviruses AcUL18, AcUL19 AcUL26, AcUL26.5, and AcUL38, or (C) with 5 p.f.u./ml AcPAK6 were centrifuged at 40,000 r.p.m. for 20 minutes through a 12ml 5-40% sucrose gradient in a Sorvall TsT41 rotor. The presence of the capsids were observed by overhead illumination. The positions of the A, B and C capsids produced by HSV-1-infection (A) and recombinant baculoviruses-derived HSV-1 capsids (B) are indicated (›). The positions of the baculovirus capsids are indicated (»).

(figures 22C, 23D, E and H) are present alongside densely staining particles resembling thoses made with AcUL19 and AcUL38 alone (figures 26C and D). The most likely explanation for the differences between the two patterns is that VP5 interacts preferentially with the AcUL26.5 protein, pre-VP22a, but that if processing by the protease has taken place then this interaction is inhibited leaving VP5 available to interact with VP19C. The importance of the carboxy terminal 25aa to capsid formation has been described in Kennard *et al.* (1995) and is covered in more detail in the discussion.

3.4.6 AcUL18, AcUL19, AcUL38

When the recombinant baculoviruses expressing only the outer shell proteins (VP23, VP5 and VP19C) were co-infected, large numbers of structures resembling the partial shells formed in the absence of the scaffolding protein encoded by AcUL26.5 were present (compare figures 26H and 23K). However no complete shells resembling those shown in figure 23K were observed. This demonstrates that the components of the capsid shell have some intrinsic ability to self-assemble in the absence of the scaffold proteins and also demonstrates the effect of VP23 in modulating the interactions of the other two proteins; ie. partial shells were formed when VP5, VP19C and VP23 were present in preference to 70nm spherical particles which were observed in the presence of VP5 and VP19C (figure 26C and D).

It is not certain whether all the structures described represent possible intermediates in HSV-1 capsid assembly or were formed as a consequence of the overexpression of the proteins in the baculovirus system. A summary of the structures formed using different combinations of the recombinant baculoviruses is shown in figure 27.

3.5 CAPSID PURIFICATION

To prepare capsids, suspension cultures of Sf21 cells were infected with 5 p.f.u./cell of each of the recombinant baculoviruses and were incubated at 28°C for a period of 48-72h. If required for analysis of the distribution of the capsid proteins throughout the gradient, proteins were labelled with [^{35}S]methionine from 30h p.i. Capsids were prepared from these cells and from HSV-1 strain 17 or *ts*1201-infected BHK cells as described in Methods. Cell extracts were centrifuged through 5-40% sucrose gradients after which the position of the capsids was visualised by overhead illumination. Examples of such gradients are shown in figure 28. Lysates from HSV-1 strain 17-infected BHK cells yield three light-scattering bands which correspond to the type A, B and C capsids described by Gibson and Roizman (1972). Atypically, few C capsids were observed in the preparation shown here as demonstrated by the weakness of the third, lower band. Capsids made from all six recombinant baculoviruses and those

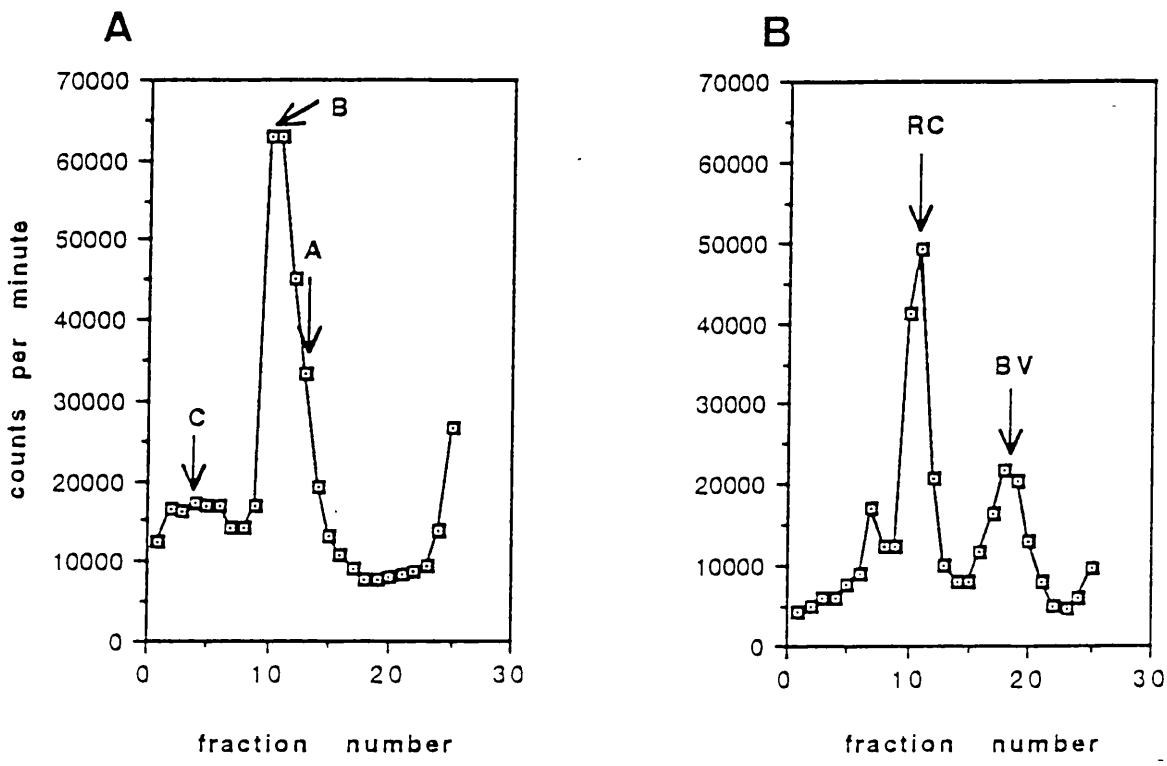
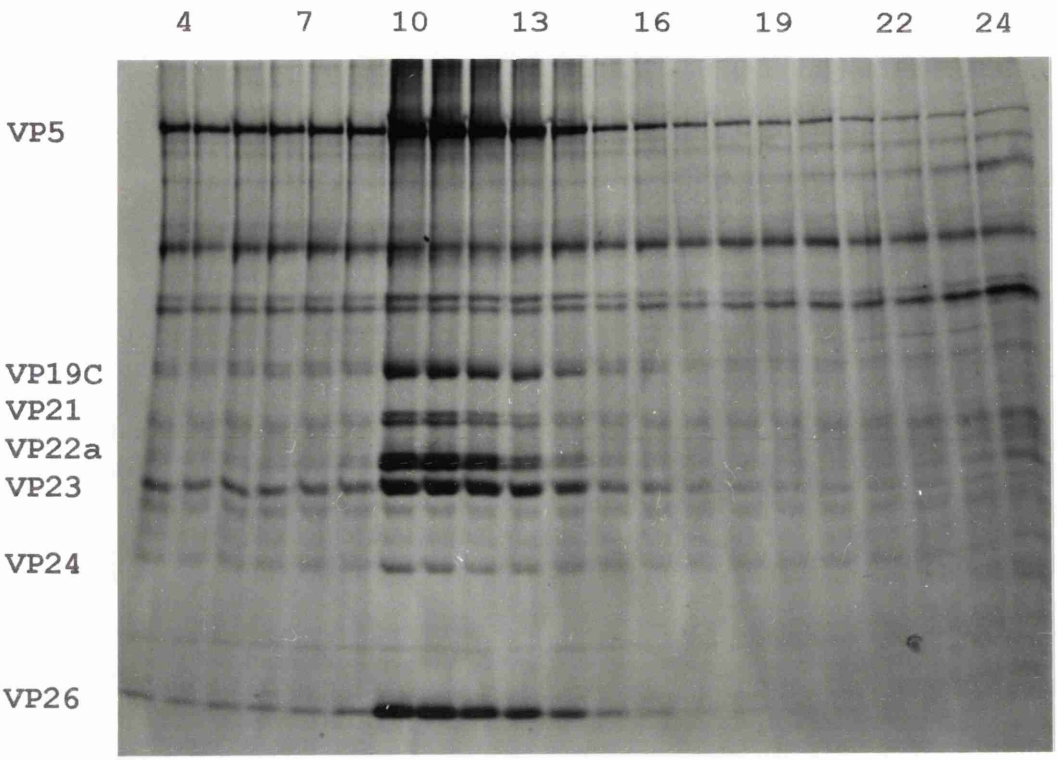

A
B
70000
60000
50000
40000
30000
20000
10000
0
A
C
counts per minute
0
10
20
30
fraction number
B
70000
60000
50000
40000
30000
20000
10000
0
RC
BV
0
10
20
30
fraction number

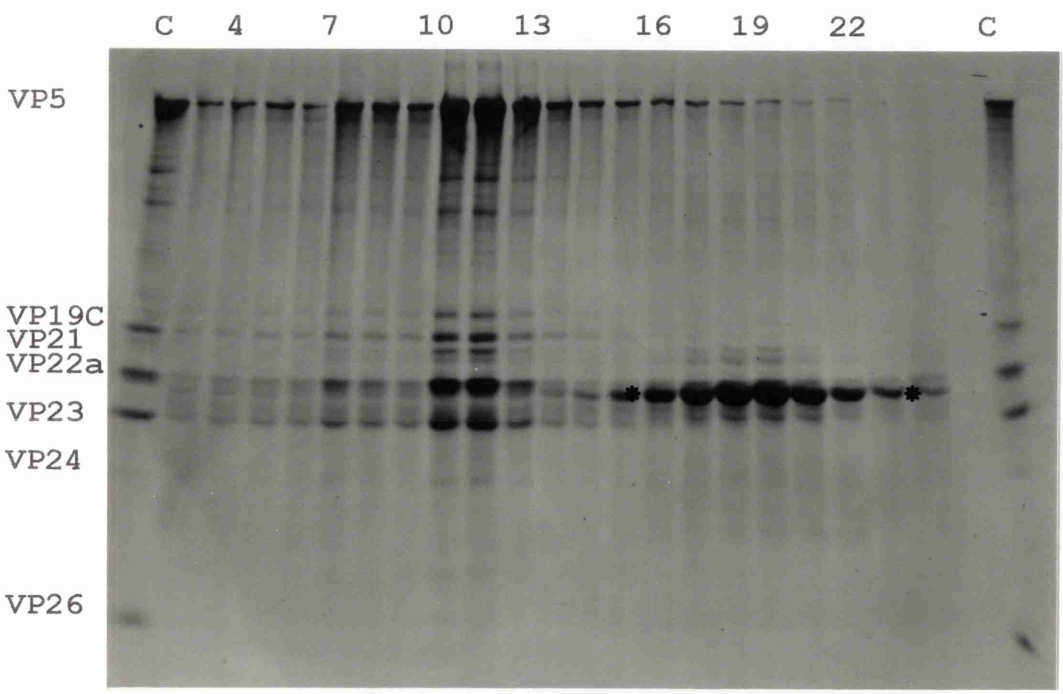
Figure 29:- Distribution of [^{35}S]methionine label across sucrose gradients of intranuclear capsids

[^{35}S]methionine-labelled capsids from HSV-1 strain 17-infected BHK cells (A) and from Sf21 cells co-infected with 5 p.f.u/ml of the recombinant baculoviruses AcUL18, AcUL19, AcUL26, AcUL26.5, and AcUL38 (B) were isolated and sedimented through a 5-40% sucrose gradient as described in Methods. Successive eight-drop fractions were collected from the bottom of the gradient. Aliquots (50ul) of each fraction were taken for TCA precipitation and scintillation counting. The amount of [^{35}S] contained in each sample is expressed as counts per minute and fraction 1 represents the first fraction collected from the bottom of the gradient. The position of HSV-1 A, B and C capsids is indicated on A. On B the peak of baculovirus capsids is marked BV and the peak of recombinant baculovirus-derived HSV capsids is marked RC.

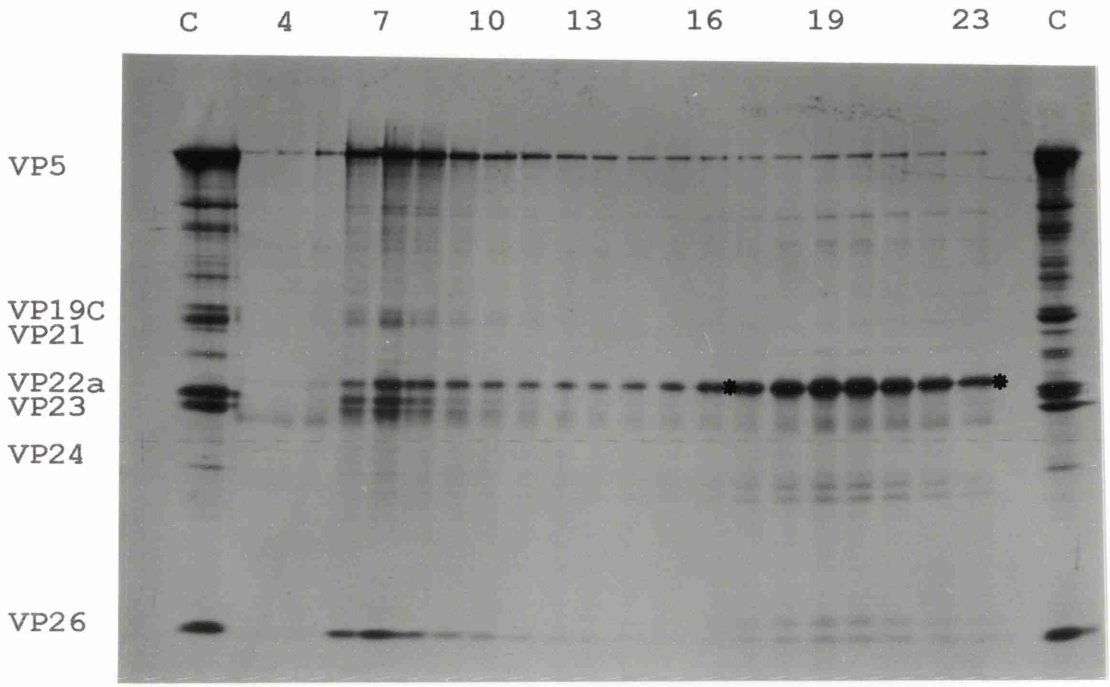
A


4
7
10
13
16
19
22
24
VP5
VP19C
VP21
VP22a
VP23
VP24
VP26

B


C
4
7
10
13
16
19
22
C
VP5
VP19C
VP21
VP22a
VP23
VP24
VP26

C


C
4
7
10
13
16
19
23
C
VP5
VP19C
VP21
VP22a
VP23
VP24
VP26

D

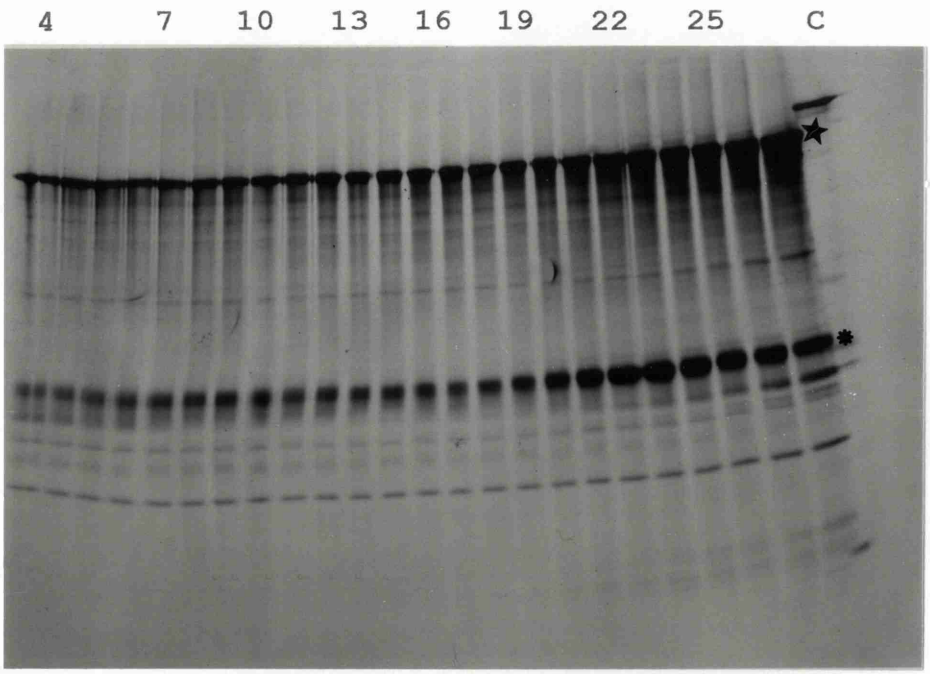

4
7
10
13
16
19
22
25
C

Figure 30:- SDS-PAGE analysis of gradient-banded capsids

(A) 50ul of fractions 4-24 of the HSV-1 gradient shown in figure 29A and (B) 50ul of fractions 3-23 of the baculovirus gradient (^AcUL35) shown in figure 29B were analysed on identical 5-15% polyacrylamide gels. The protein content of (C) 50ul of fractions 3-23 of capsids produced from infection with all six recombinant baculovirus and (D) 50ul of fractions 4-27 from a gradient produced from AcPAK6-infected Sf21 cells were analysed in a similar manner. Purified HSV-1 B capsid samples (lanes **C**) were run as markers. The positions of the HSV-1 capsid proteins are shown to the left of each gel. In B a protein which may represent the HSV-1 capsid protein VP21 is present in lanes 17-20. In B, C and D the major baculovirus capsid protein is indicated (✸). In D the AcPAK6-encoded β-galactosidase protein is indicated (★).

made in the absence of AcUL35, banded in the same position as HSV-1 B capsids (figure 28B). A faint band near the top of the gradient corresponds to the position of the baculovirus capsids shown in the control AcPAK6 gradient (figure 28C). The best yield of HSV-1 type capsids made using the baculovirus system was obtained from a longer incubation of 72h.

