An Investigation into Factors that Influence the Incorporation of Proteins into the HSV-1 Tegument.

by

Jenny Leslie

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Department of Virology,
Church Street,
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Abstract

The tegument of herpesvirus particles is an amorphous region between the capsid and envelope and consists of a complex array of virus-encoded polypeptides. It has no apparent regular structure but, along with the envelope components, the tegument proteins of herpes simplex virus type 1 (HSV-1) can assemble to give non-infectious particles termed light particles that lack nucleocapsids. Although most of the HSV-1 genes that specify tegument proteins have been identified, little is known about the mode of entry of the proteins into virus particles. To examine the processes controlling incorporation into the tegument, manipulatable experimental systems that would enable the characterisation of sequences that direct proteins to the tegument were developed.

Initial studies involved the construction of a fusion gene composed of the sequences from a tegument gene (UL41, vhs) linked to a non-viral, non-structural gene (chloramphenicol acetyltransferase (CAT)). The resultant fusion product, vhs-CAT, had an apparent molecular weight of 84KDa and retained CAT activity. By analysis of fractions across Ficoll gradients which were used to purify virions and light particles, the fusion protein was shown to be present in both types of particle. The protein could be detected by both Western blotting and enzymatic assays but was not incorporated in quantities that could be measured by staining methods. Subsequently, the commercial antibody which was used to detect the CAT component of the fusion protein was found to have poor avidity for the CAT protein and gave unreliable data. This necessitated the production of a new CAT monoclonal antibody which delayed progress and prevented further use of CAT as a marker polypeptide.

To examine the processes controlling incorporation into the tegument, studies were initiated on VP22, a major tegument protein encoded by UL49. Using a virus vector called 1802, a HSV-1 recombinant, vUL49ep, was constructed that expresses two copies of UL49; one copy specified the unmodified form of VP22 under the control of the native promoter while the second was placed under the control of the human cytomegalovirus (HCMV) immediate early (IE) promoter. To distinguish between the two versions of VP22, the inserted copy was tagged at the C-terminus with an epitope from the HCMV UL83 gene product. In cells infected with the recombinant virus, the overall levels of VP22 synthesised were about 5-fold higher than for wild type virus and this increase was due to the high levels of expression of the tagged protein. Comparison of the polypeptide compositions of particles revealed that the amount of VP22 in the tegument was approximately 2- to 3-fold higher in recombinant virions and light particles than in particles produced by wild-type virus. To demonstrate that the tag sequence did not influence the incorporation of VP22 into the tegument, a HSV-1 recombinant virus containing the epitope

linked to CAT was constructed. In this case, the distribution of epitope-tagged CAT across Ficoll gradients did not correspond with that of virions and light particles made by the virus and therefore epitope-tagged CAT could not be classified as a structural component. The high abundance of VP22 in vUL49ep virus particles correlated with a reduction in the quantities of VP13/14 present in virions and light particles and there was a decrease in the amount of untagged VP22 that was incorporated. In addition, the recombinant virions and light particles had noticeably increased migration on Ficoll gradients. These results provided the first evidence that, for certain proteins, the level of polypeptide synthesis could act as a positive controlling factor for the amount of protein incorporated into the tegument.

In order to identify a sequence or motif within the polypeptide sequence of UL49 that might be responsible for directing the protein into the tegument, a set of insertion and deletion mutants derived from the epitope-tagged UL49 gene was constructed. Oligonucleotides encoding four amino acid sequences were inserted into specific sites along the gene, thus making small changes in the coding sequence of the protein. As a result of the design of the oligonucleotides, a series of UL49 mutants lacking portions of the coding sequence also was derived from the insertion mutants. Viruses expressing these mutant forms of UL49 were constructed in an identical manner to vUL49ep. Thus, they expressed not only the mutated form of VP22 but also the endogenous version which had not been modified. Of the four insertion mutants, three (at amino acid positions 60, 159 and 267) displayed identical properties to vUL49ep. Thus, these mutant forms of VP22 incorporated efficiently into virus particles. The fourth VP22 mutant which had an insertion at position 194 showed reduced levels of incorporation of tagged VP22 and there was also a visible increase in the abundance of untagged VP22. The insertion at position 194 lies within a region of the UL49 ORF which is highly conserved among the homologues of UL49 encoded by other α-herpesviruses. This conserved region extends from approximately amino acids 178 to 257 and, by computer analysis, is the only portion of the VP22 protein for which secondary structure can be predicted. Thus, the insertion at position 194 may perturb the structural conformation of VP22. These results would suggest that this conserved part of VP22 may be more sensitive to mutation as compared to those regions that are poorly conserved.

The mutagenesis strategy also allowed the construction of four UL49 deletion mutant viruses. Analysis of the particles made by these viruses revealed that removal of the C-terminal 34 amino acids from the UL49 ORF had no effect on the ability of VP22 to incorporate into the tegument. This suggested therefore that the C-terminal region of VP22, which is not highly conserved, was not required for incorporation into tegument. By contrast, removal of residues 120-192 did significantly reduce the amount of mutated VP22 present in virus particles but sufficient protein did incorporate to remain detectable on stained gels. To further analyse whether the sequences that permitted incorporation of

VP22del120-192 resided within either the N- or C-terminal regions of the protein, mutant forms of VP22 were constructed that contained exclusively one or other of these regions of the polypeptide. A polypeptide that consisted of the N-terminal 120 residues could be detected to a very limited extent by Western blotting while the protein that comprised residues 192-301 was not found in virus particles. The levels of expression of VP22del120-301 and VP22del1-192 were somewhat less than that of VP22del120-192 in infected cells. However the data does suggest that the sequences which permit the incorporation of VP22del121-192 do not reside exclusively in either the N- or C-terminal regions of the molecule but presumably rely upon cooperation or structural integrity imparted by sequences at both ends of the polypeptide.

During the course of this work, data from Dr. P. O'Hare and his group (Marie Curie Research Foundation, Oxted, Surrey) had shown that VP22 interacts with VP16, another major component of the tegument. In short-term transfection assays, this interaction is manifested by the appearance of novel structures termed tegument bodies which are composed of VP22 and VP16. In a collaborative study using the VP22 constructs described above, it was shown that there is a correlation between the ability of VP22 to incorporate into tegument and the formation of tegument bodies in the presence of VP16. Evidence obtained following the completion of this study has suggested that VP22 is also capable of oligomerisation (J. McLauchlan, personal communication) and thus tegument body formation may require both VP16/VP22 and VP22/VP22 interactions. Since the virus recombinants constructed express unmodified versions of both VP16 and VP22, this implies that the mutant versions of VP22 may incorporate into virus particles by interaction with either of these proteins.

Abbreviations

A	adenine
Amp	ampicillin
APS	ammonium persulphate
ATP	adenosine-5'-triphosphate
BHK	baby hamster kidney
bp	base pair
BSA	bovine serum albumin
C	cytosine
CAV	cell associated virus
cDNA	complementary DNA
Ci	Curies
cm	centimetre
CPE	cytopathic effect
cpm	counts per minute
CS	calf serum
Da	Daltons
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dNTP	2'-deoxynucleoside-5'-triphosphate
dUTP	2'-deoxyuridine-5'-triphosphate
DBP	DNA binding protein
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DTT	dithiothreitol
E	early
EBV	Epstein Barr Virus
EDTA	sodium ethylendiamine tetra-acetic acid
EHV	equine herpes virus
EM	electron microscopy
ep	epitope-tag sequence
FCS	foetal calf serum
FITC	fluorescein isothiocyanate conjugate

G	guanine
gD	glycoprotein D
HCMV	human cytomegalovirus
HEPES	N-2 hydroxyethlypiperazine-N'-2-ethanesulphonic acid
HHV	human herpesvirus
hr(s)	hour(s)
HS	heparin sulphate
hsp	heat shock protein
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
IE	immediate early
Ig	immunoglobin
IR	internal repeat
l	litre
L	late
LAT	latency assiciated transcripts
LpA	late poly adenylation site
M	molar
mi	mock infected
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
moi	multiplicity of infection
MW	molecular weight
mRNA	messenger RNA
N	unspecified nucleotide or amino acid
ng	nanogram
nm	nanometre
NP40	Nonindent P40
OD	optical density
ORF	open reading frame
ORI	origin of DNA replication
^{32}P	phosphorous-32 radioisotope
P	pellet
PAA	phosphonacetic acid
PBS	phosphate buffered saline
PEG	polyethylene glycol

pfu	plaque forming unit
PRV	pseudorabies virus
PT	permissive temperature
R	purine moiety
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RR	ribonucleotide reductase
RT	room temperature
SDS	sodium dodecyl sulphate
SN	supernatant
T	thymidine
TBS	tris buffered saline
TCA	trichloroacetic acid
TEMED	n,n,n',n'-tetramethylethylene diamine
TK	thymidine kinase
TR	terminal repeat
Tris	tris(hydroxymethyl)aminomethane
ts	temperature sensitive
UV	ultra violet
V	volt
vhs	virion host shutoff
vol	volume
Vmw	apparent molecular weight of virus-induced protein
$^{v}/_{v}$	volume/volume
VP	virion protein
VZV	varicella zoster virus
$^{w}/_{v}$	weight/volume
wt	wild type
Y	pyrimidine moiety