For capsid preparations which were radioactively labelled, 8-drop fractions were collected as described in Methods. The distribution of the label throughout the gradients was determined by TCA precipitating and scintillation counting of 50ul of each aliquot and the results are presented in graphical form in figure 29. Two peaks of radioactivity were present in capsids extracted from HSV-1-infected cells (figure 29A) and correspond to the lower (C capsid) band and the middle (B capsid) band. The position of the upper (A capsid) band is represented by a shoulder on the large B capsid peak. Capsids made by the recombinant baculoviruses (recombinant capsids) in the absence of AcUL35 (figure 29B), produced a peak at the same position (fraction 11) as the HSV-1 B capsids (figure 29A). In addition there was a smaller peak at fraction 17 representative of the baculovirus capsids.

3.6 PROTEIN COMPOSITIONS OF CAPSIDS

To determine the protein composition of these capsids and their distribution throughout the gradient, 50ul aliquots of the gradient fractions were analysed by SDS-PAGE. Figure 30A and B show the gradients from nuclear extracts of HSV-1 and baculovirus (^AcUL35) infected cells respectively. Since these were prepared and spun at the same time they thus provide a direct comparison of the profiles of the capsid proteins. The intensity of each protein varies across the gradient and peaks can be seen which correspond to the capsid bands. In the HSV-1 gradient (figure 30A) all seven capsid proteins can be seen in lanes 10-12 which represent the peak of the B capsid band. In lanes 13 and 14, which represent the position of the A capsid band, VP21 and VP22a are present in lower quantities. The presence of these proteins, which are absent from A capsids, is due to contaminating B capsids resulting from poor separation of the two peaks in this experiment There is no clear profile of the capsid proteins in lane 6, the position of the C capsids and this reflects the small number of C capsids in this gradient (figures 28A and 29A).

Recombinant capsids made in the absence of AcUL35 peak in the same position (fractions 10-12) of the gradient and have a similar protein composition with the exception of VP26 (figure 30B). In a separate experiment, when the virus AcUL35 was included in the infection, VP26 was present in the capsid band (figure 30C). The elecrophoretic mobilities of the capsid proteins were the same for both HSV-1 and baculovirus derived capsids. Thus, VP21, VP24 and VP22a were of the sizes expected

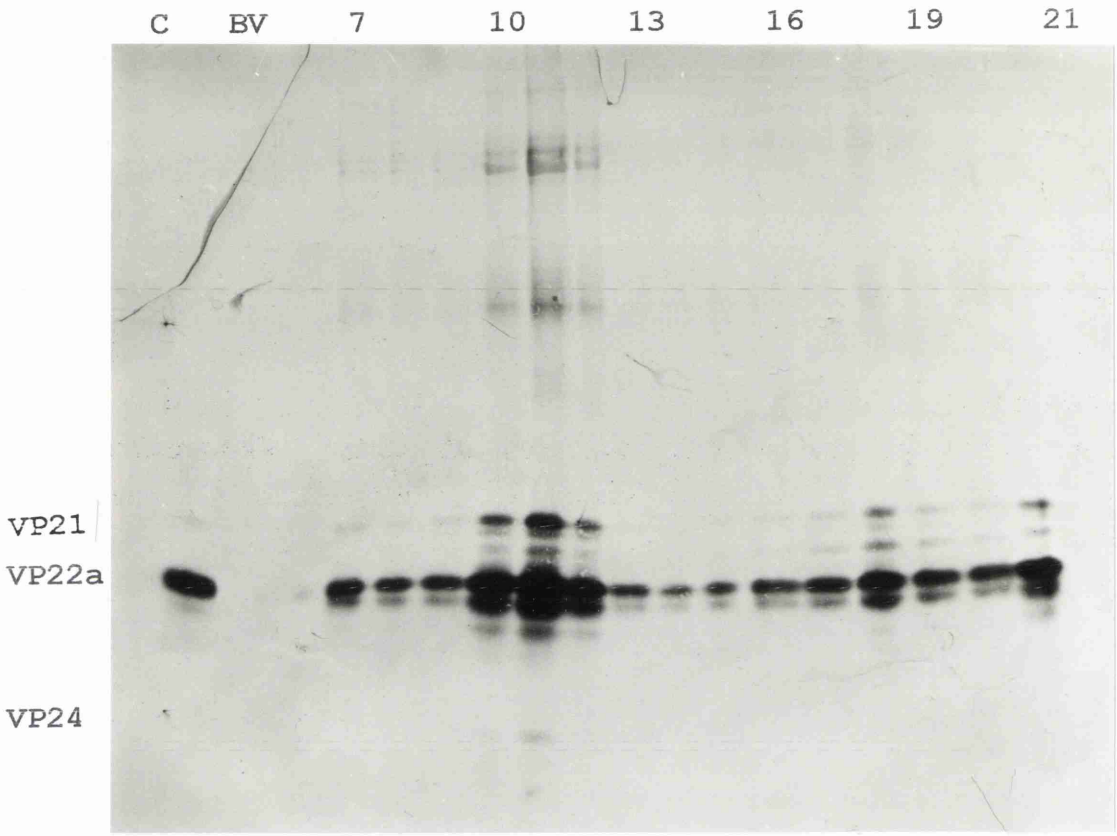

C
BV
7
10
13
16
19
21
VP21
VP22a
VP24

Figure 31:- Western blot analysis of VP21 and VP22a in a gradient of baculovirus-derived capsids.

50ul of fractions 7-21 (lanes 7-21) of the baculovirus gradient (^AcUL35) shown in figure 30B were resolved by SDS-PAGE on a 9% polyacrylamide gel. A purified HSV-1 B capsid sample (lane C) and an AcPAK6-infected cell extract (BV) were run alongside as controls. The proteins were transferred to a nylon membrane and were probed with a 1:200 dilution of the monoclonal antibody MCA 406 which recognises epitopes in both VP21 and VP22a. Antigen-antibody complexes were detected using a 1:100 dilution of Protein A coupled to colloidal gold and the signal was amplified with a silver enhancing solution. The positions of VP21 and VP22a are indicated to the left the gel.

for the processed forms demonstrating that normal cleavages had occurred. Of particular interest was the fact that the relative abundances of the capsid proteins (in particular the ratios of VP22a to VP21 and VP24) appeared approximately similar in HSV-1 and recombinant baculovirus-derived capsids. This suggests that the factors controlling the incorporation of these proteins into the capsid are inherent in the capsid structure itself and are not influenced by external factors or protein abundance.

Several other protein bands are present in the recombinant capsid profile. Some of these represent breakdown products of the capsid proteins, in particular of VP5. The lability of HSV-1 capsid proteins which have been stored for along time is demonstrated by comparing the HSV-1 capsid profiles (lane C) in figure 30C with that in 30B. These represent the same sample following storage at -70°C between the running of gels B and C. A protein profile of a similar gradient of AcPAK6-infected cells is shown in figure 30D and indicates that the other proteins found in gradients 30B and 30C are contaminating Sf21 cellular or baculovirus proteins that sediment in a similar region of the gradient. The most prominent band is that of the 39kDa baculovirus capsid protein which peaks near the top of the gradient but is present throughout the gradient (figure 30D). This protein was often found to co-migrate on gels with VP22a. Another abundant protein is the AcPAK6-encoded 116kDa β-galactosidase protein which can be seen throughout the gradient migrating just below the HSV-1 VP5 protein (figure 30, lane C). However this protein is not made by any of the recombinants.

3.7 WESTERN BLOT ANALYSIS OF VP21 AND VP24

An unexpected feature of gradient 30B is the presence of two bands above the baculovirus capsid bands which migrate to the same position as VP21 in lanes 17-19. To determine whether these corresponded to VP21, immunoblot analysis was performed (figure 31). Fractions 7 to 21 (figure 31, lanes 7-21) of the ^AcUL35 gradient shown in figure 30B, were separated by SDS-PAGE and were subsequently transferred onto a nitrocellulose membrane. HSV-1 capsid polypeptides (figure 31, lane C) and an AcPAK6-infected cell extract (figure 31 lane BV) were run as controls. The membrane was incubated with 1:200 dilution of the primary antibody, MCA 406. This mouse monoclonal antibody recognises an epitope common to VP22a, the processed product of UL26.5 and VP21, the protein encoded by the carboxy-terminal portion of UL26. Antigen-antibody complexes were detected using a 1:100 dilution of Protein A coupled to colloidal gold particles and the signal was amplified with a silver enhancing solution as described in Methods. This demonstrated the presence of the VP22a and VP21 proteins in the control capsid sample (figure 31 lane C). VP22a could be detected throughout the gradient but was most abundant in lanes 10-12, which represent the position of B capsids in the gradient. A second peak of this protein was apparent in

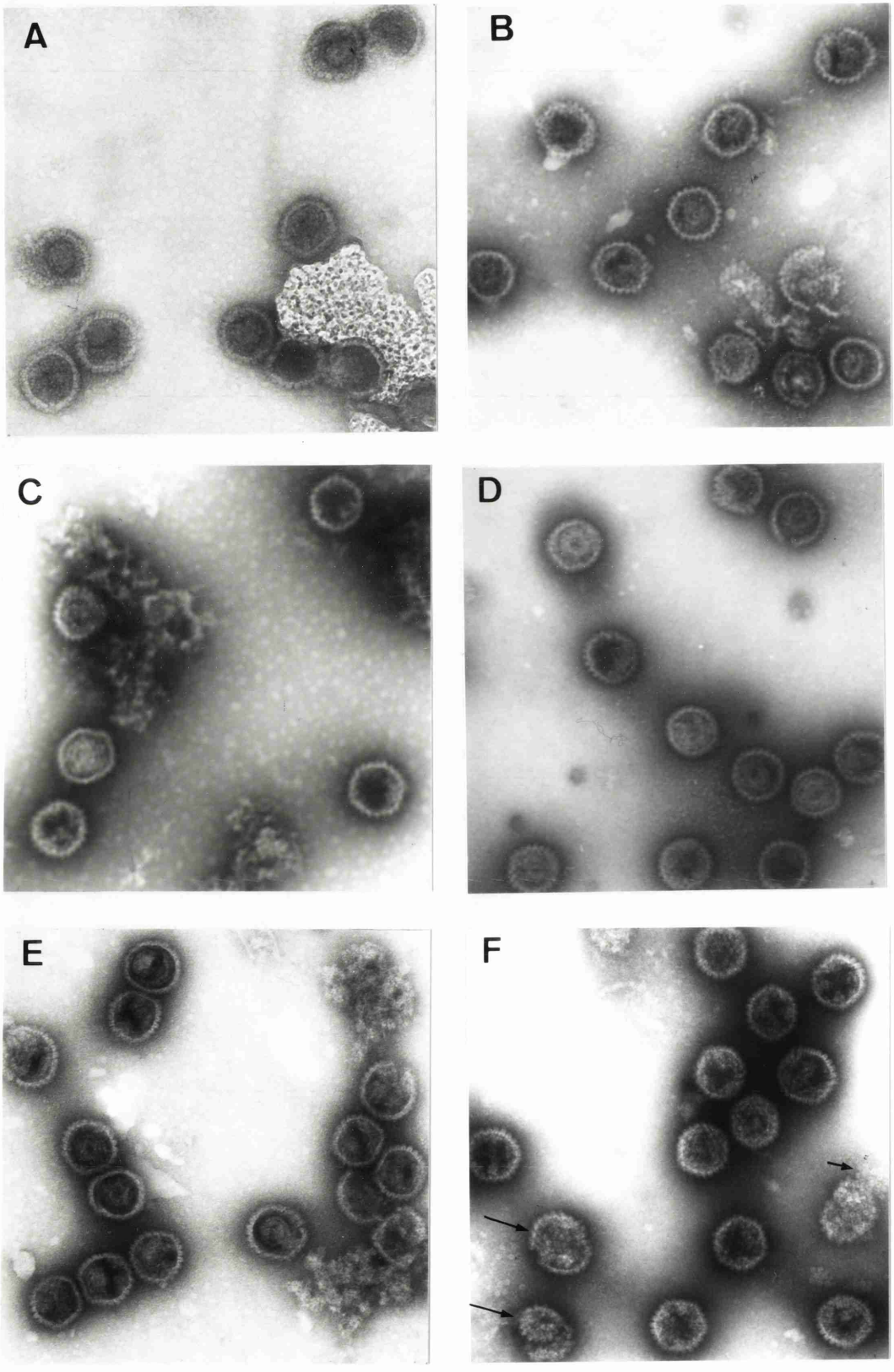

A
B
C
D
E
F

Figure 32:- Comparison of capsids by negative staining and electron microscopy

Intranuclear capsids were extracted from Sf21 cells infected for 72 hours with 5 p.f.u./cell of each of the recombinant baculoviruses in the following combinations: all (AcUL18, AcUL19, AcUL26, AcUL26.5, AcUL35 and AcUL38), ^AcUL35 (AcUL18, AcUL19, AcUL26, AcUL26.5 and AcUL38), ^AcUL26 (AcUL18, AcUL19, AcUL26.5, AcUL35 and AcUL38) and ^AcUL26.5 (AcUL18, AcUL19, AcUL26, AcUL35 and AcUL38) as described in Methods. HSV-1 B capsids were prepared from HSV-1 strain 17-infected cells and from *ts*1201-infected BHK cells as described in Methods. Capsids were sedimented at 40,000 r.p.m. in a TsT41 rotor for 20 minutes through a 5-40% sucrose gradient. The band containing the capsids was removed, the capsids diluted in buffer and pelleted at 24,000 r.p.m. for 1h. The capsid pellet was resuspended in 100ul PBS complete, 5ul of each preparation was negatively stained with 1% phosphotungstic acid and examined in the electron microscope. (A) HSV-1 strain 17, (B) All, (C) *ts*1201, (D) ^AcUL26 (E), ^AcUL35 and (F) ^AcUL26.5. In panel F, broken capsids (⟶) and free capsomers (→) are indicated. The size bar represents 200nm.

lanes 17-21. Similarly, VP21 showed peak abundances in lanes 10-12 and 17-19. The peak at 17-19 suggests that VP21 and VP22a form assemblies which band at a similar position to the baculovirus capsids. These are most likely to be the scaffolds described earlier although their presence could not be determined by negative staining and electron microscopy. The higher molecular weight bands in lanes 10-12 may represent forms of the full-length protease which have been observed previously in Western blots (Preston *et al.*, 1994).

3.8 COMPARISON OF HSV-1 AND BACULOVIRUS-DERIVED CAPSIDS BY NEGATIVE STAINING

To compare the appearance of capsids made from HSV-1 infection of BHK cells with those made in the baculovirus system, capsids were pelleted from sucrose gradients, resuspended in PBS, negatively stained and viewed in the electron microscope as described in Methods. Type B capsids prepared from HSV-1 strain 17-infected BHK cells were of typical appearance, approximately 100nm in diameter and the capsomers could be identified on the outside of the capsid shell (figure 32A). Capsids made using all six recombinant baculoviruses (figure 32B) and those made in the absence of VP26 (^AcUL35; figure 32E) appeared identical to the HSV-1 capsids. This suggests that capsids made from the baculovirus system are very similar in structure to authentic HSV-1 capsids and that the absence of VP26 does not significantly affect the stability of the capsid or its gross appearance.

Capsids made in the absence of the protease (^AcUL26; figure 32D) resembled those made by the *ts*1201 mutant, which has a defective protease, at the NPT (figure 32C). Although the difference in core size observed between HSV-1 strain 17 and *ts*1201 capsids by examination in thin section is not readily distinguishable with negative staining these latter capsids do appear to exhibit more extensive core material.