One and Three Letter Abbreviations for Amino Acids

Amino acid	Three letter code	Single letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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The Herpesviruses

Section 1

Host range

Herpesviruses are widely distributed in nature and have been isolated from a wide range of vertebrate species. Man is the natural host for at least seven herpesviruses; herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella zoster virus (VZV), Epstein Barr virus (EBV), human cytomegalovirus (HCMV), human herpesvirus 6 (HHV-6) and human herpesvirus 7 (HHV-7). Recent evidence also has identified novel herpesvirus sequences present in Kaposi's sarcoma tissue, leading to the conclusion that there is an eighth human herpesvirus which has been termed Kaposi's sarcoma herpesvirus (KSHV) (Chang *et al.*, 1994). However, to date, no virus corresponding to KSHV has been isolated (Schalling *et al.*, 1995). These viruses are each able to cause diseases of varying severity. As a group, herpesviruses display a wide tropism ranging from infections of epithelial tissue, internal organs including the central nervous system and white blood cells. A common feature of infection by herpesviruses is their ability to latently infect different cell types. In latently-infected cells the viral DNA is not integrated and there is limited expression of the viral genome. Reactivation from latency results in expression of the viral genes and leads to the production of virus progeny which may cause visible signs of infection.

In addition to those that infect humans, herpesviruses are causative agents of disease in a wide range of animals, many of which are of significant economic importance. These include Marek's Disease virus (MDV) which infects chickens, causing lymphocyte infiltration of nerves with demyelination and paralysis, as well as lymphoid tumours. Infectious virus is present in oral secretions and feather follicles. However, MDV has been successfully controlled with a live virus vaccine (reviewed by Hirai, 1988). Pseudorabies virus, (PrV), another economically important herpesvirus, is responsible for a severe encephalitis in piglets (reviewed by Gustafson, 1981). The equine herpesviruses, in particular EHV-1, give rise to a range of debilitating conditions in horses which include respiratory disease, abortion, and neurological disorders (Bryans & Allen, 1989). The bovine herpesviruses, including BHV-1, cause infectious rhinotracheitis and pustular vulvovaginitis often leading to abortion in cattle (Wyler *et al.*, 1989).

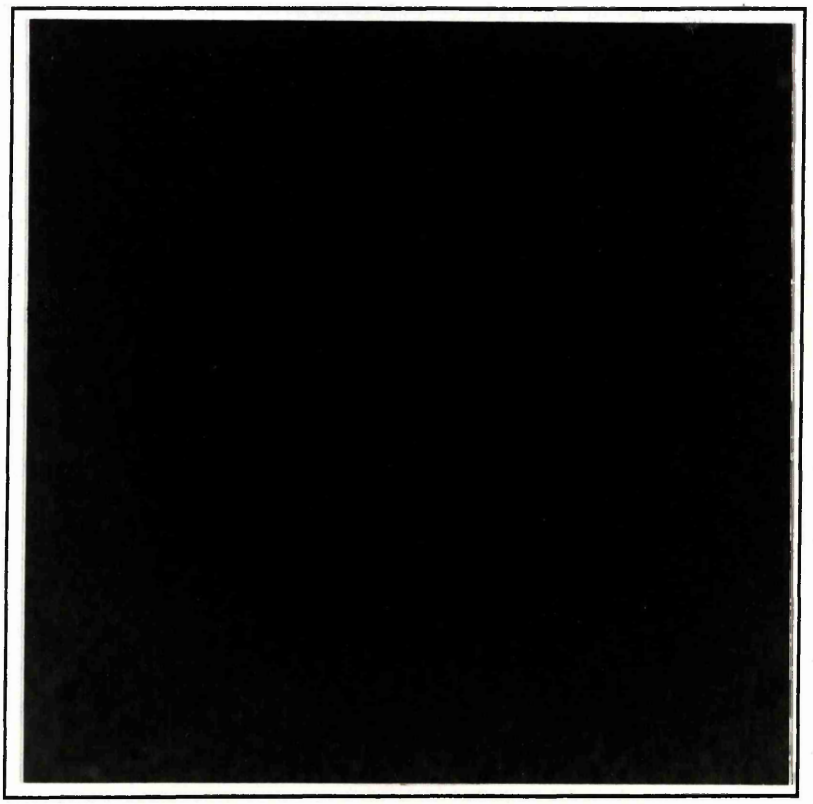


Figure 1. Cryo-electron micrograph of a HSV-1 virion.

Section 2

Characteristics and Classification of the Herpesviruses

All members of the herpesvirus group have a characteristic virion structure composed of three morphologically distinct components termed the nucleocapsid, tegument and envelope. The nucleocapsid is icosahedral in shape and contains the virus genome. The tegument is an amorphous region that surrounds the nucleocapsid and is in turn bounded by the envelope which contains lipid and exhibits glycoprotein spikes on the surface (Fig. 1). Nucleocapsids contain a simple linear double stranded DNA genome. This ranges in different herpesviruses from 80 to 150 x10^6 molecular weight, and there is great variation in G + C content and genome arrangements. The most widely used classification is based on the biological properties of the viruses and comprises three sub-families, designated α-herpesvirinae, β-herpesvirinae and γ-herpesvirinae (reviewed by Roizman, 1990).

HSV-1 and HSV-2 are members of the α-herpesvirinae, which as a group are characterised by their relatively short reproductive life cycle (usually less than 24 hours), cytolytic infection and the ability to establish latent infection in sensory ganglia. Other members of this subfamily include VZV, EHV-1, 3 and 4, and PRV

The β-herpesvirinae, such as HCMV and HHV-6, in tissue culture have a restricted host range and a relatively long reproductive cycle. Infection often results in the formation of enlarged cells and latent infections can be established in various tissues such as secretory glands and lymphoreticular cells. Members of the γ-herpesvirinae, such as EBV, have a very limited host range with a tropism for lymphocytes. The length of the reproductive cycle is variable and latent infections are frequently established in lymphoid tissue.

Comparison of genome sequences is being increasingly used to classify herpesviruses into groups and also determine the evolutionary relationships among members of the herpesviridae. To date, the complete genome sequences of nine herpesviruses have been published (summarised in Table 1) while for many other herpesviruses, partial sequence data exist. Although the genomes of the sequenced mammalian herpesviruses differ widely in gene content from 70 genes up to 200 and in their length, G+C content and arrangement, a subset of around 40 genes are conserved in all α, β and γ-herpesviruses. This implies a common ancestry followed by extensive divergence (Davison & Taylor, 1987; McGeoch, 1989; Chee *et al.*, 1990; McGeoch *et al* ., 1993).

Classification of herpesviruses by sequence homology largely confirms the assignment of viruses on the basis of their biological properties. Thus, the genomes of α-herpesviruses HSV-1, HSV-2, VZV, PRV and EHV-1 are largely co-linear (Davison & Wilkie 1983c), while comparison of VZV and the γ-herpesvirus EBV reveals regions of

Table 1. The genomes of Herpesviruses sequenced to date.

Virus	Base pair residues	Reference
Epstein-Barr virus (EBV)	172 282bp	(Baer *et al.*, 1984)
Varicella-Zoster virus (VZV)	124 884bp	(Davison & Scott, 1986)
Herpes Simplex type 1 (HSV-1)	152 260bp	(McGeoch *et al.*, 1988b)
Human cytomegalovirus (HCMV)	229 354bp	(Chee *et al.*, 1990)
Equine herpesvirus type 1(EHV-1)	150 223bp	(Telford *et al.*, 1992)
Herpesvirus saimiri (HVS)	112 921bp	(Albrecht *et al.*, 1992)
Equine herpesvirus type 2 (EHV-2)	184 427bp	(Telford *et al.*, 1995)
Human herpesvirus type 6 (HHV-6)	159 321bp	(Gompels *et al.*, 1995)
Channel catfish virus (CCV)	134 226bp	(Davison 1992)