Capsids that were prepared in the absence of the scaffolding protein, pre-VP22a (^AcUL26.5) as shown in figure 32F, have an outer shell resembling those made with the full complement of proteins but do not have an obvious central core. These represent the closed shells which were observed in small numbers in thin section. A number of the capsids have a broken appearance and look like capsids which are in the process of breaking up suggesting that these capsid shells are more fragile and it is possible that the majority of these band elsewhere on the gradient, which yielded a diffuse light-scattering band. Some of these may resemble the partial shells forms observed in thin section. Clumps of capsomers and isolated capsomers were also observed. The presence of complete capsomers suggests that the interactions that occur normally between the shell proteins (VP5, VP19C, VP23 and VP26) in capsids also occur in the absence of pre-VP22a. That these interactions are sufficient to maintain the

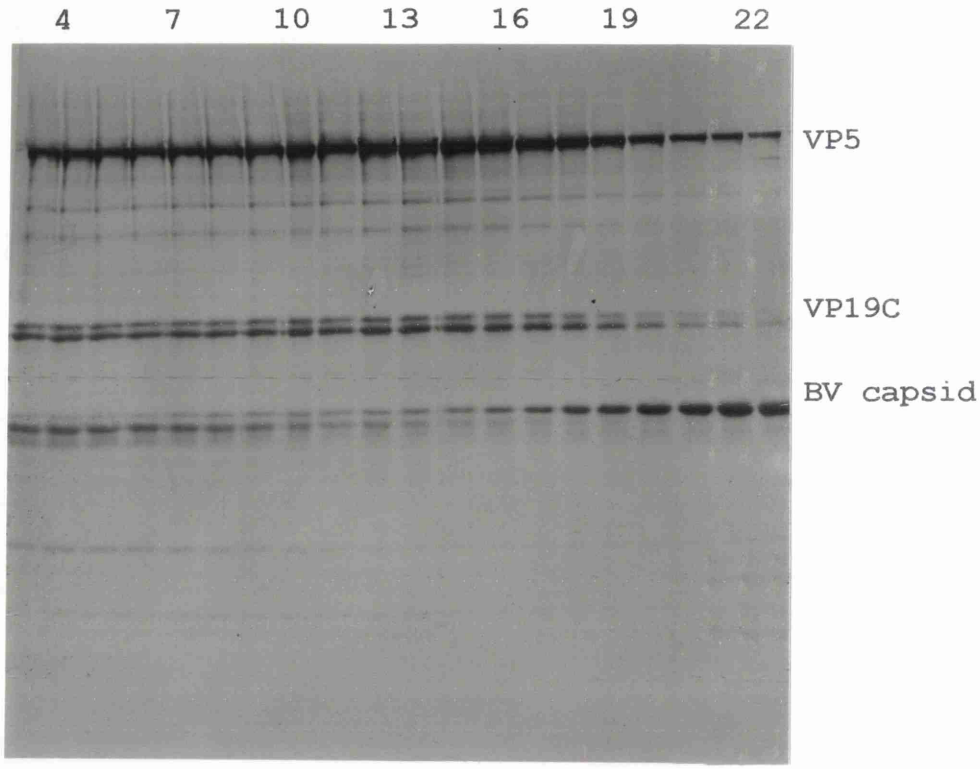

4
7
10
13
16
19
22
VP5
VP19C
BV capsid

Figure 33:- SDS-PAGE analysis of gradient-banded VP5/VP19C particles

Sf21 cells co-infected with 5 p.f.u./cell each of AcUL19 and AcUL38 were labelled with [^{35}S]methionine from 30h p.i. After a further 18h incubation at 28°C the cells were harvested and treated as for preparation of capsids (described in Methods). The cell extracts were centrifuged at 40,000 r.p.m. in a TsT41 rotor for 20 minutes on a 5-40% sucrose gradient which was collected in eight drop fractions. 50ul of fractions 3-22 were analysed on a 9% polyacrylamide gel and the positions of the proteins identified by fluorography. The positions of the HSV-1 capsid proteins VP5 and VP19C and of the major baculovirus (BV) capsid protein are shown to the right of the gel.

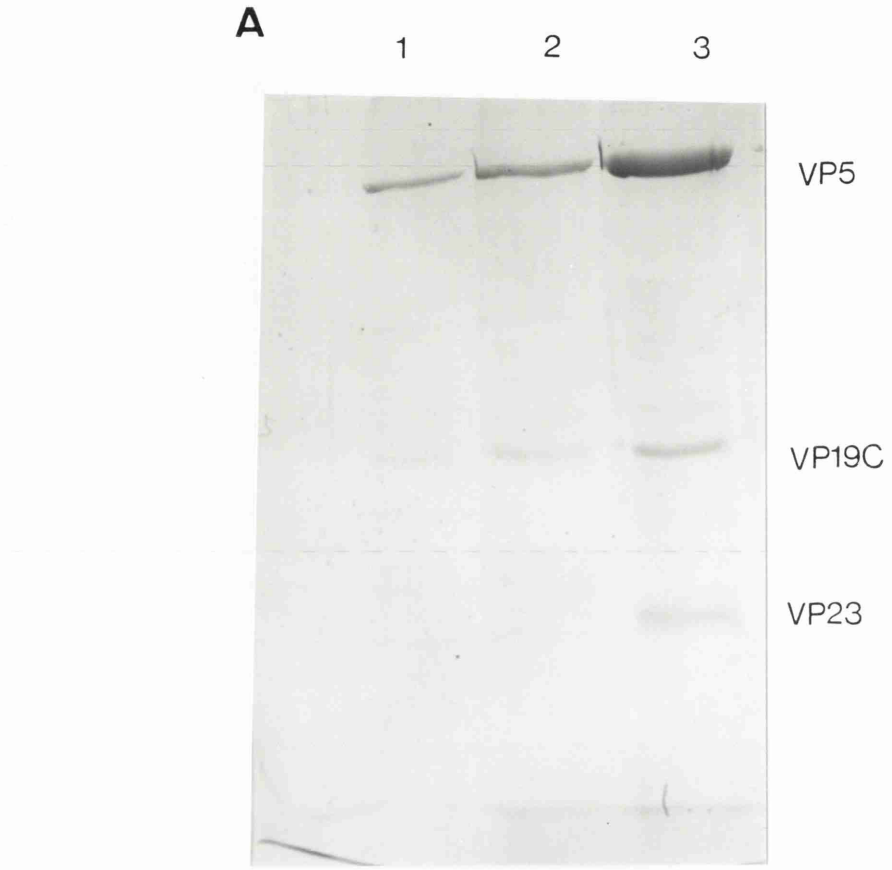

A
1
2
3
VP5
VP19C
VP23
B
C

Figure 34:- Composition and structure of VP5/VP19C particles

Particles formed from co-infection of Sf21 cells with AcUL19 and AcUL38, each at an m.o.i. of 5 p.f.u./cell, were banded on sucrose gradients as described in figure 33. The two bands observed were harvested from the gradient and the particles present in each were pelleted by centrifugation at 24,000 r.p.m. for 1h in a TsT41 rotor before being resuspended in 100ul PBS complete as described in Methods. The protein content (A) of 10ul of particles from the upper band (lane 1) and lower band (lane 2) were analysed by SDS-PAGE on a 9% polyacrylamide gel and were compared to the protein profile of HSV-1 type A capsids (lane 3). The positions of the capsid proteins are indicated to the right of the gel. 5ul of particles from the upper band (B) and from the lower band (C) were negatively stained as described in Methods and examined by electron microscopy. The bar represents 200nm.

integrity of the capsid shell structure during extraction and sedimentation is demonstrated by the presence of complete shells. However, the fragility of these structures indicates that the proteins VP21 and VP24 encoded by UL26 are not compensating completely for the scaffolding function provided by the UL26.5-encoded protein.

3.9 THE NATURE OF THE VP5 AND VP19C 70NM PARTICLES

To investigate the nature of the 70nm densly staining particles shown in figures 26C and D, suspension cultures of insect cells were co-infected with 5 p.f.u./cell of AcUL19 and AcUL38 recombinant baculoviruses. The cells were harvested and treated as for capsid purification. After sedimentation through a sucrose gradient; two diffuse bands, an upper and a lower band, were observed in the middle portion of the gradient. In the experiment shown in Figure 33, the proteins were labelled with [^{35}S]methionine and the gradient was collected in 25 eight-drop fractions. The proteins in fractions 3-22 were separated on a 9% acrylamide gel and their distribution was analysed by fluorography (figure 33). Although VP5 and VP19C were found throughout the gradient they appeared to be concentrated in the middle region of the gradient (figure 33). This pattern of distribution presumably results from the diffuse banding pattern observed on the gradients.

To examine further the nature of the particles formed between VP5 and VP19C, each of the bands was collected from an unlabelled gradient, the particles were pelleted by centrifugation and resuspended in 100ul PBS complete. 10ul of the resuspended samples was analysed by SDS-PAGE on a 9% polyacrylamide gel (figure 34A). Both the upper (figure 34, lane 1) and lower (figure 34, lane 2) bands were composed predominately of VP5 and VP19C which had the same electrophoretic mobilities as their counterparts in A capsids isolated from HSV-1 strain 17-infected BHK cells (figure 34, lane 3). The structure of the particles was examined by negative staining and electron microscopy. The majority of the particles purified from the upper band (figure 34B) resembled complete shells which were smaller in diameter (approximately 70nm) than the 100nm capsids formed from all six recombinant baculoviruses (figure 32B), although some partial shells were also observed. The particles isolated from the lower band (figure 34C) consisted almost exclusively of partial shells which appeared to be composed of organised capsomeres similar to those detected for native HSV-1 capsids. These partial shell-structures appeared to interact and form clumps which would explain their higher rate of sedimentation. The appearance of the closed shells is more interesting since it suggests that VP5 and VP19C interact to form self-contained structures and thus may provide us with some clues about capsomer/triplex interactions. These structures are currently being examined by W. Chiu using cryo-electron

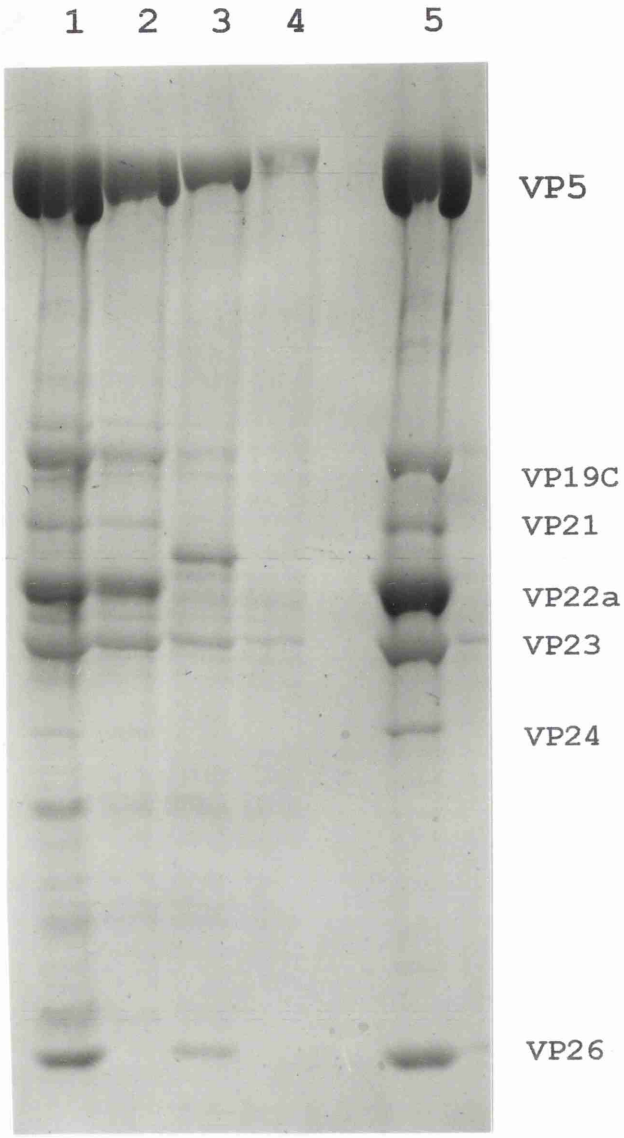

1
2
3
4
5
VP5
VP19C
VP21
VP22a
VP23
VP24
VP26

Figure 35:- SDS-PAGE analysis of purified capsids

Intranuclear capsids were extracted from Sf21 cells infected with 5 p.f.u./cell of each of the recombinant baculoviruses in the following combinations: all (AcUL18, AcUL19, AcUL26, AcUL26.5, AcUL35 and AcUL38), ^AcUL35 (AcUL18, AcUL19, AcUL26, AcUL26.5 and AcUL38), ^AcUL26 (AcUL18, AcUL19, AcUL26.5, AcUL35 and AcUL38) and ^AcUL26/^AcUL35 (AcUL18, AcUL19, AcUL26.5 and AcUL38) as described in Methods. HSV-1 B capsids were prepared from HSV-1 strain 17-infected BHK cells. Capsids were sedimented at 40,000 r.p.m. for 20 minutes through a 5-40% sucrose gradient in a TsT41 rotor. The bands containing the capsids were removed, the capsids diluted in buffer and pelleted at 24,000 r.p.m. for 1h in the above rotor. The capsid pellet was resuspended in 100ul PBS and 10ul of each preparation was analysed by SDS-PAGE on a 5-15% polyacrylamide gel and the proteins visualised by staining with Commassie Brilliant Blue. The polypeptide profiles of baculovirus-derived capsids 'all' (lane 1), ^AcUL35 (lane 2), ^AcUL26 (lane 3) and ^AcUL26/^AcUL35 (lane 4) are compared to HSV-1 B capsids (lane 5). The positions of the capsids proteins are indicated to the right of the gel.

microscopy and since no icoshedral symmetry has yet been found it is unlikely that the fine structure can be determined.

3.10 HIGH RESOLUTION STRUCTURAL ANALYSIS OF CAPSIDS MADE USING THE BACULOVIRUS EXPRESSION SYSTEM

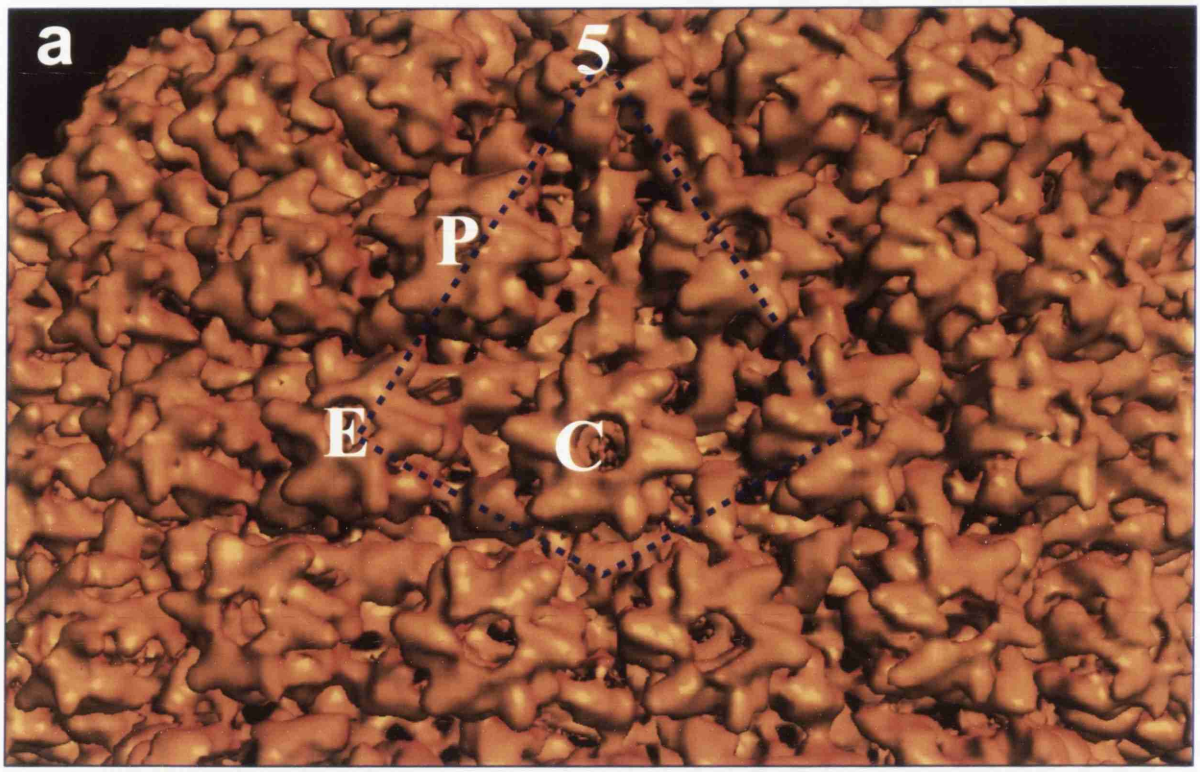
Analysis of the fine structure of capsids made in the baculovirus system was carried out in collaboration with W. Chiu and H. Zhou at Baylor College of Medicine, Houston, Texas.