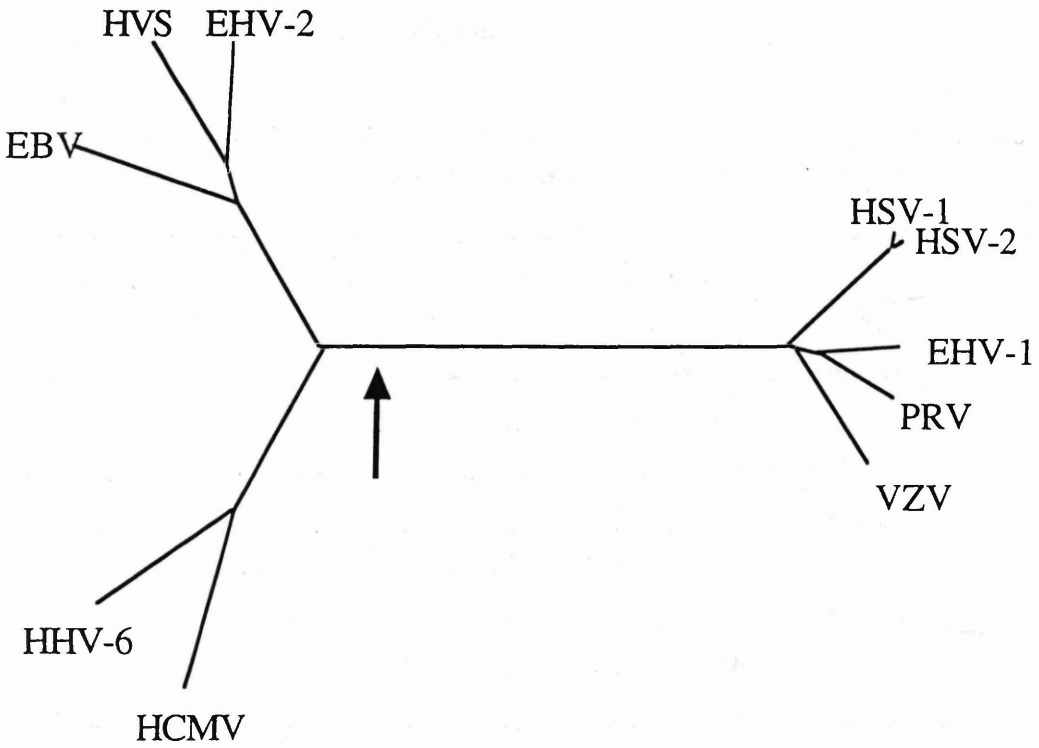


Figure 2. Broadly based phylogenetic tree. The GC2 neighbour-joining distance tree is taken from McGeoch *et al.*, (1995). The arrow indicates the approximate root position.

rearrangements and divergence (Davison & Taylor, 1987). The biological properties of EHV-2 and EHV-5 supported their provisional classification as β-herpesviruses (Plummer *et al.*, 1969), however sequence analysis of fragments derived randomly from the genomes showed that EHV-2 and EHV-5 are distinct γ-herpesviruses (Telford *et al.*, 1993). In addition, using genomic analysis, MDV, originally considered to be a γ-herpesvirus principally because of its lymphotropism, was shown to be phylogenetically more closely related to the neurotrophic α-herpesviruses (Buckmaster *et al.*, 1988). This classification has been further supported by Brunovskis & Velicer (1995) who sequenced the entire MDV U_S sequence and identified seven homologues exclusive to α-herpesvirus S component genes. Finally, HHV-6, also originally classified as a γ-herpesvirus because of its tropism for lymphocytes (Downing *et al.*, 1987), has since been shown to possess more characteristics in common with β-herpesviruses, sharing both encoded amino acid sequence similarities and overall gene organisation with HCMV (Lawrence *et al.*, 1990; Neipel *et al.*, 1991; Gompels *et al.*, 1992; Nicholas & Martin, 1994).

The recent sequencing of the entire genome of channel catfish virus (CCV), previously thought to be an α-herpesvirus, has been shown to have no detectable significant homology with any known herpesvirus sequence. This resulted in the suggestion that this virus should be assigned to a separate subfamily, or even removed from the family of herpesviridae altogether (Davison, 1992).

Sequence information has enabled detailed phyolgenetic analysis of the mammalian members of the family herpesviridae. Phylogenetic trees including timescales have been inferred from alignments of sets of sequences. In all cases, the three recognised subfamilies (α, β & γ), and major sublineages in each family were clearly evident. Also multiple gene sets were assembled to give a broadly based tree which, assuming there was a constant molecular clock, suggested that the three sub-families arose approximately 180-220 million years ago, and that major sublineages within subfamilies were probably generated 60-80 million years ago. Fig. 2 illustrates the broadly based phylogenetic tree constructed by McGeoch *et al.* (1995).

Section 3.
Biological properties of human herpesviruses

Infections in humans are spread by direct contact between infected and uninfected individuals, particularly at mucosal tissues, and are characterised by their ability to cause latent infection. HSV-1 generally causes lesions to develop at mucosal membranes around the mouth or face, including the eyes often resulting in a corneal infection. HSV-2 typically infects the genital and anal region. The clinical situation most harmful to humans results from infection of the central nervous system (CNS) causing acute necrotising encephalitis (Corey & Spear, 1986). Neonatal infection also has a high mortality rate and is usually due to a primary HSV-2 infection in the mother, when no maternal antibody is present for protection (Timbury, 1978). In addition, HSV-2 has been implicated in the development of cervical cancer from evidence obtained from seroepidemiological studies (Nahmias *et al.*, 1974) and due to the detection of some sequences of HSV-2 DNA in cervical cancer specimens (Macnab *et al.*, 1985). However, a direct link between HSV-2 and the induction of cervical cancer remains controversial, and the development of malignancy may be due to a synergistic effect with other factors such as certain human papilloma viruses (zur Hausen, 1982).

VZV, also a neurotrophic α-herpesvirus, is the causative agent for chickenpox (varicella) in children. Following primary infection, the virus spreads through viraemia and ascends to the dorsal root ganglia of sensory nerves supplying the affected skin areas and remains in a non-infectious latent state after recovery. The primary site of latency appears to be the satellite cells surrounding the neuron, although some neurons may harbour latent virus (Gilden *et al.*, 1983; Hyman *et al.*, 1983; Coen *et al.*, 1989). Unlike HSV, VZV reactivation either does not occur in most individuals or is limited to a single recurrence of infection (Straus, 1989). Accumulating evidence has implicated a role for cell mediated immunity (CMI) in controlling the ability of the virus to reactivate. Weakening of the CMI by age or immunosuppression increases the likelihood of reactivation (Weigle & Grose, 1984; Hayward *et al.*, 1991; Wilson *et al.*, 1992). Upon reactivation, virus spreads by lytic infection within the ganglion and via axonal transport infects the skin enervated by the neurons, giving rise to a crop of vesicles rich in virus (zoster), known as shingles.

HCMV, a lymphotrophic β-herpesvirus, asymptomatically affects the majority of the population. However, in immunocompromised individuals it causes a wide range of chronic respiratory infections, frequently affecting immunosuppressed transplant and AIDS patients often resulting in viral pneumonia (Meyers *et al.*, 1986). HCMV has also been implicated in causing neonatal abnormalities (Microcephaly; Ho, 1991).

EBV is a γ-herpesvirus that infects at least two cell types, B lymphocytes and stratified pharyngeal epithelial cells (Lemon *et al.*, 1977; Morgan *et al.*, 1979). It causes infectious mononucleosis (glandular fever), and has been associated with two types of human tumour, Burkitts Lymphoma (BL) and nasopharyngeal carcinoma (NPC) (reviewed by Epstein & Achong, 1979; Klein, 1989). BL predominantly affects young males in Sub-Saharan Africa between the ages of 3 and 9, causing severe swelling in the jaw, testes, stomach, etc. NPC is found specifically in Asian men over 60 years old living in a distinct region of Southern China. The localisation of NPC and BL within 'hot-spots' suggests the requirement of specific cofactors for the development of EBV-associated cancer. The true site of latent infection has been the subject of debate over several years but current evidence suggests that EBV persists in B-cells. As with all the human herpesviruses the viral genome is not integrated and transcription during latency is minimal (Speck & Strominger, 1989).

Although recently identified (Downing *et al.*, 1987), HHV-6 is now known to affect up to 90% of the population. Infection occurs in infants which may be either asymptomatic or cause a mild skin rash (exanthem subitum), however the significance of HHV-6 as a pathogen is unclear (Yamanishi *et al.*, 1988; Suga *et al.*, 1989). Its tropism for CD4+ lymphocytes has led to speculation that it may be involved in the development of AIDS or other immunodeficiencies (Levy *et al.*, 1990). At present there is only limited understanding of the properties of HHV-6.

HHV-7, recently isolated from prelipheral blood lymphocytes (Frenkel *et al.*, 1990), remains unclassified, as HHV-7 DNA did not show any hybridisation with probes derived from other herpesviruses, except to a limited degree with HHV-6 (Frenkel *et al.*, 1990). HHV-7 does show significant antigenic dissimilarity to HHV-6, sufficient to allow seroepidemiological discrimination (Wyatt *et al.*, 1991).

KSHV, the most recently discovered human herpesvirus (Chang *et al.*, 1994), is yet to be fully characterised and indeed the virus has not yet been isolated. Kaposi's sarcoma (KS) is the most common neoplasm occurring in individuals with AIDS, rarely affecting immunocompetent individuals (Beral *et al.*, 1991). Due to the epidemiology of KS sufferers it was suspected that the lesions were due to an infectious agent, and samples of KS tissues were shown to contain DNA sequences that had homology to a number of herpesviruses, including EBV, BHV-4, HHV-6, HCMV, HHV-7 and herpesvirus samiri (HVS). The sequence comparisons were sufficiently divergent to suggest the existence of a new human herpesvirus (Chang *et al.*, 1994; Schalling *et al.*, 1995).

Herpes Simplex Virus type 1

HSV-1 is the most widely investigated herpesvirus and is the prototype for α-herpesviruses. In addition, since it was also the virus used in this study, a more detailed overview of its properties is described.

Section 1. Pathogenesis and latency

Following infection, the virus multiplies at the site of entry, where it invades nerve endings and is able to travel through sensory nerves via axonal flow to the nucleus of the sensory neuron where it establishes latency. Latency is a condition or state by which the virus can persist in infected individuals and involves the maintenance of multiple copies of circular extrachromosomal viral DNA in the nuclei of infected cells (Mellerick *et al.*, 1987). The viral DNA in neuronal cells originally was suggested to have a similar structure to that of cellular chromatin (Griffith, 1975; Cremisi *et al.*, 1976; Ponder *et al.*, 1978). However, a report on the state of HSV-1 DNA in the CNS of acutely infected mice indicated that the majority of viral DNA was not organised into nucleosomes (Muggeridge & Fraser, 1986).

Reactivation from the latent state involves virus being transported back along the nerve axon to the original site of entry where it infects cells and causes lesions. In some individuals, periodical successful reactivation is frequent and painful lesions at the site of initial infection cause extreme discomfort to the sufferer. The processes involved in reactivation are not fully understood but can be triggered by a range of stimuli including exposure to UV light, stress, pregnancy, menstruation, etc. (Hill, 1985).

During latent infection, viral genes associated with lytic replication are not expressed but in cells such as peripheral sensory neurons, RNAs called latency associated transcripts (LATs) are expressed from the long repeat region of the genome (Deatly *et al.*, 1987; Stevens *et al.*, 1987; Dobson *et al.*, 1989). The LATs encoded by these regions of the genome are antisense to the mRNA of the immediate early (IE) regulatory protein Vmw110 (*Introduction; Chapter 3, Section 3.3.c*); Stevens *et al.*, 1987; Mitchell *et al.*, 1990). Annealing of LAT RNA to IE110 mRNA may lead to inhibition of synthesis of Vmw110, a protein which appears to play a critical role in reactivation of virus from latency (Russell *et al.*, 1987; Cai & Schaffer, 1989; Harris *et al.*, 1989; Leib *et al.*, 1989a). In addition, the prevention of immediate early gene transcription during latency was suggested by Latchman and co-workers to be a result of the repression of the formation of the complex assembly implicated in the initiation of immediate early gene transcription (complex formation is described in detail in *Introduction; Chapter 3, Section 3.2.a*, IE promoters) (He *et al.*, 1989;

Kemp *et al.*, 1990; Lilycrop *et al.*, 1991).

LAT RNAs of 2.0 and 1.5Kb accumulate in the nuclei of latently-infected cells, and are frequently used as markers for cells harbouring latent virus. A number of studies have indicated that the LATs are dispensable for the establishment and maintenance of latent infection, though their presence may increase the efficiency of these processes (Leib *et al.*, 1989b; Steiner *et al.*, 1989). The LATs have been implicated in efficient reactivation of the virus from its latent state, but the mechanism of activity remains obscure (Hill *et al.*, 1990; Trousdale *et al.*, 1991; Fraser *et al.*, 1992; Fareed & Spivack, 1994; Perng *et al.*, 1994). However recent evidence does not support this function for LATs (Ecob-Prince & Hassan, 1994). In these elegant studies, reactivation induced by the explantation of latently infected dorsal root ganglia into culture rarely occurred in cells still expressing LATs.

The most useful animal model systems in which latent infection is examined are mice, guinea pigs and rabbits. In the mouse, following infection of the corneal epithelium, lip, footpad or ear, latent infection is established, but the latent virus does not spontaneously reactivate (Stevens & Cook, 1971; Blyth *et al.*, 1976; Hill *et al.*, 1978; Blue *et al.*, 1981; Hill *et al.*, 1982). However, in rabbits and guinea pigs reactivation of latent virus occurs spontaneously (Nesburn *et al.*, 1967). Currently there are a number of *in vitro* models for studying HSV latency and these rely upon the ability to artificially suppress viral replication. Several replication-permissive non-neuronal cell lines sustain non-productive infection at temperatures not optimal to HSV replication, with reactivation being induced following superinfection with temperature sensitive mutants of HSV-1 or with HCMV (Russell & Preston, 1986). The *in vitro* models with the most obvious relevance to latency in animals and humans utilise fetal neurons from rats or primates (Wigdahl *et al.*, 1983, 1984; Wilcox & Johnson, 1987, 1988; Wilcox *et al.*, 1990). However, non-primary tissue culture cells including human fetal lung fibroblasts have also been used (Wigdahl *et al.*, 1981, 1982; Harris & Preston, 1991).

In cells in culture, approximately half of the 75 known different genes are not essential for virus replication. However, the majority of these genes are required for efficient viral replication in experimental animal models. Mutations in a small number of virus genes have been shown to decrease the ability of the virus to access the CNS or to multiply efficiently and these genes play a key role in defining the pathogenic properties of the virus. The virulence functions that enable neuronal growth include the viral thymidine kinase (TK) gene (Field & Wildy, 1978). TK phosphorylates pyrimidine nucleosides and analogues and is essential for the replication of viral DNA. Cellular TK present in dividing cells is able to complement TK negative virus in cell culture, but as neuronal cells are non-dividing and lack TK function, virus replication in these cells requires TK expression (Tenser, 1991).

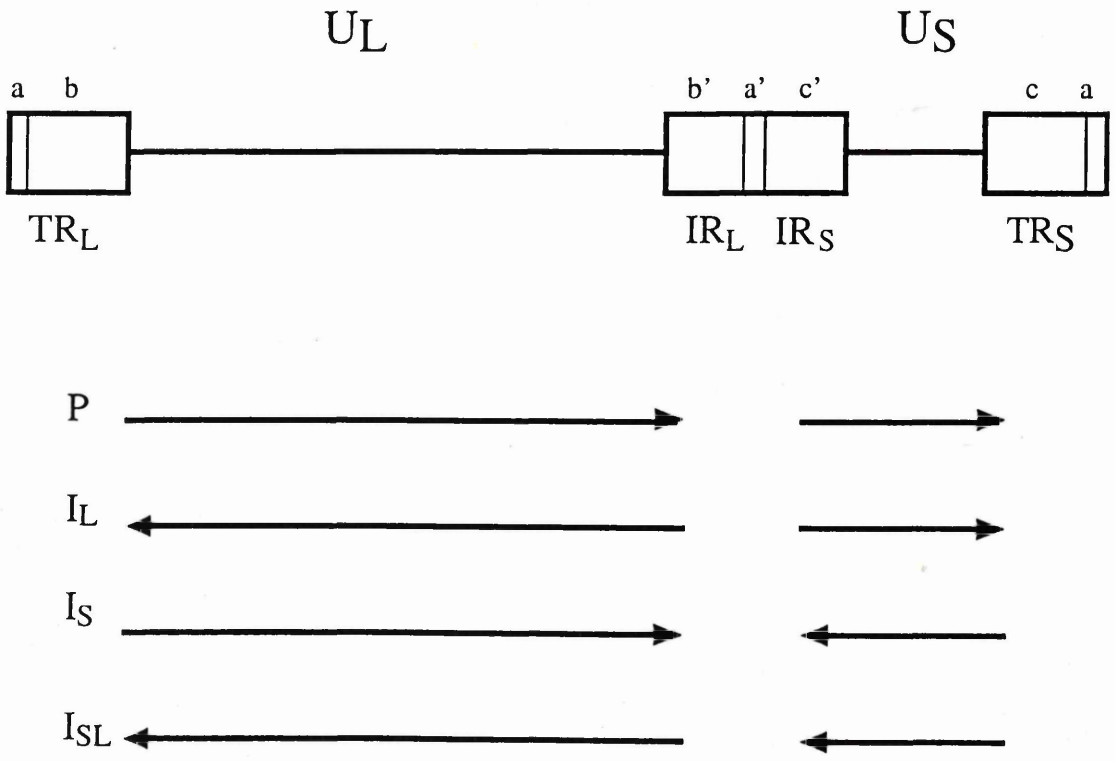
ICP34.5, encoded by gene RL1, influences intra-cerebral virulence (Chou *et al.*, 1990). The host response to stress caused by viral infection is to shutoff protein synthesis inducing cellular apoptosis. ICP34.5 functions to preclude this response enabling viral growth. Deletion or mutation of this gene results in variants that grow as well as wild type virus in dividing cells of many established cell lines, but show impaired replication in non-dividing cells (Chou *et al.*, 1990; McGeoch *et al.*, 1991; Bolovan *et al.*, 1994). In mice ICP34.5 mutants are incapable of replicating in the CNS and do not cause encephalitis (Javier *et al.*, 1987; Chou *et al.*, 1990; Taha *et al.*, 1990; MacLean *et al.*, 1991). This ability of ICP34.5 mutants to grow in dividing cells but not in non-dividing cells has led to studies that show that neuroattenuated HSV-1 mutant 1716, with a 759-bp deletion in ICP34.5 (MacLean *et al.*, 1991), was a safe and effective therapeutic agent for intracranial murine melanoma (Randazzo *et al.*, 1995).