3.10.1 Purification and protein composition

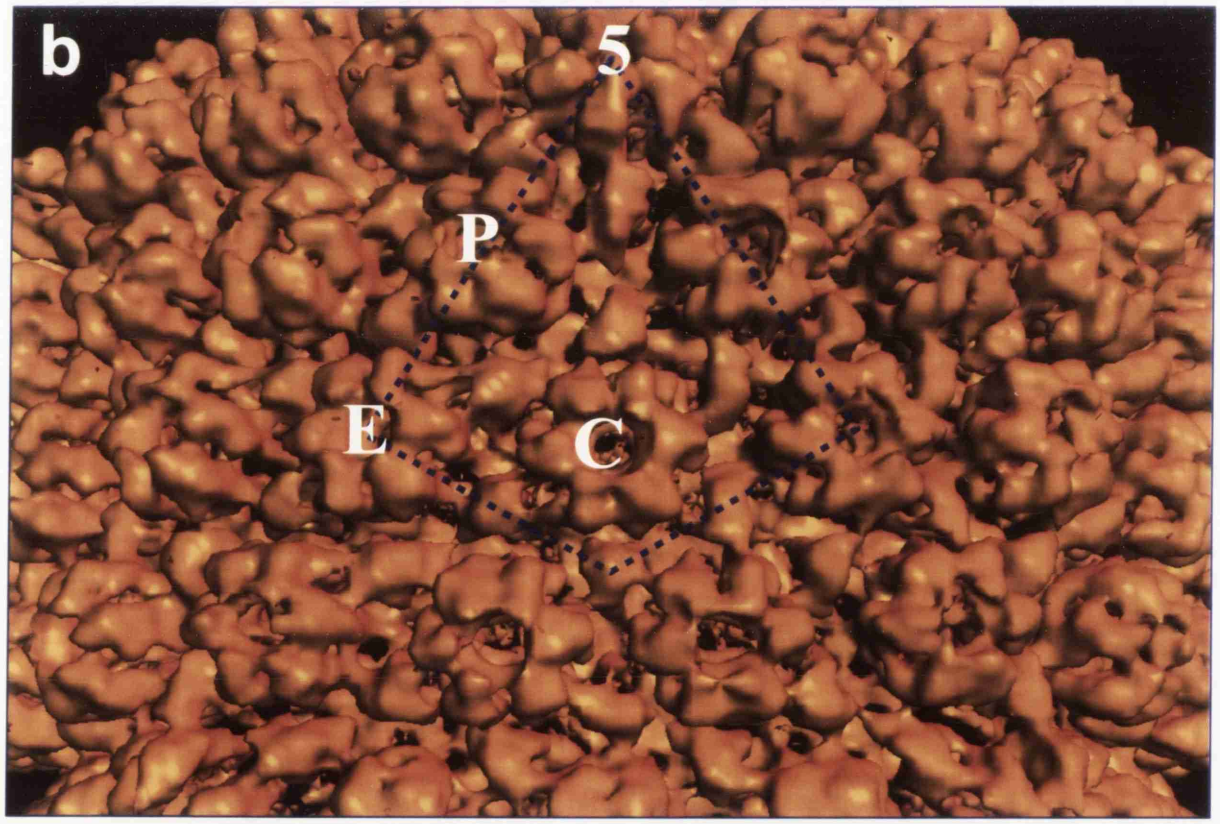
Cells were infected with combinations of the recombinant baculoviruses, capsids extracted from the cells, purified on sucrose gradients and concentrated as described in Methods. Capsids were made using the following combination of viruses: ALL (AcUL18, AcUL19, AcUL26, AcUL26.5, AcUL35 and AcUL38), ^AcUL35 (AcUL18, AcUL19, AcUL26, AcUL26.5 and AcUL38), ^AcUL26 (AcUL18, AcUL19, AcUL26.5, AcUL35 and AcUL38) and ^AcUL26/^AcUL35 (AcUL18, AcUL19, AcUL26.5 and AcUL38). The protein composition of the capsids was verified using SDS-PAGE analysis. Figure 35, lanes 1-4, shows the protein profiles of these capsid preparations resolved on a 5-15% gradient acrylamide gel. Type B capsids made from HSV-1 infection of BHK cells were prepared in a similar manner and the protein profile, which demonstrates the positions of all seven capsid proteins, is shown for comparison in figure 35; lane 5. As shown in lane 1, capsids made from co-infection of all seven recombinant baculoviruses have the same profile as HSV-1 B capsids confirming that all the proteins are present in the correct amounts. VP26 is the only capsid protein absent in ^AcUL35 capsids (figure 35, lane 2). In capsids made in the absence of the protease (^AcUL26; figure 30, lane 3) not only are VP21 and VP24 absent, but as expected, the UL26.5 product, pre-VP22a, is unprocessed and migrates more slowly than VP22a. The yield of capsids produced in the absence of AcUL26 and AcUL35 was considerably lower in this particular infection (figure 35, lane 4). The integrity of the capsid samples was also checked by negative staining and electron microscopy (not shown).

3.10.2 Three dimensional reconstructional analysis

The remainder of the capsid samples described above were frozen at -70°C and were sent to our collaborators W. Chiu and H. Zhou in Houston, Texas. This group had used spot-scanning electron cryomicroscopy and computer reconstruction techniques to determine, to a resolution of ~2.6nm, the three-dimensional structure of type A capsids



Naturally Occurring HSV-1 B-capsid



Recombinant VP26- Capsid

Figure 36:- Three-dimensional structures of naturally occurring HSV-1 B capsids and of baculovirus-derived VP26- capsids

Three-dimensional electron cryomicrographs were obtained (a) from preparations of naturally occurring HSV-1 B capsids and (b) from capsids derived from Sf21 cells infected with the five recombinant baculovirus: AcUL18, AcUL19, AcUL26, AcUL26.5 and AcUL38 (VP26-). Each three-dimensional map was reconstructed to a nominal resolution of 1.9nm from over 300 particle images. One of the 60 asymmetric units of the capsid is outlined by a dashed line and one of each of the P-, E- and C-hexons is labelled. One penton is indicated (5). These images were kindly provided by Wah Chiu at Baylor College of Medicine, Houston, Texas.

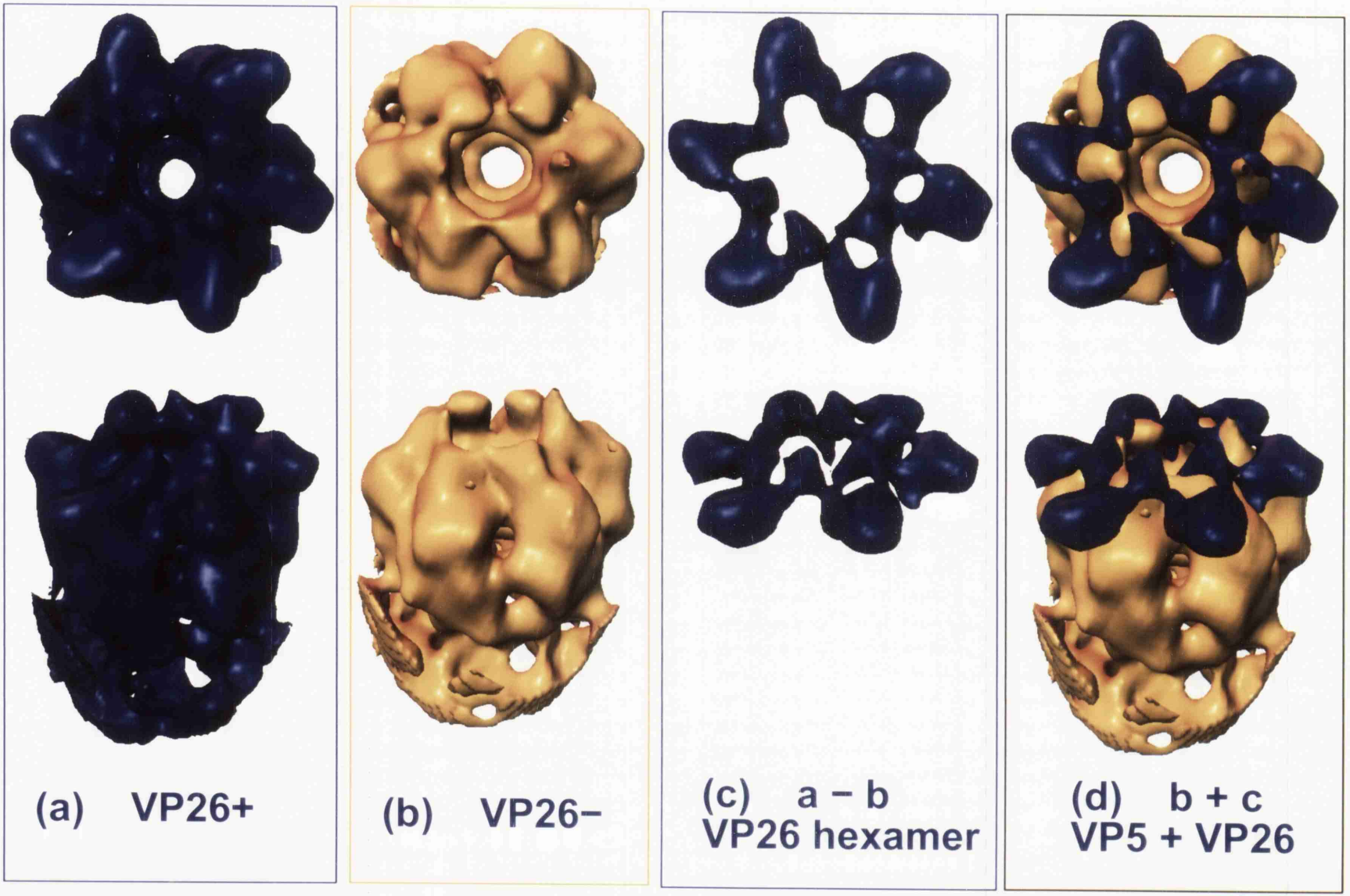

(a) VP26+
(b) VP26–
(c) a – b
VP26 hexamer
(d) b + c
VP5 + VP26

Figure 37:- Difference map of P-hexons of HSV-1 B capsids and of VP26- capsids

P-hexons were mathematically isolated from the three-dimensional map (figure 36) of (a) naturally-occurring HSV-1 capsids (VP26+) and (b) from recombinant VP26- capsids. (c) Difference map of P-hexons from capsids with and without VP26. (d) The difference map (blue) is overlaid on the VP26- hexon to reveal the molecular boundary between VP5 and VP26. The upper panel shows the top views of the hexon whilst the bottom panel shows the side views. These images were kindly provided by Wah Chiu at Baylor College of Medicine, Houston, Texas.

made during HSV-1 infection of BHK cells and had defined structural features of the outer capsid shell (Zhou *et al.*, 1994). The hexons and pentons, which are comprised of VP5, are connected by triplexes which are composed of VP19C and VP23. One unexpected difference between the hexons and pentons was the presence of a horn-shaped mass, present on the tips of the hexons but absent from the pentons, which had been attributed to VP26.

Since reconstruction analysis of capsids made with all six recombinant baculoviruses determined that these were indistinguishable from normally occurring HSV-1 B capsids (W. Chiu; personal communication) we were able to exploit this system to try to establish the structural contributions of individual proteins to the capsid. Initial studies have concentrated on determining the position and structure of VP26.

HSV-1 strain 17 B capsids and baculovirus-derived capsids made in the absence of VP26 (^AcUL35; hereon referred to as VP26- capsids) were analysed and the three-dimensional structures determined to a resolution of ~1.9nm. HSV-1 derived capsids (figure 36a) and ^AcUL35 capsids (figure 36b) share an identical lattice organisation of pentons, hexons and triplexes. However, the horn-shaped mass densities observed at the upper distal end of each hexon subunit (but absent from the penton subunit) in the naturally occurring capsid are not seen in the VP26- recombinant capsid. Except for the presence of one additional subunit present in each hexon, the domain feature of the penton subunit is very similar to that of the hexon subunits in the VP26- reconstruction.

To further determine the nature of the VP26 protein in relation to the hexon, P-hexons were mathematically isolated from the 3-dimensional map of naturally-occurring B capsids (figure 37a) and VP26- capsids (figure 37b). The top-view (upper panel) in figure 37B shows that the horn-shaped density at the upper distal end is completely missing from the VP26- capsid. Conversely, the side views (lower panel) reveal a striking similarity in the bulk of the hexons of the two types of capsids. A difference map of P-hexons with or without VP26 was compiled and clearly shows the horn-shaped density at the distal end thus confirming the exact location of VP26 (figure 37c). It also serves to elucidate the structure of VP26 which appears to consist of a major and a minor domain which attach to the face and vertice respectively of the upper domain of VP5. The major and minor domains of the VP26 appear to associate with each other to form an intact ring-like hexamer cap formed by six copies of VP26. The difference map (blue) was overlaid with the VP26- P-hexon to show the molecular boundary between the VP5 hexon and VP26 (figure 37d).

These reconstructions have thus provided evidence of the exact location of VP26; that is on the tips of the hexons, and have confirmed that the extra mass density on hexons as compared to pentons is due this protein. It has also demonstrated that VP26 exists as a

hexamer and has served to reveal the interactions between VP26 and VP5 in more detail.

Subsequent to the period of this project, the capsid structures were refined to a nominal resolution of 1.5nm. This gave us an even clearer picture of the difference between wt B capsids and recombinant VP26- capsids. These results are shown in Hong *et al.* (1995) which is included at the back of this thesis.

Reconstructions using the other baculovirus-made capsids mentioned above are presently underway and will hopefully elucidate more of the details of the capsid structure. The application of this reconstruction work demonstrates the usefulness and potential of the baculovirus expression system to the understanding of the constitution, structure and assembly of HSV-1 capsids.1. .

CHAPTER 4

DISCUSSION

BACKGROUND

After a considerable amount of study the protein composition of HSV-1 capsids and the genes encoding them have been identified (Zweig *et al.*, 1979a; Cohen *et al.*, 1980; Rixon *et al.*, 1990; Davison *et al.*, 1992; McNabb and Courtney, 1992a; Person *et al.*, 1993; Weinheimer *et al.*, 1993). The 7 HSV-1 capsid proteins are encoded by the genes: VP5 (UL19), VP19C (UL38), VP21 (UL26), VP22a (UL26.5), VP23 (UL18), VP24 (UL26) and VP26 (UL35) as shown in table 2. The isolation of viruses containing mutations in most of these genes has allowed their importance for capsid assembly to be determined. Capsids failed to be formed in the absence of functional UL18, UL19 and UL38 genes (Weller *et al.*, 1987; Pertuiset *et al.*, 1989; Desai *et al.*, 1993). Viruses with mutations in either UL26 or UL26.5 did produce capsids but these were of abnormal appearance. Capsids with a large core, as compared to the small core of B capsids were formed by UL26 mutant viruses (Preston *et al.*, 1983; Gao *et al.*, 1994). A UL26.5 mutant produced capsids with little or no material in their cores (Matusick-Kumar *et al.*, 1994). Mutants lacking both UL26 and UL26.5 failed to assemble intact capsids (Desai *et al.*, 1994). Although the identification of such mutants have provided information as to whether the genes are essential for production of capsids and infectious virions they do not provide much information regarding the individual role of the proteins. Therefore it was important to develop a non-HSV system which would enable manipulation of the individual proteins so as to identify the sites and nature of the viral protein interactions and also determine the contributions of the individual proteins to the stages of capsid assembly. Furthermore, a system was required which would enable capsid assembly to be studied in the absence of other HSV-1 proteins so as to determine whether other auxillary HSV-1 proteins are required for capsid assembly. For the reasons outlined below, the baculovirus system was chosen for the analysis of the structure and assembly of the HSV-1 capsid and has proved a very useful tool.

2. THE BACULOVIRUS EXPRESSION SYSTEM

Autographa californica Nuclear Polyhedrosis Virus (AcNPV) is the prototype of the family *Baculoviridae* and is a very important tool, serving as a vector for foreign gene expression (reviewed by King and Possee, 1992), as a biological pesticide (Miller *et al.*, 1983) and as a model system for the molecular biological analysis of baculoviruses (Doerfler, 1986).