The lack of virus gene expression in the latent state presumably is one mechanism that permits the virus to avoid detection by the immune system. Recent studies have implicated certain virus gene products in additional mechanisms following reactivation which also may facilitate infected cells avoiding immune surveillance. For example, investigation of UL41 negative mutants by Hill *et al.*, (1995) revealed that this gene product, which possesses a host shutoff function and is a component of the virion, was responsible for the reduction or abolition of the synthesis of HLA class 1 molecules in cells infected with HSV-1. More recently, expression of the HSV-1 immediate early protein Vmw12 (ICP47) has been shown to block the presentation of viral polypeptides in MHC class I restricted cells. Current evidence suggests that Vmw12 acts by binding to the transporter protein associated with antigen processing (TAP) thereby inhibiting peptide transport across the endoplasmic reticulum membrane (York *et al.*, 1994; Fruh *et al.*, 1995; Hill *et al.*, 1995).

Another mechanism by which the virus is able to evade the immune system is through cell to cell spread of the virus across cell junctions in a manner resistant to neutralising antibodies. This mechanism is facilitated by the gE-gI glycoprotein complex (Dingwell *et al.*,1994) that promotes cell to cell spread of the virus which enables the virus to avoid the extracellular environment and consequently the humoral antibody response.

Figure 3. The structure of the HSV-1 genome.

The HSV-1 genome is shown, with unique sequences as solid lines (U_S & U_L). The repeats TRL, IRL, IRS and TRS are illustrated as boxes with their component parts a, b, c and a', b', c' designated. Below the genome represenation, the isomerisation of the HSV-1 genome is illustrated. The four isomers are: P (prototype), IL (L inverted with respect to P), IS (S inverted with respect to P), and ISL (S and L inverted with respect to P).



Section 2. Genome structure and gene content

HSV-1 DNA is a linear double-stranded molecule that consists of two distinct covalently linked sequences, designated the long (L) and the short (S) segments. Both the L and S segments consist of a unique region (U_L and U_S) flanked by a pair of inverted repeat sequences (TR_L, IR_L, TR_S and IR_S) as shown in Fig 3. The termini of each molecule possess a redundancy of some 400 base pairs, termed the *a* sequence which is also located internally at the joint between the L and S segments in the opposite orientation (Sheldrick & Berthelot, 1974; Wadsworth *et al.*, 1975; Wagner & Summers, 1978). The *a* sequence is present as one or more copies at the L terminus and the joint but only as a single copy at the S terminus and is essential for packaging of replicated DNA into capsids. As a consequence of their model, Sheldrick and Berthelot (1974) suggested that recombination events occurring between the terminal and internal repeat sequences might give rise to four isomeric arrangements of the genome differing in the relative orientations of the unique regions as shown in Fig. 3. Partial denaturation studies by electron microscopy, and restriction enzyme analyses of HSV-1 DNA (Delius and Clements, 1976; Wilkie & Cortini, 1976) confirmed this prediction, indicating that all four possible genome arrangements occur with equal frequency in the DNA from virions. One isomer, chosen arbitrarily, is designated as the prototype for genetic map representations (Fig 3; Roizman, 1979b).

The complete HSV-1 genome sequence has been determined (McGeoch *et al.*, 1988b) and the interpretation of these sequences indicate at least 75 protein coding sequences. The HSV-1 genes identified to date and their functions are shown in Table 2. The genome is arranged in a compact form with tightly packed open reading frames (ORF's) for example, 89% of U_L codes for protein. The genes are orientated in both directions, and there is little overlap between coding sequences.

At least 3 genes are completely contained within the repeat sequences, R_S and R_L, that flank the unique segments, and there are therefore two copies of each of these genes in the genome. Examples of diploid genes include those encoding the transcriptional control proteins Vmw175 and Vmw110 (Rixon et al., 1982; Perry *et al.*, 1986).

The transcripts encoding genes are often arranged as nested families of mRNAs which share common 3' termini but have unique 5' ends and promoter sequences (Wagner, 1985). This arrangement is exemplified in the U_S region of the genome where 11 of the 13 mRNAs (encoding 12 proteins) are arranged into four nested families (Rixon & McGeoch, 1984, 1985; McGeoch *et al.*, 1986b). In contrast to cellular and most other viral mRNAs, the majority of HSV-1 transcripts are not spliced. Of the 75 proposed distinct genes, only IE1 (Perry *et al.*, 1986), IE4 and IE5 (Watson *et al.*, 1981; Rixon & Clements, 1982),

UL15 (Costa *et al.*, 1985; McGeoch *et al.*, 1988) and the latency associated transcripts (LATs) (Wagner *et al.*, 1988; Weschler *et al.*, 1988) are known to generate mature mRNA by splicing.

Table 2. The HSV genes identified to date

Gene	Protein/Function	Status
RL1	Neurovirulence factor (ICP34.5)	ne
ORF P	Encodes a protein of 248 amino-acids, funtion unknown	ne
RL2	IE protein; transcriptional regulator (Vmw110)	e/ne
UL1	Glycoprotein L, role in cell entry	e?
UL2	Uracil DNA glycosylase	ne
UL3	Function unknown	ne
UL4	Function unknown	ne
UL5	Component of DNA helicase-primase	e
UL6	Associated with virion capsid and involved in packaging of nascent DNA	e
UL7	Function unknown	--
UL8	Component of DNA helicase primase, necessary for DNA replication	e
UL8.5	Encodes a protein of 486 amino-acids (OBPC), potential role in HSV DNA replication	--
UL9	*Ori*-binding protein essential for DNA replication	e
UL10	Glycoprotein M	ne
UL11	myristylated tegument protein, role in the envelopment and egress of virions	ne
UL12	Deoxyribonuclease, role in maturation/packaging of nascent DNA	e
UL13	Tegument protein, protein kinase	ne
UL14	Function unknown	--
UL15	Role in the cleavage of concatameric DNA	e?
UL16	Function unknown	ne
UL17	Function unknown	e
UL18	Capsid protein triplexes (VP23)	e
UL19	Major capsid protein (VP5); constitutes the hexons and pentons	e
UL20	Integral membrane protein, role in egress of virions	e/ne
UL21	Function unknown	--
UL22	Virion surface glycoprotein H, role in virion entry	e
UL23	Thymidine kinase, DNA replication	ne
UL24	Function unknown	ne
UL25	Virion protein; role in capsid maturation and DNA packaging	e
UL26	Proteinase, the N-terminal portion is capsid protein VP24,	

	C-terminal is VP21, involved in capsid assembly	e
UL26.5	Scaffolding protein of B capsids (VP22a), processed by UL26 proteinase	e
UL27	Virion surface glycoprotein B, role in cell entry	e
UL28	Role in capsid maturation/DNA packaging	e
UL29	ssDNA binding protein (ICP8), essential for DNA replication	e
UL30	Catalytic subunit of replicative DNA polymerase	e
UL31	Nuclear phosphoprotein, function unknown	--
UL32	Role in capsid maturation/DNA packaging	--
UL33	Role in capsid maturation/DNA packaging	e
UL34	Membrane associated phosphoprotein	--
UL35	Capsid protein (VP26), present on tips of hexons	e
UL36	Tegument protein (ICP1/2, VP1/2, Vmw273)	e
UL37	Tegument phosphoprotein, function unknown, may have DNA binding role, binds to ICP8	--
UL38	Capsid protein (VP19C), component of triplexes	e
UL39	Ribonucleotide reductase large subunit (R1)	e/ne
UL40	Ribonucleotide reductase small subunit (R2)	e/ne
UL41	Located in the tegument, Virion host shutoff	ne
UL42	Subunit of replicative DNA polymerase, increases processivity	e
UL43	Function unknown, probable integral membrane protein	ne
UL44	Virion surface glycoprotein C, role in entry	ne
UL45	Envelope protein, mediates cell fusion	ne
UL46	Tegument protein (VP11/12, Vmw90/92)	ne
UL47	Tegument protein (VP13/14, Vmw81/82), modulates IE gene transactivation by VP16	ne
UL48	Major tegument protein (VP16, Vmw65, αTIF), transactivator of IE genes	e
UL49	Tegument protein (VP22)	--
UL50	Deoxyuridine triphosphatase	ne
UL51	Function unknown	e/ne
UL52	Component of DNA helicase-primase	e
UL53	Glycoprotein K, role in cell fusion	e?
UL54	IE protein, post-transcriptional regulator of gene expression (ICP27, Vmw63)	e
UL55	Function unknown	ne
UL56	Function unknown, role in intraperitoneal virulence	ne
LAT	Family of transcripts expressed during latency, function unknown, protein coding capacity uncertain	ne

RS1	IE protein (ICP4, Vmw175), transcriptional regulator	e
US1	IE protein (ICP22, Vmw68), transcriptional regulator	e/ne
US2	Function unknown	ne
US3	Protein kinase	ne
US4	Glycoprotein G	ne
US5	Glycoprotein J	ne
US6	Glycoprotein D, role in entry	e
US7	Glycoprotein I, complex together with gE, role in cell-to-cell spread and is receptor for Fc of IgG	ne
US8	Virion surface glycoprotein E, complex together with gI, role in cell-to-cell spread and is a receptor for Fc of IgG	ne
US8.5	Function unknown	ne
US9	Tegument protein	ne
US10	Virion protein	ne
US11	Virion protein, ribosome associated in infected cells	ne
US12	IE protein, turns off TAP to block the presentation of viral polypeptides to MHC class I restricted cells (ICP47, Vmw12)	ne

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e: essential for virus growth in culture cells

ne: non-essential

e/ne: necessity depends on the culture conditions or temperature

e?: the data regarding status is not conclusive

--: absence of information regarding the status

Adapted from:

Addison *et al.*, 1984, 1990; Al-Kobaisi *et al.*, 1991; Baines *et al.*, 1995; Baines and Roizman, 1993; Baradaran *et al*, 1994; Barker and Roizman, 1992; Barnett *et al.*, 1992; Chang and Roizman, 1993; Goeorgopoulous *et al.*, 1993; Haanes *et al.*, 1994; Hill *et al.*, 1995; Lagunoff and Roizman, 1994; Liu and Roizman, 1991a; McGeoch *et al.*, 1985, 1986, 1988a, 1993; Overton *et al.*, 1994; Fruh *et al.*, 1995; Patel and MacLean, 1995; Purves *et al.*, 1993; Rosen-Wolff and Darai, 1991; Schmitz *et al.*, 1995;

The Lytic Life Cycle of Herpes Simplex Virus Type 1

The lytic life cycle of HSV-1 involves a series of complex stages which, although interconnected, can be identified as separable topics for descriptive purposes. Therefore, the following sections describe the key events which take place during productive infection. These are 1) attachment and entry of the virus particle into the infected cell 2) transcription and translation of viral mRNA 3) replication of viral DNA 4) the assembly of mature virus particles including the packaging of replicated DNA, and finally 5) egress of virus particles from the infected cell.

Section 1. Attachment and Entry

(i) Attachment of HSV-1 particles to cells

The initial stages of herpesvirus infection involve the attachment of the virus particle to the cell surface. Current research has identified many of the viral and cellular components that are involved in the attachment and penetration processes but the precise molecular mechanisms involved in these processes are still to be determined. The processes of attachment for HSV-1, HSV-2, PRV and BHV-1 are similar, however each of these herpesviruses uses distinct cell surface receptor molecules (Vahlne *et al.*, 1979; Wahren *et al.*, 1984). The attachment of the HSV-1 virus particle to the cell involves interactions between the virion glycoproteins and the cell surface. Initial attachment is via gC and/or gB to the cell surface heparin sulphate (HS) proteoglycans (WuDunn & Spear, 1989; Mettenleiter *et al.*, 1990; Shieh *et al.*, 1992; Gruenheid *et al.*, 1993; Herold *et al.*, 1995). These glycoproteins form the most prominent envelope spikes on the virus particle (Stannard et al., 1987), and therefore are likely to provide the first virus contact with the cell. gC, non-essential for virion infectivity, is principally responsible for the initial adsorption of HSV-1 virions to cell surface HS (Langeland et al., 1990; Herold *et al.*, 1991). The role played by gC is suggested to be similar to that of the gC homologue, gIII, of PRV in that it may help to mediate stable attachment of the virion to the cell surface (Schreurs *et al.*, 1988; Zuckermann *et al.*, 1989). gB has heparin binding activity independent from gC, and mutant virions lacking both gC and gB are practically devoid of the ability to bind to HEp-2 cells (Herold *et al.*, 1991 & 1994). In addition, *in vitro* studies allowing the analysis of formation of complexes between virion proteins and cell surface components supported evidence that gC and gB play roles in viral attachment (Johnson *et al.*, 1984; Fuller & Spear,

1985; Khun *et al.*, 1990).

Initial attachment is followed by a stable form of attachment which allows close association of the virus with the cell in a manner that is more resistant to removal by heparin or high ionic strength washes (Fuller & Lee, 1992; McCain & Fuller, 1994). It is believed that this stable form of attachment is mediated by gD, interacting with a number of cell surface molecules including 275KDa and 46KDa mannose-6-phosphate receptors (Burnetti *et al.*, 1994). Evidence for this second form of attachment comes from biochemical analyses which show that gD neutralised, or gD null virus binds to cells but does not penetrate (Highlander *et al.*, 1987; Ligas & Johnson, 1988). gD has also been shown by *in vitro* studies to be able to form complexes with cell surface components, suggesting a role in viral attachment (Khun *et al.*, 1990).

(ii) Penetration by HSV-1 particles

Following stable attachment, HSV-1 particles have been shown by electron microscopy to enter the cell by fusion with the plasma membrane (Morgan *et al.*, 1968; Para *et al.*, 1980). In order for this fusion to occur multiple interactions involving virion glycoproteins and cell surface components are required. The study of HSV mutants has shown that several virion glycoproteins including gD, gB and gC known to be involved in virus attachment to the cell surface, are also necessary for viral entry into the cell. As a result of gB and/or gC interacting with HS moieties, conformational changes in the glycoproteins or the HS may trigger further events either in the virus particle or the cell surface, that result in fusion of the virion envelope with the plasma membrane. With regards to gD, a model of HSV-1 cellular entry proposed by Fuller and Lee (1992) suggests that gD performs at least two functions. Firstly, as described in the previous section, gD interacts with cellular components to bring the virus envelope and cell membrane closer together, and secondly, this glycoprotein interacts with a viral component which is directly involved in fusion initiation. The conformational change of gD following step one would result in a change that allows or triggers the second function. Evidence supporting this dual function of gD comes from the observation that there are distinct epitopes that can be neutralised by anti-gD antibodies, that either block attachment or that block penetration (Eisenberg *et al.*, 1985a; Cohen *et al.*, 1986).

In addition, gH and gL are known to be essential for cell fusion and virus entry into cells (Gompels & Minson, 1986; Cai *et al.*, 1987; Ligas & Johnson, 1988; Forrester *et al.*,1992; Roop *et al.*, 1993; Burnetti *et al.*, 1994). HSV-1 virus mutants that lack any one of these glycoproteins are able to bind to cells at normal efficiency but fail to penetrate. The

involvement of gH in fusion was demonstrated by the inactivation of virus penetration by anti-gH neutralising antibodies. gL interacts with gH forming a heterodimer which appears to be essential for the proper processing and intracellular transport of both proteins (Hutchinson *et al.*, 1992a). gL negative mutants failed to incorporate gH into the virion envelope, and were unable to enter cells (Roop *et al.*, 1993). Therefore gH, probably complexed with gL, seems to participate in the initiation of fusion but not the subsequent steps associated with the entry of the nucleocapsid into the cell (Fuller & Lee, 1992).

More recently, gK has been implicated in the process of viral penetration. Viruses lacking functional gK exhibit a syncitial phenotype which resulted in the suggestion that gK might play an important role in regulating membrane fusion (Bond & Person, 1984; Pougue-Geile & Spear, 1987; Hutchison *et al.*, 1992b; Ramaswamy & Holland, 1992). Recent evidence suggests that the fusion of infected cells mediated by gK is indirect as gK is not exhibited on the surface of infected cells (Hutchinson *et al.*, 1995).

The potential role of non-glycosylated proteins in viral attachment and fusion have been suggested. Previous investigations have shown that virus attachment can occur when glycosylation is inhibited, suggesting that other viral proteins are important in binding (Svennerholm *et al.*, 1982). The tegument protein encoded by UL25 has been suggested to play a role in penetration following the isolation of a temperature sensitive (*ts*) mutant with a defect in gene UL25. The *ts* mutant at non-permissive temperature binds to cells but is impaired in its ability to penetrate (Addison *et al.*, 1984).