AcNPV infects insects, mainly those of the order Lepidoptera, and is generally propogated in cultures of *Spodoptera frugiperda* (Sf) cells. Two types of virus progeny

are produced during AcNPV infection. Extra cellular virus (ECV) is produced during the early stages of infection and is highly infectious for cultured cells whereas Occluded virus (OV) is produced during the terminal stages of infection (reviewed by Summers and Smith, 1987). These OV are embedded in proteinaceous viral occlusions, called polyhedra, the main structural component of which is the 29kDa polyhedrin protein. This protects the embedded virus particles in the environment between susceptible hosts. Polyhedra are ingested by larvae which feed on contaminated plants and the occlusions then dissolve in the insect gut releasing virus which invade and replicate in the cells of the midgut tissue. Secondary infection spreads to other insect tissues by the ECV form. The life-cycle, structure and assembly of AcNPV is reviewed in detail by Harrap (1972a; b; c) and is only described briefly here. Virus particles enter the cell by endocytosis or fusion and the viral ds DNA is uncoated in the nucleus. DNA replication begins at about 6h p.i. and from 10h p.i. ECV is released from the cell by budding. Viral occlusions are detected by 24h p. i., and extra cellular virus levels reach a maximum between 36 and 48h p. i. However, the polyhedrin protein continues to accumulate for 4-5 days until the infected cells lyse.

The regulation of baculovirus gene expression is described in detail by Friesen and Miller (1986). Both the polyhedrin protein and the p10 protein, which forms extensive fibrillar structures found in the nucleus and in the cytoplasm of infected cells (Van der Wilk *et al.*, 1987), are hyper-expressed and produced from very late genes. The polyhedrin gene has been mapped and sequenced and has been shown to be non-essential for infection and replication of virus. Deletion or insertional inactivation of the polyhedrin gene results in the production of occlusion negative viruses that form plaques (occ-) that are distinctly different from those (occ+) produced by wild type viruses. Genetic manipulation has resulted in the replacement of the polyhedrin gene by a number of marker genes, such as the B-galactosidase gene, which facilitate the identification and selection of recombinant viruses. Various transfer vectors have been designed which utilise the polyhedrin or p10 promoters to produce large amounts of recombinant protein at very late times in infection. In the course of the work for this thesis, pAcCL29.1 (Livingston and Jones, 1989) which is derived from pAcYM1 (Matsuura *et al.*, 1987), was used. This vector contains the complete 5' non-coding leader sequence of the polyhedrin gene which is essential for high level of expression from this promoter as well as the first nucleotide of the translation initiation codon. pAcCL29.1 also has the M13 intergenic region necessary for ss DNA production and a multicloning region to facilitate cloning. Foreign DNA is recombined into the virus by a co-transfection reaction. A variety of baculovirus transfer vectors have been constructed which exploit either the polyhedrin or p10 promoter or both (reviewed by Bishop, 1992; King and Possee, 1992). Multiple gene transfer vectors have been

developed to facilitate the insertion of 3 or 4 foreign genes into the AcNPV genome by means of a single transfection (Belyaev and Roy, 1993).

2.1 THE ADVANTAGES OF THE BACULOVIRUS SYSTEM IN EXPRESSION OF RECOMBINANT PROTEINS

The AcNPV genome, which is circular and consists of approximately 128kbp, can accommodate relatively large segments of foreign DNA without affecting normal replication and DNA packaging; the nucleocapsid expands lengthways to accommodate the extra DNA. It is a safe virus to work with as it only replicates in invertebrate cells and large volumes of infected culture can be produced. Another advantage is that because the polyhedrin (and p10) gene products are expressed from strong promoters at very late times in infection, after the maturation of budded, infectious virus then synthesis of potentially cytotoxic proteins will not adversely affect virus replication. This appeared to be a problem when previous studies were undertaken to express the HSV-1 protease from vaccinia virus (Nicholson, 1992).

Vaccinia virus has also been widely used as an expression vector (reviewed by Mackett *et al.*, 1985; Mackett and Smith, 1986; Piccini *et al.*, 1987). For example, virus-like particles have been assembled from proteins expressed by vaccinia virus for hepatitis A virus (Winokur *et al.*, 1991), for Aleutian mink disease parvovirus (Clemens *et al.*, 1992), and for human and bovine papilloma viruses (Hagensee *et al.*, 1993; Zhou *et al.*, 1993). However, previous studies using vaccinia virus to clone HSV-1 capsid genes proved difficult; providing a low efficiency of recombination as well as plaques that were hard to identify (Nicholson, 1992). Although the HSV-1 genes, UL18, UL19, UL26.5 and UL38 were eventually successfully cloned and their protein products expressed, it proved impossible to express the protease encoded by UL26. This protein has since been expressed to high levels in baculovirus (Preston *et al.*, 1994; Tatman *et al.*, 1994; Thompson *et al.*, 1994) which suggests that either the protease is more toxic to the animal cells in which vaccinia virus replicates, or that production at very late times in infection in the baculovirus system overcomes such potential problems. A further difficulty encountered by Nicholson (1992) was that it proved difficult to get cells multiply infected with recombinant vaccinia viruses.

Another advantage in using baculovirus, this time over prokaryote expression systems, is that the insect cells which are used in the propagation of baculovirus appear capable of accomplishing most post-translational modifications that are required to process many foreign gene products into their biologically active forms (reviewed by King and Possee, 1992). For example, phosphorylation of baculovirus expressed proteins has been demonstrated for the SV40 large T antigen and for mouse p53 (O'Reilly and

Miller 1988). It does appear, however, that glycosylation takes place via a different pathway than in vertebrate cells; glycoproteins produced in insect cells have relatively simple unbranched sugar side chains with a high mannose content which results in greater mobility in denaturing polyacrylamide gels (Lanford *et al.*, 1989). It also appears that cellular targeting and secretion of proteins synthesised by recombinant baculovirus occurs as would be expected for the normal protein in its natural host; most signal peptides are recognised and cleaved in the endoplasmic reticulum and the resulting protein is targeted to the membrane or secreted as appropriate (Kang *et al.*, 1987).

It has also been shown that tertiary and quaternary structures of proteins and protein complexes can be formed correctly by baculovirus expressed proteins. Recombinant proteins purified from the baculovirus system are able to ellicit similar immune responses to the native protein. (Ghiasi *et al.*, 1992; Roy *et al.*, 1990; Oldfield *et al.*, 1990; Saliki *et al.*, 1992). Recombinant proteins expressed in insect cells also appear to be capable of assembling into multi-protein complexes. Sub-viral particles synthesised from a single protein species have been generated for the surface and core antigens of Hepatitis B virus (Takehara *et al.*, 1988). Active enzymatic complexes can be formed since when baculovirus expressing the UL5, UL8 and UL52 genes of HSV-1 were co-infected, a functional helicase-primase complex was formed which was shown to have the authentic enzyme activities (Dodson *et al.*, 1989; Stow, 1992). In the case of poliovirus type 3, expression of the entire coding region resulted in the production of non-infectious, empty poliovirus capsids containing distinct VP0, VP1 and VP3 proteins whereas expression of the region encoding the capsid proteins only resulted in the unprocessed precursors of these structural proteins. This demonstrated that the proteolytic processing events required for the maturation of the poliovirus virion, with the exception of the final cleavage of VP0 to VP2 and VP4 which is autocatalytic upon encapsidation of the virus RNA genome, can function in this system (Urakawa *et al.*, 1989).

2.1.1 The formation of sub-viral particles using the baculovirus system

Studies most pertinent to this thesis describe the formation of discrete sub-viral particles formed from infection of insect cells with a combination of recombinant baculoviruses. A number of Blue Tongue Virus (BTV) genes have been cloned under baculovirus promoters and the resulting recombinant baculoviruses used to analyse protein interactions and to produce recombinant particles. Since the BTV model is particularly relevant to the HSV system it will be described in some detail.

BTV is an RNA virus, the genome of which is contained within a inner core composed of two major proteins, VP3 and VP7 and three minor proteins; VP1, VP4 and VP6. The inner core is surrounded by an outer capsid consisting of the proteins VP2 and VP5. Expression of VP3 and VP7 from a dual recombinant baculovirus results in the assembly of the core like particles (CLP) which are produced in BTV-infected cells and has demonstrated that neither the minor core proteins nor the BTV non-structural proteins are required to assist or direct the formation of the CLPs (French and Roy, 1990). Cryo-electron microscopy has been used to further determine their structure (Hewat *et al.*, 1992). Construction of a baculovirus expressing the genes coding for the outer capsid proteins VP2 and VP5 and co-infection of this recombinant virus with that expressing VP3 and VP7 resulted in the formation of double-shelled virus like particles (VLPs) (French *et al.*, 1990). Antibodies raised against these VLPs have the ability to neutralise the homologous BTV serotype. VLPs have also been assembled from recombinant baculoviruses which express all of the four proteins, VP2, VP3, VP5 and VP7 (Belyaev and Roy, 1993). This work has been explored further by the addition into the system of a recombinant baculovirus expressing the VP1 protein (a component of the viral RNA-directed RNA polymerase). It has proved possible to incorporate this protein into both CLP and VLP (Loudon and Roy, 1991). Thus it has been possible to demonstrate that CLP are composed of an icosahedral subcore of VP3 which forms a scaffold for the assembly of VP7 capsomers and that the site of interaction of VP1 and the CLP is on VP3. CLP produced from baculovirus-infected cells have also been used to determine ss RNA binding activity, which is now attibuted as a function of VP3 (Loudon and Roy, 1992). Another advantage of the baculovirus system is the flexibility it allows, for example, it has made possible the assembly of heterologous VLP using VP2 and VP5 of different serotypes (Loudon *et al.*, 1991).

Sub-viral particles have also been formed using the baculovirus system for feline leukaemia virus (Thomsen *et al.*, 1992), polyomavirus (Montross *et al.*, 1991), influenza virus (St. Angelo *et al.*, 1987), rotavirus (Tosser *et al.*, 1992; Crawford *et al.*, 1994; Zeng *et al.*, 1994) and human papilloma virus (Kirnbauer *et al.*, 1992; Rose *et al.*, 1993). For the reasons described above it was decided to employ the baculovirus expression system to study HSV capsid assembly. Since the aim was to study the process of capsid assembly it was important to know whether the baculovirus system mimicked the natural system. Fortunately, the large amounts of information obtained from the isolation of HSV-1 *ts* mutants has enabled comparisons of structures formed with these mutants to those formed from different combinations of recombinant baculoviruses. In addition, the determination by cryo-electron microscopy of the fine structure of the capsids formed by both systems has demonstrated that the baculovirus system effectively mimics what has been observed in HSV-1 infected cells. Similarities between the processes of HSV-1 assembly and those of several bacteriophages have

been documented and use of the baculovirus system has allowed such parallels to be studied in greater depth.

3 HSV-1 CAPSID PROTEINS EXPRESSED BY RECOMBINANT BACULOVIRUSES

This thesis describes the construction of recombinant baculoviruses expressing HSV-1 capsid protein genes; VP19C (AcUL38), VP23 (AcUL18) and VP26 (AcUL35). Baculoviruses expressing VP21 and VP24 (AcUL26) and pre-VP22a (AcUL26.5) were a gift of V. Preston and their construction is described elsewhere (Preston *et al.*, 1994). The construction of a partially purified VP5-expressing baculovirus (AcUL19) is described by Nicholson (1992). Four of these capsid proteins were found to migrate at the position of the equivalent protein in a profile of purified HSV-1 capsids. Hence, AcUL18, AcUL19, AcUL38 and AcUL35 produced proteins migrating at the positions of VP23, VP5, VP19C and VP26 respectively (figure 20). However, in cells infected with AcUL18, a second protein was observed running at about <24kDa which appears to be a breakdown product of VP23. This might be explained by the observation by Lemaster and Roizman (1980) who mention that VP23 undergoes cleavage in preparations of purified virions and who also suggested that cleavage occurs by an enzyme dependent on Mg^{++} for activation. In AcUL19-infected cells, several breakdown products of VP5 were observed which also appear in preparations of capsids that have been stored for prolonged periods. Due to a baculovirus-encoded 12.5 kDa basic protein produced late in infection (Tweeten *et al.*, 1980; Wilson *et al.*, 1987) the 12kDa VP26 capsid protein proved more difficult to resolve by electrophoresis although it could be separated on a higher concentration of polyacrylamide gel. However, *in vitro* transcription/translation of the UL35 gene under the control of the SP6 promoter produced a protein of similar size to the native HSV-1 UL35 gene product (figure 14) and verified that the initial construct of the UL35 gene used in construction of the baculovirus construct was correct. The apparent similarity in size between the native HSV-1 proteins and those expressed by the recombinant viruses demonstrates that the HSV proteins are being correctly made in the baculovius system.

The products of UL26 and UL26.5 do undergo significant post-translational processing (Liu and Roizman, 1991b; Preston *et al.*, 1992). Two proteins were produced in AcUL26-infected cells and are formed as a result of self-cleavage of a precursor protein into the 2 smaller forms, VP21 and VP24, which represent the C- and N-terminal portions of the UL26 gene product respectively. This demonstrates that the protease remains active when produced in the baculovirus system. The lack of the precursor

form of the protease demonstrated that cleavage is rapid and agrees with other work on the kinetics of this enzyme (Preston *et al.* 1994; Thomsen *et al.*, 1994). Due to the absence of the protease in these single infections, the 2 novel proteins produced by AcUL26.5 (designated ICP35c and d by Liu and Roizman (1991b), and referred to throughout this thesis as pre-VP22a) are larger than those found in B capsids. However, when AcUL26 and AcUL26.5 are present in the same infection, the UL26.5 gene product is processed to the 2 smaller forms (designated ICP35e and f, Liu and Roizman (1991b) and generally referred to as VP22a) as is found in HSV-1 infection. Again this demonstrated that post-translational processing occurs in the baculovirus system but also confirmed that this cleavage can take place in the absence of capsid assembly.

3.1 RECOMBINANT BACULOVIRUS-DERIVED CAPSIDS

To determine whether the 7 capsid proteins would suffice for capsid assembly, insect cells were co-infected with all 6 recombinant baculoviruses. Examination of thin sections of co-infected cells revealed the presence of capsid structures which resembled B capsids found in HSV-1 infection of BHK cells (figure 21). Determination of the protein composition of purified recombinant capsids showed that the proteins were present in the same molarity as those in wt B capsids. Analysis of these recombinant capsids by cryoelectron microscopy has determined that, at a resolution of 1.7nm, these are indistinguishable from B capsids produced from wt HSV-1 infection (H. Zhou, personal communication). Thus the baculovirus system has already produced three important results. Firstly, although it has been previously determined through the use of DNA inhibitors (Nii *et al.*, 1968b) and the study of DNA- mutants (Schaffer *et al.*, 1974; Atkinson *et al.*, 1978) that capsid assembly can occur in the absence of viral DNA synthesis it was previously not known whether assembly was mediated by the transient association of any auxiliary proteins. Thus this system has proved that the 7 capsid proteins contain all the information necessary to direct capsid assembly. Secondly, that the interactions of the proteins in the baculovirus system mimic the interactions in wt virus thus providing an effective model for capsid assembly and thirdly, that HSV-1 capsid assembly is a highly regulated event and that despite the different amount of each recombinant protein produced in insect cells, each capsid protein is incorporated in the correct molarity within the capsid.

3.2 THE EFFECT OF OMITTING INDIVIDUAL CAPSID PROTEINS

As has been previously mentioned, the isolation of mutants in HSV-1 capsid genes has enabled us to determine which genes are essential, either for capsid assembly, or for production of infectious progeny virus. Thus to determine whether omission of individual capsid proteins from the baculovirus system would reflect the scenario in HSV-1 infection and in an attempt to determine protein-protein interactions and to assess the contribution of each protein to the integrity of the capsid, different combinations of the recombinant baculoviruses were used. Initially only individual viruses were omitted.

3.2.1 VP26- Recombinant Capsids

In the case of the UL35 gene, no mutants have been identified. Since in other respects the baculovirus system appeared to mimic what happens in wt infection, it provided a potential means of determining a role for this protein. In fact, omission of AcUL35 from the system had no obvious effect on the formation and appearance of capsids in thin sections or negatively stained sections. Capsids closely resembled those produced from co-infection with all 6 recombinant baculoviruses or from infection with wt HSV-1 (figure 23). Although it had previously been shown, using 2M GuHCl, that VP26 could be removed from the surface of mature capsids without destroying the outer capsid shell (Newcomb *et al.*, 1993), it was not known whether VP26 was required for capsid assembly. Production of VP26- capsids in the baculovirus system has therefore determined that this protein is not required for capsid assembly although it did not shed any light on whether it was required for the production of infectious virions.

VP26 has previously been localised by difference mapping of wt B capsids and capsids from which VP26 had been removed by denaturants, to the outer tips of the hexons (Booy *et al.*, 1994). However, since this study was carried out with pentonless capsids it was not possible to determine whether this protein was also present on the pentons. Difference maps of hexons and pentons derived from reconstructions of intact capsids suggested that VP26 was not present on pentons (Zhou *et al.*, 1994). However, a means of proving the exact position of VP26 was provided by the baculovirus system. Comparison by cryoelectron microscopy of the VP26- capsids described above with HSV-1 B capsids determined that, to a resolution of 1.9nm, the only difference was the absence of a horn shaped mass of density at the distal tip of the hexons on the VP26- capsids (Zhou *et al.*, 1995). This reconstruction also provided information as to the structure of VP26. Each VP26 molecule consists of a large and small domain which attach to the face and vertex respectively of the upper domain of VP5. The fact that VP26 does not attach to the pentons confirms the previous observation using antibodies

that separately labelled hexons and pentons (Trus *et al.*, 1992), that VP5 is folded in a different conformation in the pentons. It also suggests that the stability of the VP26 on the hexon may depend on the formation of a six-fold oligomer. A similar reconstruction to a resolution of 2.7nm also showed this but less information could be obtained on the conformation and nature of the interactions between VP5 and VP26 (Trus *et al.*, 1995). These observations support the calculations of Newcomb *et al.*, (1993) that VP26 forms a 1:1 complex with VP5, although only in hexons.

Contrary to the evidence reported here for the external location of VP26, Thomsen *et al.* (1994), also using recombinant baculoviruses to express the HSV-1 capsid proteins, observed that the absence of VP26 resulted in an increase of the number of free cores and suggested that VP26 interacted with the core so as to stabilise the capsid structure. In light of the work described here (Tatman *et al.*, 1994) and that described pertaining to the external location of VP26, it seems more likely that their observation was a result of poor infection with some of the other recombinant baculoviruses since the large numbers of viruses involved can lead to problems with reduced yield of each individual protein and can also result in cells that fail to be infected with all the recombinant viruses.

Thus, manipulation of the protein constitution of capsids by the baculovirus system has proved valuable in localising the precise position of VP26 in the capsid and in determining its site of interaction with VP5. It is not clear when this protein is added to the capsid structure, although Zhou *et al.* (1995) suggested that VP26 polymerises into hexameric assemblies before attaching to hexameric, but not pentameric arrays of VP5 subunits. It is unlikely that it is added after the basic capsid structure is completed since VP26, which is found distributed throughout the cell when expressed alone (figure 16), has been shown in co-localisation experiments to locate to the nucleus only in the presence of VP5 (F. Rixon, personal communication) and therefore has the intrinsic ability to complex to VP5 prior to capsid formation.

Although the baculovirus system has demonstrated that VP26 is not required for capsid assembly, it is still not known whether this protein is essential for virus growth or what its role in the virus life cycle could be. VP26 has been reported as a potential DNA binding protein which could function in the condensation and/or packaging of the viral genome (McNabb and Courtney 1992b). However, this binding may be of a non-specific nature due to the highly basic properties of this protein. Its external location on the tips of the hexons is more suggestive of a role in forming interactions with tegument or cellular proteins that are important for capsid maturation or transport. Construction of HSV-1 mutants with lesions in this gene should provide more information on the role of VP26.

3.3 INTERACTIONS BETWEEN THE OUTER SHELL PROTEINS

Before the baculovirus system allowed manipulation of individual proteins, it was not possible to determine in such detail the protein interactions that could occur between the individual capsid proteins.

3.3.1 The formation of aberrant capsid shells

Co-infection of cells with AcUL18, AcUL19 and AcUL38 (i.e. omission of all of the scaffold protein genes), resulted in the formation of sheet and spiral structures which appear to represent interwoven, aberrant capsid shells (Tatman *et al.*, 1994; figure 26H). These are presumably formed by extensive self-assembly of the capsid subunits to yield structures larger than capsid shells. Whereas it has been shown via the chemical cross-linking of purified HSV-1 capsids that complexes between VP5, VP19C and VP23 exist (Desai *et al.*, 1994) the baculovirus results have demonstrated that the outer shell proteins VP5, VP23 and VP19C have the intrinsic ability to self assemble in the absence of the scaffolding proteins. However, the aberrant and incomplete appearance of these structures suggests an important role for the scaffold in ensuring that the process is correctly modulated. A similar phenomenon was observed in the presence of VP26 (Thomsen *et al.*, 1994).

Structures of similar nature have been observed for phage P22 where, in infections with mutants in the scaffolding protein, aberrant aggregates of the coat protein were observed (King *et al.*, 1973; Lenk *et al.*, 1975; Earnshaw and King 1978). In addition, purified coat protein formed aberrant shell structures *in vitro* but only when present in higher concentrations than were necessary to form normal proheads in conjunction with the scaffolding protein (Fuller and King, 1981). This demonstrates further parallels between the assembly of HSV capsids and the proheads of this bacteriophage. It could be argued that the aberrant shell structures formed from the HSV-1 shell proteins are a consequence of their overproduction in the baculovirus system, except that similar structures have been observed in cells infected with a UL26/UL26.5 null mutant virus (Desai *et al.*, 1994). Schaffer *et al.* (1974), who also observed similar structures in the nucleus of cells infected with several HSV *ts* mutant viruses, suggested that their appearance was due to a thermal sensitivity of the virions of these mutants at the NPT which resulted in abnormal nucleocapsid assembly and accumulation of aberrant capsid material. It is pertinent to note the absence of these structures following infection with the mutant *ts*G3, which contains a lesion in the UL19 gene (Schaffer *et al.*, 1974) since this is reflected in the baculovirus system. Similar structures were observed as early as 1959 (Morgan *et al.*, 1959) when it was assumed that such particles were in the process of capsid formation. However, close examination of cells at sequential intervals after infection determined that they were only present at the terminal stages of infection

suggesting that they were examples of aberrant capsid differentiation (Nii *et al.*, 1968a, b, c). Now, with the use of the baculovirus system, the presence and composition of these structures can be explained.

3.3.2 70nm dense particles are formed from interactions between VP5 and VP19C

When baculoviruses expressing VP5 and VP19C were co-infected, densely staining particles 70nm in size were observed in thin section (figure 26). Examination of purified preparations of these particles by negative staining (figure 34) determined that many of them had the appearance of complete spherical shells. The capsomers could be clearly observed on the particle surface. Although di-sulphide bond interactions between these proteins has previously been suggested by 2-dimensional gel electrophoresis (Zweig *et al.*, 1979a) and immunofluorescence localisation studies have determined a role for VP19C in localising VP5 to the nucleus (F. Rixon; personal communication), the observation of these particles in the baculovirus system suggests that VP5 and VP19C interact to form self-contained structures. However, they appear to be more fragile than capsids due to the number of broken shell structures that were also observed in these preparations. Unfortunately, since it appears that these structures do not have a centre of symmetry, high resolution determination of their structure is not yet possible by cryo-electron microscopy (W. Chiu; personal communication). These particles were also observed in ^AcUL18/^AcUL35 infections together with arrays of cores (Tatman *et al.*, 1994; figure 23D and 26G). In contrast, Thomsen *et al.* (1994) described them as aberrant capsid shells and concluded that VP5 and VP19C alone were required for the assembly of aberrant capsids and that VP23 and VP26 served to stabilise the structure. However, from the results described in this thesis it appears more likely that VP23 serves to modulate the interaction between VP5 and VP19C, to produce the typical shell structure; perhaps by forcing these proteins into a different conformation. Since VP21 and VP22a did not associate with the VP5 and VP19C structures in the absence of VP23, Thomsen *et al.* (1994) suggested that VP23 either acts directly or indirectly to mediate the interactions of the scaffolding proteins with the outer shell proteins. However in this instance, since it was the processed form of the scaffolding protein that was present, then an interaction would not be expected (discussed below). Furthermore, data from immunofluorescence studies have shown that there is no direct interactions between VP23 and the core proteins (Nicholson *et al.*, 1994).

Unlike the other sub-capsid structures described, there does not appear to be any mention of these particles in natural HSV-1 infection although their presence may have been overlooked. However, due to the parallels observed between the structures formed

from expression of HSV-1 proteins in insect cells and those observed in wt and mutant HSV-1 infection, it is possible that such structures between VP5 and VP19C are formed at some time during capsid assembly.

It has now been suggested as a result of 3-dimensional cryo-electronmicroscopy of purified HSV-1 B capsids, that VP19C complexes with VP23 to form the trimers which contribute to the floor of the capsid (Zhou *et al.*, 1994). However, it appears from immunofluoresence studies that interactions occur between VP5 and VP19C, VP19C and VP23 and between VP5 and pre-VP22a (summarised in figure 25) and suggests that certain domains of these proteins (in paticular VP5) are important in the interaction with the scaffolding proteins. Since the baculovirus system allows not only alteration of the combination of capsid proteins but also manipulation of the individual proteins it provides an ideal system for analysing the interactions which occur between the shell proteins and scaffolding proteins.

3.4 THE STRUCTURAL IMPORTANCE OF THE UL26 AND UL26.5 GENE PRODUCTS

The only two recombinant baculoviruses which produced visible strucutures in electron micrographs of infected cells were AcUL26 and AcUL26.5 (figure 22). Expression of the UL26.5 gene product, pre-VP22a revealed a range of forms from fibrous material to scaffold-like particles, with structures intermediate between these extremes predominating. By contrast AcUL26 formed only large aggregates of fibrous material (Preston *et al.*, 1994).

The different forms of the UL26 and UL26.5 proteins observed in the baculovirus system might help explain the observations of Miyamoto (1971) who described similar filamentous material and aggregates of granules of relatively uniform size (40nm) and shape in HSV-1-infected cells. The dense amorphous material, shown to have viral antigenicity (Nii *et al.*, 1968c), appeared to be closely related to the formation of the granules since the material was frequently localised near the granular aggregates. Miyamoto (1971) suggested that each of these granules represented a subunit and that several adjoining subunits constituted the requirement for formation of a single capsid core.

3.4.1 Core Structures

40-60nm ring-like structures, resembling the internal scaffold or core found in B capsids were found regularly as a consequence of co-infections of AcUL26 and AcUL26.5 (figure 22). It has been confirmed, using an antibody to VP22a that free core structures are comprised of either VP22a and/or VP21 (Preston *et al.*, 1994). These structures

were occasionally observed in cells infected with all 6 recombinant baculoviruses (figure 21) and were also found in the cytoplasm, presumably as a result of overproduction in the cell (figure 23). Since there have been a number of reports of such structures occurring as part of normal HSV-1 and HSV-2 infections (Schwartz and Roizman, 1969; Nii *et al.*, 1968a; Atkinson *et al.*, 1978) this again demonstrates that the baculovirus system mimics processes occuring during HSV-1 infection.

Core structures were also observed in infections in which AcUL18, AcUL19 or AcUL38 were omitted. This suggests that the presence of the remaining outer shell capsid proteins does not hinder formation of free cores. As has been mentioned previously the baculovirus system reflects the situation that occurs with some *ts* mutants. Rings 25nm in diameter which have also been suggested to represent free cores have been described as a feature of cells infected with certain UL19 *ts* mutants of HSV-1 which are defective for capsid assembly (Schaffer *et al.*, 1974; Celluzzi and Farber, 1990). It is possible that their smaller size reflects the method of preparation and quality of microscopy. Localisation of VP5 at the PT by immunoflouresence corresponded with areas in the nucleus where these ring-like structures were most commonly detected at the NPT and suggested that the loss of functional VP5 may result in their aberrant accumulation (Celluzzi and Farber, 1990). It is surprising that no similar structures have been described for mutants in UL18 and UL38 which also fail to assemble capsids (Pertuiset *et al.*, 1989; Desai *et al.*, 1993). The ability of core structures to exist as discrete entities has been shown by Newcomb and Brown (1991), who prepared VP22a from purified HSV-1 B capsids that were extracted with 2M GuHCl *in vitro*. This treatment resulted in the removal the core and pentons as well as the VP22a and VP26 proteins. Subsequent dialysis to remove the GuHCl enabled the core structures to be separated by centrifugation, thus demonstrating the ability of VP22a to self-assemble into 60nm spheres resembling the B capsid core from which the protein was derived. The baculovirus system provides the confirmation as to the composition of the structures that have been observed in HSV-1 infection. Unfortunately it has not yet proved possible to purify these baculovirus-derived cores by centrifugation through a sucrose gradient, although Western blot analysis (figure 31) suggests the presence of VP22a near to the top of the gradient, providing further evidence that these cores do exist as discrete particles.

The ability of the core proteins of HSV-1 to self assemble demonstrates parallels with the assembly of the scaffold of bacteriophage T4 and this is discussed in further detail in section 4.2.

3.4.2 UL26-Recombinant Capsids (^AcUL26/^AcUL35)

When AcUL26 was omitted from the infections, capsids with a large core phenotype were observed which resembled those observed in cells infected with the HSV-1 mutant *ts*1201 at the NPT (Tatman *et al.* 1994). The *ts*1201 lesion maps within UL26, at aa 30 which is in the region encoding VP24, and results in a virus defective in serine protease activity (Preston *et al.*, 1983; 1992). Thus the protease is unable to cleave itself to release VP21 and VP24 and to release the C-terminal 25aa from itself and from pre-VP22a (Liu and Roizman, 1991b; 1992; Preston *et al.*, 1992). The baculovirus system effectively mimics this situation since, in the absence of AcUL26, the capsids contain only unprocessed pre-VP22a and display the same phenotype of large capsid cores. The subsequent construction of a inactive protease expressed in the presence of the other capsid proteins in the baculovirus system produced a similar result (Thomsen *et al.*, 1995). This supports the conclusion that the large cored phenotype is due to the presence of unprocessed pre-VP22a. The baculovirus system provides additional information to that from the study of *ts*1201 since it demonstrates that capsids can be made in the complete absence of the UL26 proteins. A similar result has since been obtained with a HSV-1 UL26 null mutant (*m*100) in non-complementing cell lines (Gao *et al.*, 1994). As with *ts*1201, this virus failed to produce infectious progeny demonstrating that functional UL26 is essential for viral growth. In both these mutants, viral DNA replication proceeded at normal levels but was not processed to unit length or encapsidated.

3.4.3 Structures formed in the absence of AcUL26.5

When baculoviruses expressing the capsid proteins with the exception of AcUL26.5 (Thomsen *et al.*, 1994) and of AcUL26.5 plus AcUL35 (Tatman *et al.*, 1994) were co-infected (figure 23), a small number of apparently intact capsids were obtained although the majority of the structures resembled the aberrant shells formed from VP5, VP19C and VP23. The intact capsids were similar in appearance to the type A (empty) capsids made in HSV-1 infection which have been described as an abortive product of DNA packaging (see Rixon *et al.*, 1988). Thus the addition of AcUL26 to the three main capsid proteins appears to guide their polymerisation into closed capsid shells. Similar structures were observed in cells infected with a null UL26.5 mutant virus (Matusick-Kumar *et al.*, 1994). Small amounts of progeny virus were produced which reflects the ability of this mutant virus to cleave and package DNA into the few complete capsids that were formed. These results are surprising in view of the fact that VP22a is a major component of B capsids and with its presumed function as a scaffolding protein it would be expected to be essential for capsid assembly. Presumably this reflects the ability of the protease to cleave itself into N-terminal and C-terminal portions, the latter of which

(VP21) comprises the entire amino acid sequences of VP22a with a short N-terminal extension. The most likely explanation is that pre-VP21 can substitute for pre-VP22a during capsid assembly. This has been demonstrated directly using the baculovirus system (V. Preston; personal communication). Thomsen *et al.* (1995) suggested that since there is a lack of an inner core in these capsids then pre-VP21 serves as a scaffolding protein in a different way than does pre-VP22a. They suggested that the scaffold was either difficult to distinguish from the capsid shell or would have a more cage-like structure rather than that of a solid sphere which would also make it difficult to visualise. It is possible that the extra 59 N-terminal amino acids found on VP21 compared with VP22a sterically hinder it from forming an inner core. The substitution appears to be inefficient since the majority of the structures formed in the absence of pre-VP22a under these conditions resembled partial and distorted capsid shells. The C-terminal 25aa of pre-VP21 and pre-VP22a have recently been shown to be essential for interaction with VP5 (Hong *et al.*, 1996) and for capsid assembly (Gao *et al.*, 1994; Kennard *et al.*, 1995; Thomsen *et al.*, 1995). The inefficiency of capsid assembly in the absence of pre-VP22a may reflect rapid self-cleavage of the protease and removal the C-terminal 25aa from pre-VP21 before they can interact with VP5, or alternatively, it may be a consequence of the smaller amounts of VP21 that is made.