Following fusion with the plasma membrane, discernible changes to the tegument and envelope can be seen by electron microscopy. These changes include the condensation of the virus tegument and envelope and release of the nucleocapsid and some tegument proteins from the virion (Fuller & Lee, 1992). The de-enveloped HSV particle is transported across the cytoplasm (Batterson & Roizman, 1983), by a mechanism that probably involves host cell microtubules (Lycke *et al.*, 1984) to the nuclear membrane, where the viral genome is released from the capsid structure. The virus DNA migrates to the nucleus of the infected cell, via a nuclear pore (Hummeler *et al.*, 1969), and it quickly becomes associated with one or more host cell nuclear proteins in a pronase-phenol-sensitive complex. The release of DNA can take place in the presence of nucleic acid and protein synthesis inhibitors, indicating that *de novo* RNA and protein synthesis are not required for this process (Rice *et al.*, 1976). In the light of these observations it was proposed that a structural component of the virus or a cellular enzyme mediates the DNA release step (Hochberg & Becker, 1968). A function essential for this process has been mapped to the UL36 gene (Batterson *et al.*, 1983), which encodes the tegument protein VP1 (McNabb & Courtney, 1992b). It is possible that other tegument proteins may also be involved in the

release of DNA from the capsid.

(iii) The effects of HSV-1 infection on host cell macromolecular synthesis

Following viral infection, synthesis of a 57KDa cellular species, that corresponds to a heat shock protein, is induced in HSV-2 infected cells (La Thangue *et al.*, 1984). An accumulation of this protein was also seen in HSV-1 infected cells, however to a lower level (Patel *et al.*, 1986). In the majority of cases the viral lytic cycle is not inhibited by the stress response. However it is possible that induction of the stress response in some cell types may cause the lytic cycle to be aborted, resulting in the establishment of cellular transformation (Minson, 1984). In addition, HSV-2 has been shown to induce the expression of a cellular protein, U90, a tumour-specific polypeptide (Hewitt *et al.*, 1991).

Reduced host cell protein synthesis is brought about by the host shut-off protein (vhs), a component of the virion, and Vmw63, one of the polypeptides produced at the onset of infection. vhs acts to induce the degradation of host cell mRNAs which abrogates most host protein synthesis, while Vmw63 affects snRNP localisation and splicing. Both vhs and Vmw63 are described in more detail in *Introduction; Chapter 3, Section 4.3.c* and *Introduction; Chapter 3, Section 3.3.e* respectively.

Section 2.

HSV-1 Gene Expression

(i) General features of HSV-1 gene transcription

HSV-1 genes are transcribed by cellular RNA polymerase II, in the nucleus of the host cell (Alwine *et al.*, 1974; Costanzo *et al.*, 1977). Following transcription, the viral mRNAs share common structural features with cellular RNAs; they have a 5' cap structure, are polyadenylated at the 3' end, and are internally methylated (Bachenheimer & Roizman, 1972; Silverstein *et al.*, 1973; Bartoski & Roizman, 1976). The replication of HSV-1 is coordinated by temporal control of gene expression, in which genes are transcribed and proteins are synthesised sequentially and coordinately. The classification of genes are as follows: immediate early (IE,α), early (E,β), and late (L,γ) (Honess and Roizman, 1974). Five immediate early genes are transcribed at the onset of infection and their expression does

not require *de novo* protein synthesis. The IE proteins activate the early genes which are predominantly those that encode the proteins necessary for DNA replication. The late genes which encode mainly structural proteins can be sub-divided into leaky late ($\gamma 1$) genes that are induced shortly after the E genes and true late ($\gamma 2$) genes which are activated only after the onset of DNA replication (Honess & Roizman, 1974; Clements *et al.*, 1977). Expression within each class is determined by *cis*-acting DNA sequences within the promoter sequences of each gene and *trans*-acting factors within the infected cell, of cellular or viral origin, that interact with defined regions within the promoters.

In addition, the temporal control of gene expression appears to be influenced by the post-transcriptional processes of mRNA splicing and 3' mRNA processing. Recent studies by McGregor *et al.*, (1995) demonstrated *in vitro* that HSV-1 infection induces an activity, which requires Vmw63 gene expression that is responsible for an increase in 3' processing at the polyA sites of two late genes, UL38 and UL44. Processing efficiencies at these polyA sites appear to be inherently low in the absence of Vmw63. In contrast, the polyA sites of selected immediate early and early genes were shown to be efficient processing sites and were not affected by Vmw63 expression.

A number of reports provide evidence that Vmw63 inhibits host-cell splicing, and the repression of expression of genes has been correlated with the presence of introns (Sandri-Goldin & Mendoza, 1992). Inhibition of host cell splicing by Vmw63 is associated with the redistribution of snRNPs, with the consequent effect of promoting shutoff of host protein synthesis (Sandri-Goldin & Mendoza, 1992; Phelan *et al.* 1993; Hardy & Sandri-Goldin, 1994). This effect would be beneficial for HSV-1 as very few of its transcripts are spliced. However a recent report by Sandri-Goldin *et al.* (1995) suggests that the redistribution of snRNPs is not sufficient to inhibit host cell splicing. They postulate that this redistribution might serve to transport newly transcribed viral mRNA to the the periphery of the nucleus.

(ii) HSV-1 promoter sequences

(a) Immediate early gene promoters

Transcription of the five immediate early genes (IE1 to IE5) is mediated by both promoter and enhancer sequences. The promoter component is found within 100bp upstream of the transcriptional start site and contains elements, such as a TATA box, that is recognised by the transcription factor TFIID, and the GC box which is recognised by the transcriptional activator Sp1 (Jones & Tjian, 1985). The enhancer-like elements of IE promoters are situated far upstream from the transcription start site (-140 to -340) and include the consensus sequence 5'-TAATGARAT-3', where R is purine. The consensus sequence is present in one or more copies in either orientation and has been shown to be essential for trans-induction by the virion component VP16 (Mackem & Roizman, 1982a, 1982b; Cordingley *et al.*, 1983; Kristie & Roizman, 1984; Preston *et al.*, 1984; Gaffney *et al.*, 1985; Bzik & Preston, 1986; O'Hare & Hayward, 1987).

Transcription of the five immediate early genes is stimulated 5-10 fold by VP16 (Post *et al.*, 1981; Campbell *et al.*, 1984). The trans-inducing effect is mediated by two cellular components; OCT-1, a transcription factor implicated in the regulation of a number of cellular genes (Pruijn *et al.*, 1986; Staudt *et al.*, 1986; Fletcher *et al.*, 1987; Stern *et al.*, 1989) and the less well characterised CCF factor (Katan *et al.*, 1990). Current results indicate that CCF binds directly to VP16 in the absence of OCT-1 or DNA. The next step in complex assembly is the binding of OCT-1 to the TAATGARAT site, followed by an interaction with the VP16-CCF complex (Gerster & Roeder, 1988; O'Hare & Goding, 1988; Preston *et al.*, 1988; Stern *et al.*, 1989; O'Hare, 1993). A GA-rich element, present in close proximity to at least one TAATGARAT sequence, also confers a strong independent stimulatory response to VP16 (Bzik & Preston, 1986; Triezenberg *et al.*, 1988). The IE-regulatory sequence for the IE1 is contained within a full octamer sequence (ATGCTAATGATAT). Results have shown that this overlapping octamer site increases the activity of the IE1 promoter element by allowing tighter binding of the Oct-1/VP16 complex (Douville *et al.*, 1995).

The promoters of the IE genes 1, 2 and 3 also possess copies of a sequence identified as a binding site for Vmw175 (Faber & Wilcox, 1986, 1988; Kristie & Roizman, 1986; Muller, 1987). The binding of Vmw175 to the IE1 and IE3 promoters results in repression of the promoter in transfection assays suggesting a mechanism for the observed autoregulation of IE proteins (Gelman & Silverstein, 1987a, 1987b; DeLuca & Schaffer, 1988; Paterson & Everett, 1988a, 1988b; Roberts *et al.*, 1988; ; DiDonato & Muller, 1989; Resnick *et al.*, 1989; Everett & Orr, 1991) However, Everett & Orr (1991) have reported

that mutation of the Vmw175 binding sites in both copies of the IE1 promoter in the viral genome has no effect on IE1 expression during normal HSV-1 infection.