3.5 INTERACTIONS BETWEEN THE SHELL AND CORE PROTEINS

An area of intense interest is the interactions that occur between the outer shell proteins and the scaffold proteins since, as has been discussed previously, both are required for the formation of the capsid. Two candidates for the proteins providing the interaction between the scaffold and shell are pre-VP22a and VP5. Interactions between these proteins are known to play an important role in capsid assembly as it has been determined that pre-VP22a is involved in the transport of VP5 to the cell nucleus (Nicholson *et al.*, 1994; Matusick-Kumar *et al.*, 1994). It also appears that the interaction between VP5 and pre-VP22a is important for the correct conformation of the hexons. In cells infected with a UL26.5 null mutant HSV-1 virus, which produces a few capsid particles, a monoclonal antibody specific for the hexon form of VP5 did not recognise the VP5 protein suggesting that in the absence of the UL26.5 product, VP5 was folded in a different conformation (Matusick-Kumar *et al.*, 1994).

The baculovirus system has again proved invaluable in identifying such interactions. When cells were infected with AcUL19 and AcUL26.5, indistinct structures of approximately 40nm in diameter were observed (Kennard *et al.*, 1995; figure 26A and B). These appear different from cores and have indistinct boundaries. In some

preparations they appeared to consist of an inner ring with external projections. In all these infections, there was a noticeable absence of the fibrillar aggregates observed in infections of AcUL26.5 alone suggesting that VP5 was preventing aggregation of pre-VP22a. This supports the idea that the 40nm particles represent VP5 bound to pre-VP22a. These particles have not been definitely observed in HSV-1 infections. Particles which may resemble them, described in lysates prepared from HEp-2 cells infected with the MPdk- strain of HSV-1 (Spring *et al.*, 1968), were round bodied, 20-25nm in diameter and impervious to negative stain. They appeared to be surrounded by a beaded structure 5-8nm in width. In view of the parallels discussed previously between the structures formed as a result of HSV-1 (wt or *ts*) infection and those formed in the baculovirus system, it is possible that these pre-VP22a/VP5 particles may represent a structure that is transiently formed during capsid assembly.

Manipulation of the proteins using the baculovirus system has also allowed identification of the sites of interaction between these proteins. In infections in which the protease was present (^AcUL18/^AcUL35; figure 23D) cores and 70nm particles were formed suggesting that the action of the protease in removing the C-terminal 25aa prevented pre-VP22a and VP5 interacting thus leaving VP5 free to interact with VP19C. When recombinant viruses expressing a truncated form of pre-VP22a equivalent to VP22a (AcVP22a) and VP5 were coinfected the 40nm particles were not formed but free cores were readily observed. Furthermore, co-infection of AcVP5, AcVP19C, AcVP23 and AcVP22a did not result in capsid formation but gave rise to core arrays and aberrant capsid shells (Kennard *et al.*, 1995). Thus, VP22a is unable to interact with either VP5 or the other shell proteins. The lack of capsids suggests that the scaffold-like particles produced by VP22a in the presence or absence of shell proteins, were abortive products rather than intermediates of capsid assembly. Using an antibody to VP5, which has also been shown to co-precipitate pre-VP22a from insect cells co-infected with baculoviruses expressing these two proteins, Thomsen *et al.* (1995) also determined that this interaction was dependent upon the presence of the C-terminal 25aa. Expression of pre-VP22a as a fusion protein in bacteria has enabled the interaction of the C-terminal 25aa with VP5 to be defined. By way of deletion and substitutional analysis Hong *et al.* (1996) mapped the interaction to a minimal 12 aa and suggested that the interaction is a hydrophobic one. Since attempts to locate the cleaved 25aa in the mature virion have failed it is not known whether they are expelled from the capsid along with the scaffolding proteins or are retained. A second site of interaction between VP5 and VP22a has been described which is important in the transport of VP5 to the nucleus (Kennard *et al.*, 1995) and therefore, the interactions between these two proteins is obviously a complex one. Hopefully, further manipulation of the individual proteins will allow elucidation of this interaction.

3.6 THE IMPORTANCE OF THE PROTEASE

It is apparent that the protease plays a very pivotal role in capsid assembly. Its mechanism of interaction is particularly interesting since its elucidation could lead to the design of inhibitors to prevent production of infectious virions. It had previously been suggested that the unusual structure of the UL26 protein, which shares its C-terminal sequences with UL26.5 but has unique N-terminal sequences, would allow it to form the connection between the scaffold and outer shell (Rixon, 1993). However, it is clear from the present experiments that interactions between the scaffold and outer shell do not depend on the presence of the unique N-terminal sequences (Gao *et al.*, 1994; Tatman *et al.*, 1994; Thomsen *et al.*, 1994). Although it is known that cleavage causes an alteration of core size and is necessary for the acquisition of DNA (Preston *et al.*, 1983) it is still not apparent when these cleavages occur. Since the UL26 protein is not a recognised component of capsids, unlike VP24 and VP21, then release must occur before capsid isolation. Since assembly cannot take place after the carboxy terminal processing of pre-VP22a, this proteolytic event must occur subsequent to assembly. This leaves us to wonder what roles VP21 and VP24 serve.

3.6.1 The function of VP24

The exact role of the self-cleavages within the protease is not clear although it is possible that the autoprocessing at the N-terminal site may serve to release the catalytic domain and enable it to exert its function during capsid formation. Alternatively, VP24 could play a structural role in addition to having enzymic activity since VP24 lies within the cavity of the mature capsid. In addition it has been shown that the release of VP24 from the UL26 gene product is necessary for viral growth and that processing of pre-VP22a alone is not sufficient to support viral replication (Matusick-Kumar *et al.*, 1995b). A very plausable explanation for the role for this N-terminal cleavage is that it might serve to ensure that VP21 becomes free so as to allow its removal along with VP22a, from the capsid during packaging of the DNA.

3.6.2 The function of VP21

It has been suggested that one function of pre-VP21 is to interact with the other capsid proteins during the formation of capsids. As it may have a conformation different from that of pre-VP22a prior to its release from the full-length protease, as suggested by Gao *et al.* (1994), then it could interact with the capsid proteins in a different manner to the way in which pre-VP22a interacts and as such would lend an important role to this protein. However, in light of the observation of capsid formation in the complete

absence of UL26, it seems that VP21 is unnecessary for the assembly of the basic capsid structure (Gao *et al.*, 1994; Tatman *et al.*, 1994; Thomsen *et al.*, 1994). Self-cleavage at the C-terminus might only occur because it shares sites with the UL26.5 gene product rather than having a separate biological function. Using plasmids expressing either pre-VP22a, VP24, or protease, Gao *et al.* (1994) demonstrated that VP24 on its own was unable to locate to the nucleus unlike either pre-VP22a or the full-length protease. This suggested that the function of the VP21 domain of the protease was to direct the catalytic domain, VP24 to the nucleus. The product of UL26 was also found in the nucleus in single infection by baculovirus expressing this gene (Preston *et al.*, 1994). This would suggest that the N-terminal release site of the protease is protected from autoproteolysis when synthesised in the cytoplasm or that VP24 and VP21 remain associated after cleavage. It could be either rapidly transported into the nucleus prior to its autoproteolysis or perhaps it is protected by the formation of a complex between the protease and a cellular protein. It is unlikely that another viral protein is involved since the protease is the only viral protein present in the transfection system or baculovirus system (Gao *et al.*, 1994; Preston *et al.*, 1994). A third possibility is that it is held in a different, inactive, conformation in the cytoplasm.

3.6.3 The C-terminal 25 amino acids

One of the advantages of the baculovirus system is to allow manipulation of individual capsid genes and to determine the effect that they have on capsid assembly. As described above, construction of a recombinant baculovirus expressing VP22a (i.e. a truncated form of the UL26.5 protein lacking the carboxy terminal 25 aa), resulted in an absence of capsids demonstrating that VP22a is unable to participate in capsid assembly. However, if the N-terminal cleavage site of the protease was made inactive but cleavage of the C-terminal 25aa remained unaffected, then capsids were formed (Kennard *et al.*, 1995; Thomsen *et al.*, 1995). These observations are consistent with the proposal that cleavage of pre-VP22a occurs during or after capsid assembly or when pre-VP22a is in a complex with one or more capsid shell proteins. Construction of a HSV-1 mutant virus expressing a C-terminal 25aa truncated form of the protease also determined that these terminal amino acids were essential for viral growth (Matusick-Kumar *et al.*, 1995a). *Trans*-complementation experiments determined that pre-VP22a, but not VP22a was able to complement the growth of this mutant virus. Together these results demonstrated that growth of HSV-1 requires the C-terminal 25aa of either the protease or its substrate, pre-VP22a.

Studies, using the baculovirus system, on the kinetics of cleavage by the protease have determined that the C-terminal 25aa were cleaved rapidly from both the full length protease and from pre-VP22a being removed when the internal cleavage of the protease

had not occurred (Thomsen *et al.*, 1995). C-terminal cleavage by unattached VP24 was much slower, and this suggests that in line with the observation by Gao *et al.* (1994) that the protease is transported to the nucleus in its full length form allowing cleavage of the 25aa to occur once the capsid is formed. It also suggests that *trans*-cleavage of pre-VP22a should be slower as it is not attached to VP24. When the protease was expressed in bacteria it was found that it was processed more rapidly especially at the N-terminal cleavage site, in the presence of the UL26.5 product than in its absence (Deckman *et al.*, 1992). These results suggest that the protease and pre-VP22a function together to regulate the kinetics of cleavage and to allow optimum capsid formation. However, to gain a comprehensive understanding of the exact role of the protease a more detailed analysis is required. It is hoped that the baculovirus system will provide a means for this.

4 SIMILARITIES BETWEEN THE ASSEMBLY OF BACTERIOPHAGE PROHEADS AND OF THE HSV-1 CAPSID

Our understanding of the mechanism of herpes virus capsid assembly has been enhanced by comparison with the assembly of various bacteriophages. Most of what is known about double stranded bacteriophage assembly has evolved from analysis of gene mutations and from examination of the products of in *vitro* assembly of purified proteins and phage subunits.

As has been mentioned previously, there are several similarites between the assembly of the bacteriophage head and of the HSV-1 capsid. Formation of the phage head into an icosahedron procedes via a head that contains a protein core. This structure, termed the prohead, resembles the HSV-1 type B capsid; it is built as a double shelled structure comprising an outer shell composed of coat (or shell) subunits and an inner shell composed of scaffolding or assembly proteins. Also in a manner similar to the HSV-1 B capsid, the majority of the scaffolding proteins leave the prohead concomitant with the packaging of the DNA. In the case of the *Salmonella typhimurium* bacteriophage P22 (reviewed by Georgopoulos *et al.*, 1983), the scaffolding proteins exit intact and are recycled (King *et al.*, 1973; King and Casjens, 1974) while in *E. coli* bacteriophage T4 (reviewed by Black and Showe, 1983) the scaffolding proteins are cleaved by a phage-encoded protease and are not reused (Laemmli, 1970). Other similarities exist: like HSV, DNA is packaged into pre-formed capsids and is cleaved into concatemeric units (Frenkel, 1966) and other auxiliary proteins, such as the packaging proteins of HSV-1, are required for the packaging of the DNA (King *et al.* 1973). Thus from comparison of the mechanisms of assembly of each of P22 and T4, it appears that HSV-1 assembly

shares processes in common with either or both of these phages. However, there are limitations on the correlations which can be drawn between the different systems. For example, entry of DNA into the head of phage P22 results in a 15% linear expansion of the head (Earnshaw *et al.*, 1976). Such expansion does not appear to be a feature of the HSV capsid. Also, HSV capsids do not have a tail, or as far as is known, portal proteins.

4.1 BACTERIOPHAGE P22

The P22 outer shell is composed of 420 molecules of the viral coat protein gene product 5 (gp5) and the inner shell is composed of 200 molecules of a 42kDa scaffolding protein gp8. The initial assembly of the shell proteins into the prohead depends on the presence of the scaffolding protein (Earnshaw and King, 1978). 4 minor proteins are also found in P22 procapsids: gp1, gp7, gp16, gp20. The latter 3 are not essential for prohead assembly since if they are removed by mutation, morphologically normal proheads are produced. These mutants are non-infectious due to the inability of the phage to inject their DNA into the bacterium at a later stage in the life cycle (Botstein *et al.*, 1973; Poteete and King, 1977). The fourth of the minor proteins, gp1 is also not essential for prohead assembly although it is required for encapsidation of DNA (King *et al.* 1973). It has been suggested that this protein is located at the prohead vertex through which the DNA is packaged and that it plays a role in recognition of DNA during packaging (Fuller and King, 1981). The products of the genes 2 and 3 are also required for packaging of DNA into the prohead.

Thus only 2 proteins, gp5 and gp8 are required to assemble P22 closed double shells although other proteins are required to be incorporated if these shells are to become infectious particles. In this respect the dispensability for prohead assembly of these other proteins resembles the situation with the HSV-1 UL6, UL15, UL25, UL28, UL32 and UL33 gene products which are non-essential for capsid assembly but which are required for DNA packaging (Addison *et al.*, 1984; 1990; Sherman and Bachenheimer, 1988; Al-Kobaisi *et al.*, 1991; Poon and Roizman, 1993; Tengelson *et al.*, 1993; Baines *et al.*, 1994; Patel *et al.*, 1996). In addition, the UL6 protein has been shown to be present in preparations of purified wt HSV-1 capsids (Patel and Maclean, 1995).

It is possible to isolate soluble forms of the core and coat subunits of P22 by treatment of proheads with GuHCl (Fuller and King, 1981) and it has been suggested that this treatment mimics the release of the scaffolding protein which occurs *in vivo* in that the scaffolding subunits exit through holes at the two fold axis of the coat protein lattice, which remains intact. The net result of this procedure leads to the purification of empty shells and soluble subunits of the scaffolding protein. A similar method was utilised by

(Newcomb and Brown, 1991) to remove cores from HSV-1 B capsids. The importance of the scaffolding protein in the P22 assembly process has been shown by the examination of mutants in this gene which demonstrate that in the absence of the scaffolding protein the coat proteins are unable to assemble efficiently and accumulate as aberrant aggregates (King *et al.*, 1973; Lenk *et al.*, 1975; Earnshaw and King, 1978). Since the coat protein aggregates appear similar to the HSV-1 aberrant shells that are produced from the copolymerisation of 3 of the shell proteins; VP5, VP19C and VP23 in the baculovirus system (Tatman *et al.*, 1994; Thomsen *et al.*, 1994) it appears that, at least in this aspect, the mode of assembly of HSV-1 capsids resembles that of the P22 prohead. Hence, the scaffolding proteins of both P22 and HSV serve to regulate the precision of coat protein assembly. However, there is no evidence of assembly of the P22 scaffolding protein into discrete core structures in the absence of the coat protein and the P22 scaffolding protein remains as soluble subunits (Fuller and King, 1981). This is in contrast to the situation in HSV-1, where cores are regularly observed in the absence of either one or all of VP5, VP19C or VP23 in baculovirus (Tatman *et al.*, 1994; Thomsen *et al.*, 1994) and in HSV-1 wt and *ts* mutant infections (Schaffer *et al.*, 1974; Celluzzi and Farber, 1990). Therefore, unlike the situation in HSV, P22 scaffolding proteins require the presence of the shell proteins to assemble into scaffolds. It has been suggested that P22 gp8 subunits autogenously repress their own synthesis since scaffolding subunits are present only in low levels in cells infected with coat protein mutants (King *et al.*, 1978). This does not appear to be the case for HSV-1 since in cells infected with mutants in either VP5, VP19C or VP23, (Weller *et al.*, 1987; Pertuiset *et al.*, 1989; Desai *et al.*, 1993) wt levels of the scaffolding proteins are produced.

P22 proheads can be assembled *in vitro* from purified coat and scaffold subunits Since under similar conditions, neither scaffolding subunits nor coat proteins could polymerise independently of each other (King and Fuller, 1982), these results suggest that the procapsid is built by copolymerisation of the 2 protein species; gp5 and gp8, rather than initial assembly of the core and subsequent addition of the shell proteins. The proheads formed in this way are competent for DNA packaging. In a system combining purified gp5 and gp8 together with mutant-infected cell extracts lacking procapsids but competent for DNA packaging *in vitro*, the proheads that are formed from the constituent proteins can be filled with DNA and converted into viable phage (Poteete *et al.*, 1979; Poteete and Botstein, 1979; Fuller and King, 1982). Biologically active proheads of phage lambda can also be formed in a similar way (Murialdo and Becker, 1977; 1978).

Although the situation in HSV-1 is somewhat different, in that discrete cores do form in wt, mutant and recombinant baculovirus infections, it has been shown from the

mutagenesis of scaffolding proteins that these cores only form from the processed form of these proteins which are incompetent for capsid assembly (Kennard *et al*, 1995). The inportance of the interactions of VP5 with pre-VP22a and the structures observed in baculovirus-infected cells as a result suggest that assembly of HSV-1 B capsids occurs via copolymerisation a similar way to the assembly of P22 proheads.

4.2 BACTERIOPHAGE T4

The constitution of the T4 prohead is more complex than that of P22 comprising at least 11 proteins, 6 of which are essential for prohead assembly (Traub and Maeder, 1984; Black and Showe, 1983). The main shell component is the gp23 protein which forms hexamers except at the 12 icosahedral vertices. The 11 distal vertices are composed of pentamers of gp24 whereas the proximal vertex is composed of a ring of gp20 which is required for shell initiation; a process which is aided by the gp40 protein (Onatario *et al.*, 1978; Van Driel and Couture, 1978a). An auxiliary shell protein gp31 is required to aid the assembly of gp23 into shell structures (Castillo and Black, 1978). The regulation of assembly of the shell protein by gp31 resembles the situation with HSV-1 where VP23 has a noticeable effect in modulating the interactions occurring between the other two shell proteins VP5 and VP19C (figure 26).

The scaffolding core is formed from one major and seven minor proteins (Taub *et al.*, 1984). The major core protein which appears to be similar in function to the HSV-1 scaffolding protein is termed gp22 and during maturation is proteolytically cleaved by the gp21 protease (Showe *et al.*, 1976a ;1976b). Three other internal proteins (IP) which are found in the core are IPI, IPII and IPIII and are dispensable for assembly. The protein gp67 is essential for shell head assembly and has been suggested to have an important role in core construction (Traub *et al.*, 1984). Unlike the situation in bacteriophage P22, the scaffolding proteins can self-assemble into structures similar to the internal shell of the procapsid. However, the proximal vertex protein, gp20 is required to direct the assembly of core proteins into the core structure (Van Driel and Couture, 1978b). Naked core structures are formed in infections with mutants where either the production or assembly of the shell protein does not occur (Traub and Maeder, 1984; Traub *et al.*, 1984). Of the internal proteins, only gp21 and gp22 are essential for *in vivo* core formation. This indicates a marked similarity to the interactions of the UL26 and UL26.5 core proteins in HSV-1 (Preston *et al.*, 1994; figure 22) since both these cores are composed of a product of the major scaffolding protein gene and of a protease gene. The T4 protease plays an important role in assembly since in gp21 mutant infections, proheads are formed which contain an organised but incomplete core (Traub *et al.*, 1984). It is possible that this may represent the T4 equivalent to HSV-1

large cored capsids formed in the absence of a functional protease (Preston *et al.*, 1983; 1992; Tatman *et al.*, 1994). Another similarity between the T4 and HSV-1 scaffolding proteins is that they appear to have two separate domains that either interact with the major shell protein or are required for core formation. For example, a near full length amber mutation of gp22 lost its ability to interact with gp23 although it retained the ability to assemble the core (Traub and Maeder, 1984). The missing sequences might therefore play a similar role to the C-terminal 25aa of pre-VP22a which are required to interact with VP5 (figure 26 , Kennard *et al.*, 1995; Hong *et al.*, 1996).

Kuhn *et al.*, (1987), using a strain of *E. coli* in which head assembly was inhibited at low temperatures, determined that prohead assembly could be achieved *in vitro* by mixing naked cores with shell proteins. Therefore it appears that, since cores can act as intermediates in the prohead assembly pathway, it is likely that the process of T4 prohead assembly differs substantially from the copolymerisation required in P22. These researchers also demonstrated that gp20 must be attached to the prominant neck structure seen at the vertex of naked cores in order to direct the correct folding of gp23 and that subsequent shell assembly requires contact of both gp23 and gp20 with gp22 during or after its integration into the assembling core structure.

Maturation of the prohead, which involves the removal of the core proteins, appears to operate by a similar mechanism in T4 as in HSV-1 since the core proteins are either partially or totally degraded and the degradation products of some core proteins remain in the head (Showe *et al.*, 1976a ;1976b). This resembles the situation in HSV-1 where VP24 (and possibly the C-terminal 25 aa which are cleaved from pre-VP22a and pre-VP21 during processing by the protease) are retained in the mature capsid.

5 A MODEL FOR HSV-1 CAPSID ASSEMBLY

The two bacteriophages P22 and T4 differ in their mode of assembly. In T4, core assembly is independent of shell assembly (Traub and Maeder, 1984), whereas assembly of P22 proheads takes place via a copolymerisation process (Fuller and King, 1982). It is clear that in many cases these phage systems provide good models for HSV capsid assembly although there are sufficient differences to suggest that extrapolation of mechanisms should be treated cautiously. It appears that assembly of mature HSV-1 capsids shares parts of both these processes; the initial assembly of the type B capsid occurring by a process of copolymerisation similar to P22 and the mechanism for the subsequent removal of core proteins resembling T4. From consideration of the effect of HSV-1 mutants on assembly of the capsid and from the understanding of protein interactions and the assembly of sub-capsid structures formed in the baculovirus system, it is possible to draw the following conclusions and hence propose the model of HSV-1

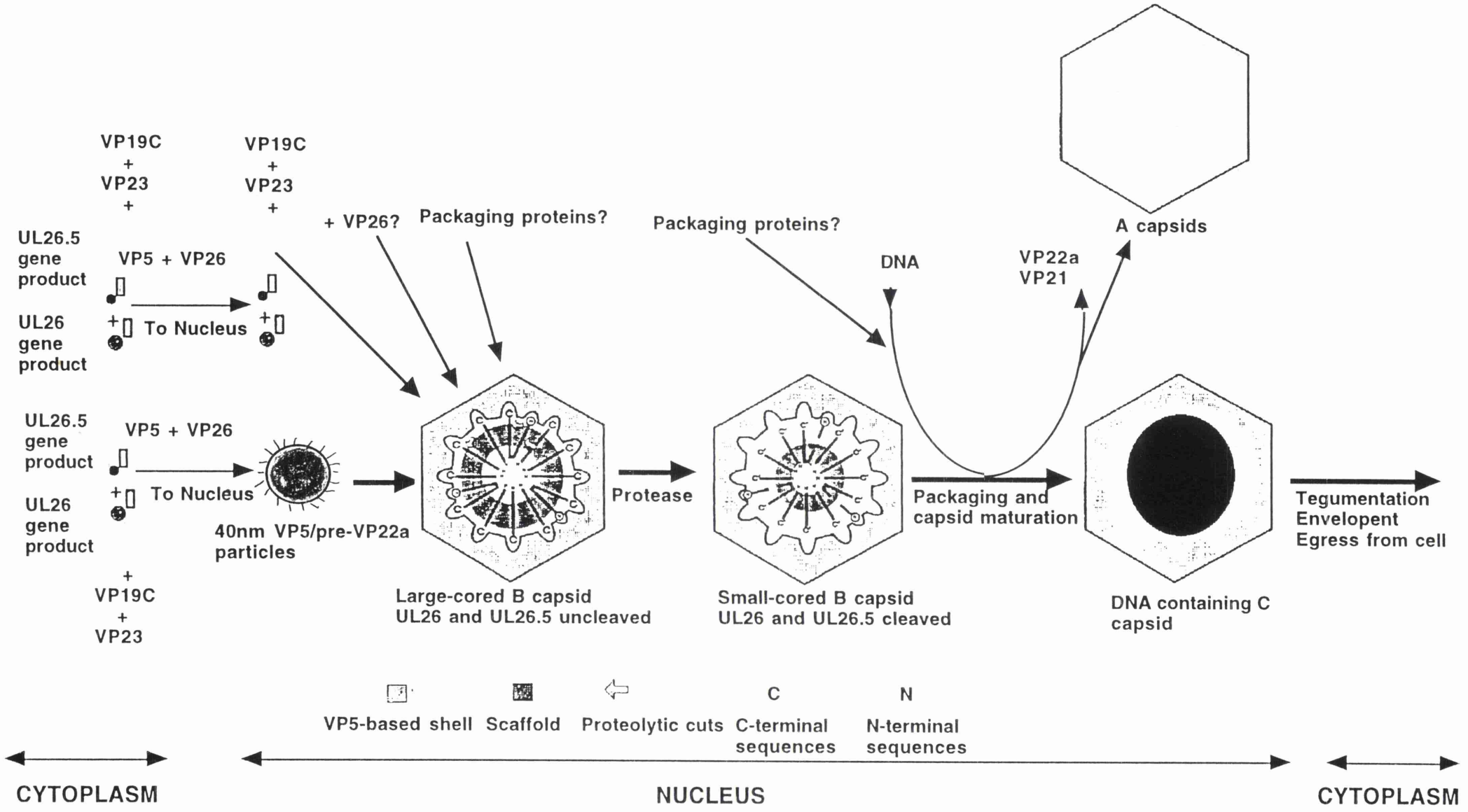

VP19C
+
VP23
+
VP19C
+
VP23
+
A capsids
UL26.5
gene
product
VP5 + VP26
UL26
gene
product
To Nucleus
+ VP26?
Packaging proteins?
Packaging proteins?
DNA
VP22a
VP21
UL26.5
gene
product
VP5 + VP26
UL26
gene
product
To Nucleus
40nm VP5/pre-VP22a
particles
Protease
Packaging and
capsid maturation
Tegumentation
Envelopent
Egress from cell
+
VP19C
+
VP23
Large-cored B capsid
UL26 and UL26.5 uncleaved
Small-cored B capsid
UL26 and UL26.5 cleaved
DNA containing C
capsid
VP5-based shell
Scaffold
Proteolytic cuts
C
C-terminal
sequences
N
N-terminal
sequences
CYTOPLASM
NUCLEUS
CYTOPLASM

Figure 38:-Pathway for HSV-1 capsid assembly

The assembly pathway is adapted from Thomsen *et al.* (1995) and is based on the results presented in this thesis and in other relevant studies. The proposed pathway suggests that VP5 associates in the cytoplasm with uncleaved forms of the UL26.5 and UL26 proteins in order to be transported to the nucleus. VP26 may also be transported to the nucleus by the complex of VP5 and scaffold proteins. VP23 is transported by VP19C. 40nm particles composed of VP5 and pre-VP22a may act as short-lived intermediates in capsid assembly to which the other capsid proteins attach to form the basic capsid structure. Alternatively, the capsid may be assembled by co-condensation of the capsid proteins. The interaction between the UL26 and UL26.5 proteins and VP5 would be via the common C-terminal 25 amino acids of the two scaffold proteins. As depicted here, the UL26 protein may also interact through its unique N-terminal sequences. Proteolytic cleavage at the C-terminal maturation site in both the UL26 and UL26.5 proteins and at the internal release site in the UL26 protein generates the forms of these proteins, VP21, VP24 and VP22a, found in small cored capsids. At some point concomitant with this cleavage event, VP21 and VP22a exit from the capsid, DNA is packaged, possibly through the pentons, to form the type C capsids. Several other HSV-1 proteins participate in the packaging event. On leaving the nucleus C capsids become tegumented, acquire an envelope and egress from the cell as infectious virions.

capsid assembly shown in figure 38, which is based on the model suggested by Thomsen *et al.*, 1995).

1. In light of the results from the studies of the *ts*1201 mutant which forms large cored capsids at the NPT and on downshift to the PT is able to package DNA and mature into infectious virions (Preston *et al.*, 1983) and with the knowledge that VP22a is unable to form capsids or to interact with VP5 (Kennard *et al.*, 1995; Thomsen *et al.*, 1995; Hong *et al.*, 1976) it is almost certain that large cored capsids represent an earlier stage in capsid assembly than do small cored B capsids. Therefore it follows that the processing of the UL26 and UL26.5 proteins occurs subsequent to such capsid assembly.

2. It seems likely that the initial interactions between the capsid proteins occur in the cytoplasm since it has been shown by co-localisation experiments that pre-VP22a can relocate VP5 into the nucleus (Matusick-Kumar *et al.*, 1994; Nicholson *et al.*, 1994; Kennard *et al.*, 1995). VP5 may also relocate VP26 (F. Rixon; personal communication). Since VP19C is the only protein which has been demonstrated to relocate VP23 (F. Rixon; personal communication) into the nucleus, then a VP23/VP19C interaction may dominate the VP5/VP19C interaction thus leaving VP5 free to interact with pre-VP22a.

3. After transport of the capsid proteins to the nucleus, pre-VP22a/VP5 complexes come together with UL26 protein/VP5 complexes and form the basis of a template on which the other capsid proteins assemble, possibly via a similar mechanism of copolymerisation to that described above for the P22 prohead. It is possible that the pre-VP22a/VP5 complex could be in the form of the 40nm particles observed with combinations of these baculovirus-expressed proteins (Kennard *et al.*, 1995). The interactions of the scaffolding proteins and shell proteins would take place via the C-terminal 25aa although the N-terminal part of VP24 may also help form the link between the scaffold and outer shell (Rixon, 1993). Clearly the activity of the protease needs to be inhibited to permit these complexes to form. The mechanism by which this inhibition is achieved is not known. It is unlikely that the interaction of VP5 with pre-VP22a or the protease serves to prevent cleavage of the latter into VP22a since this cleavage occurred in the presence of both these proteins and of the protease. Conformational changes associated with assembly of capsid structures may subsequently serve to activate the protease, so that it is adjacent to the C-terminal cleavage site of pre-VP22a and pre-VP21. Work with *ts*1201 (a reversible mutant) has shown that large cored capsids are competent for DNA packaging. Since other *ts* mutants which form small cored capsids but do not package DNA do not appear to be reversible upon temperature downshift, it is possible that small cored capsids may not be the precursors of mature capsids.

4. It is probable that the free core structures that have been observed both in HSV wt- and VP5 mutant-infected cells and from co-infections of AcUL26 and AcUL26.5 do not represent an intermediate of the assembly pathway. Free cores, like the aberrant shells, are formed when there is an imbalance of the capsid proteins and hence, like the type A capsids may represent an abortive product of capsid assembly (Friedman *et al.*, 1975; Lee *et al.*, 1988; Rixon *et al.*, 1988; Sherman and Bachenheimer, 1988; Rixon, 1993). Such 'side' products of capsid assembly may be formed from any surplus proteins so as to stop them interfering with the completed capsids.

5. At some point, possibly concomitant with the cleavage and exit of the scaffolding proteins, DNA enters the capsid. The larger size of the pentameric channels compared to the hexameric channels led to the suggestion that DNA enters through one of the former leaving the other 11 pentameric (or the hexameric) channels as the portal of exit for the scaffolding proteins (Zhou *et al.*, 1994). In support of the proposal that DNA can traverse penton channels, is the observation that DNA, observed from electron micrographs of freeze-dried type C capsids, appeared to extrude from the vertices of the capsid following treatment with low concentrations of denaturant *in vitro* (Newcomb and Brown, 1994). This raises the question of whether there is a unique vertex for packaging of DNA or whether any of the twelve penton channels are capable of allowing entry of DNA. As yet there is no evidence of a unique HSV-1 vertex. Several HSV-1 proteins have been determined as having a role in DNA packaging (Sherman and Bachenheimer, 1988; Addison *et al.*, 1984; 1990; Al-Kobaisi *et al.*, 1991; Poon and Roizman, 1993; Tengelson *et al.*, 1993; Baines *et al.*, 1994; Patel *et al.*, 1996) and one of these, the UL6 protein, has been shown to complex with capsids (Patel and Maclean, 1995).

6. Following packaging, mature capsids leave the nucleus, becoming tegumented and enveloped during the process, and egress from the cell. The location of VP26 on the outside of the capsid suggests that this protein may serve to interact with tegument or nuclear membrane proteins during the later stages of capsid morphogenesis.

At the present time it is not possible to study packaging of DNA into HSV-1 capsids using the baculovirus system due to the lack of HSV-1 DNA and packaging proteins. However, with a DNA replication system already existing in insect cell (Dodson *et al.*, 1989; Stow, 1992) and from the construction of baculovirus expression vectors with the capacity for the expression of multiple recombinant proteins (Belyaev and Roy, 1993), it may prove possible in the future to achieve packaging in the baculovirus system as well as to study interactions of other structural proteins with the capsid proteins.

6 CONCLUSION

From the work described in this thesis it is clear that the baculovirus system is a useful tool with which to study HSV-1 assembly. To date it has allowed the identification of several sub-capsid structures which appear to reflect the situation in HSV-1 infections. More importantly, it has enabled the identification of the constituent proteins of these structures and has provided a tool for the mutational analysis of the capsid proteins. From manipulation of homologous herpes virus capsid proteins it is also possible to analyse the similarities between the different viruses and it has already been demonstrated that the BHV-1 protease and scaffolding protein can substitute for the homologous HSV-1 proteins in B capsid assembly (Haanes *et al.*, 1995). Thus manipulation of the baculovirus system, whether in insect cells or *in vitro* (Newcomb *et al.*, 1994) could aid the identification of important targets for anti-viral agents which may even act against more than one of the herpes viruses.

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