(b) Early gene promoters

HSV-1 genes that are expressed early in infection include UL23 and US6 that encode TK, and gD respectively. The promoter sequence for the TK gene has been extensively studied and is comprised of a control region that extends 135bp upstream of the transcription start site and contains five regulatory elements; a TATA box, a CCAAT box, two GC box elements and an octamer binding site (McKnight *et al.*, 1981; Zipser *et al.*, 1981; McKnight & Kingsbury, 1982; Smiley *et al.*, 1983; Halpern & Smiley, 1984; Cohen *et al.*, 1986; Boni & Coen, 1989). The gD promoter requires only 83bp upstream of the transcription start site for efficient induction and, like the TK promoter, contains several regulatory elements including a TATA box and two GA-rich sequences (Everett, 1983 & 1984a).

The TK and gD promoters also contain binding sites for Vmw175 (McKnight *et al.*, 1981; Tedder *et al.*, 1989). However, the binding of Vmw175 to the TK promoter is not a prerequisite for transcription (Imbalzano *et al.*, 1990; Shepard & DeLuca, 1991), and the removal of the three binding sites in the gD promoter does not affect the efficiency of gD expression during a normal HSV-1 infection (Smiley *et al.*, 1992).

(c) Late gene promoters

The structure of a true late gene promoter is much simpler than for IE or E promoters. The only consensus sequence element identified in these promoters is the TATA box. However, several groups have identified additional *cis*-acting sequences downstream from the TATA box, but these sequences do not form recognisable consensus sequences and the specific binding of factors to these regions has not been observed (Mavromara-Nazos & Roizman, 1989; Kibler *et al.*, 1991; Steffy & Weir, 1991).

Efficient expression of late gene promoters requires the presence of IE gene products (DeLuca & Schaffer, 1985) and is also dependent on DNA replication (Mavromara-Nazos & Roizman, 1987), although it is unclear why. The onset of DNA replication may provide a factor which is necessary for transcriptional activation, or it may remove a transcriptional block.

Negative regulatory elements have been identified in late gene promoters (Costa *et al.*, 1985; Blair & Wagner, 1986). For example, upstream of the UL19 (VP5) cap site between -75 and -125 base pairs lies a region that suppresses the activity of the UL19 promoter in transient expression assays (Costa *et al.*, 1985).

(iii) Polypeptides involved in the expression of HSV genes

The principal viral factors that influence the transcription of HSV-1 genes include four of the five IE gene products, Vmw175, Vmw110, Vmw68 and Vmw63, and the major virion protein VP16. The following sections are an overview of their properties and functions:

(a) VP16

VP16 is encoded by gene UL48 and has an apparent molecular weight of 65KDa on denaturing SDS-polyacrylamide gels. The protein is synthesised during the late phase of HSV-1 replication and is phosphorylated. It is a structural component of HSV-1 virions, residing within the tegument of the virus particle. Upon infection, VP16 is released from the virus particle, whereupon it specifically trans-induces the transcription of viral IE genes in the nucleus (Post *et al.*, 1981; Batterson & Roizman, 1983; Campbell *et al.*, 1984). VP16 does not directly interact with viral DNA, rather it physically interacts with cellular factors including CCF (Preston *et al.*, 1988; Katan *et al.*, 1990; Xiao & Capone, 1990) and OCT-1 to form a complex that binds to the TAATGARAT sequence element (Gerster & Roeder, 1988; Stern *et al.*, 1989). Mutational analysis of the VP16 protein led to the identification of eight amino acids within the N-terminal portion of the protein that are involved in its interaction with OCT-1 (Hayes & O'Hare, 1993), whilst the carboxy terminal 80 residues, known as the acidic activation domain, function in IE gene transactivation (Ace *et al.*, 1988; Triezenberg *et al.*, 1988a; Walker *et al.*, 1993). The acidic activation domain is not a requirement for the formation of the OCT-1/CCF/VP16 complex (Greaves & O'Hare, 1989), instead it appears to promote the assembly of an initiation complex containing the TATA box-binding factor, also known as TFIID. The binding of TFIID to the TATA box appears to be the critical step in the formation of the pre-initiation complex which is necessary for transcription *in vitro* (Davison *et al.*, 1983; Sawadogo & Roeder, 1985; Moncollin *et al.*, 1986). However, the mechanism by which the acidic domain functions and stimulates transcription remains to be discovered.

Mutational studies have shown that the transactivating function of VP16 is separate from its requirement in virus particle assembly (Ace *et al.*, 1988). One of the most well characterised VP16 virus mutants is *in*1814 which contains a 4 amino acid insertion at position 379 in the VP16 protein. This mutated form of VP16 is able to perform its structural role but is deficient in IE transactivation (Ace *et al.*, 1989). *in*1814 is viable in tissue culture but impaired for growth, exhibiting a dramatically increased particle:pfu ratio (Ace *et al.*,1988, 1989; Harris & Preston, 1991). The virus is also avirulent after inoculation

into mice, and in an *in vitro* latency tissue culture system, it establishes a latent infection even at high multiplicities of infection (Harris & Preston, 1988). Taken together, these results suggest that the IE gene transactivating activity of VP16 is important for the onset of lytic infection. However, induced expression of UL48 mRNA in neuronal cells infected with a virus recombinant in which the UL48 gene is under the control of the metallothionein promoter demonstrated that despite the presence of VP16, latency was still established in these cells (Sears *et al.* 1991). In addition, OCT-1 expression is not detected in adult murine sensory neurons (He *et al.*, 1989), and therefore the importance of the IE transactivating activity conveyed by VP16 in the establishment of lytic infection is difficult to assess.

The second function described for VP16 is its role in the assembly of the virus particle. Its influence on this process has been deduced from studies on a VP16 deletion mutant that lacks the entire UL48 ORF (Weinheimer *et al.*, 1992). Propagation of this mutant in a VP16-expressing cell line gave yields of virus comparable to those for a wild-type strain. However, the virus did not replicate in non-expressing cells. Following infection of non-expressing cells the deletion mutant produced normal levels of viral DNA synthesis and capsid proteins, but levels of DNA encapsidation were decreased and the further steps of virion maturation were defective. Therefore the absence of VP16 protein synthesis at late times of infection prevents the production of infectious progeny virus and correlates with a profound defect in HSV-1 particle assembly.

(b) Vmw175

Belonging to the IE class of polypeptides, Vmw175 is the product of IE3 which lies entirely within R_S and is therefore present as two copies in the genome (Rixon *et al.*, 1982). The protein is post-translationally modified by phosphorylation, poly-ADP-ribosylation, adenylation and guanylation (Wilcox *et al.*, 1980; Preston & Notarianni, 1983; Blaho & Roizman, 1991), and differential modification gives rise to three electrophoretically distinct forms in extracts of infected cells (Pereira *et al.*, 1977). Phosphorylation of Vmw175 is essential for its interaction with early and late genes, but not for its interaction with IE genes (Papavassiliou *et al.*, 1991).

Vmw175 localises to the nucleus after synthesis (Pereira *et al.*, 1977) and following the onset of viral DNA replication, its distribution changes and it becomes localised to large globular structures in the nucleus (Randall & Dinwoodie, 1986; Knipe *et al.*, 1987). These structures also contain ICP8 (see *Chapter 3; Introduction, Section 4.2*) and have been called 'replication compartments' (Quinlan *et al.*, 1984).

It is a major transactivator of HSV genes, and its expression is essential for early and late protein synthesis (Marsden *et al.*, 1976; Courtney *et al.*, 1976; Watson & Clements, 1978, 1980; Preston, 1979; Dixon & Schaffer, 1980). Analyses of *ts* mutant viruses, in particular *ts*K, (Preston, 1979; Dixon & Schaffer, 1980; DeLuca *et al.*, 1984; DeLuca & Schaffer, 1985) and partial deletion mutants (DeLuca *et al.*, 1985; DeLuca & Schaffer, 1988) have shown that impairing the activity of Vmw175 results in its overproduction and that of other IE mRNAs and an inability to induce synthesis of early and late genes (Marsden *et al.*, 1976; Watson & Clements, 1980).

Vmw175 binds to DNA non-specifically (Freeman & Powell, 1982), but it also shows a significant preference for the consensus ATCGTCNNNNYCGRG, where R= purine, Y= pyrimidine, and N= any base (Faber & Wilcox, 1986). Transient transfection assays have confirmed that Vmw175 can transactivate early gene promoters (Gelman & Silverstein, 1985; O'Hare & Hayward, 1985a), however extensive studies have failed to provide any evidence for the existence of a Vmw175-specific induction sequence (Eisenberg *et al.*, 1985b; Coen *et al.*, 1986; Smiley *et al.*, 1992). Everett (1984b & 1986) has suggested that this transactivation activity is the result of synergistic interaction with Vmw110. Evidence for an interaction between Vmw175 and Vmw110 in infected cells has come from indirect immunoflourescence studies (Mullen *et al.*, 1994), and these results have been further supported by *in vitro* co-immunopreciptiation experiments (Cuifo *et al.*, 1994). In addition, Vmw175 has been shown to form a tripartite complex with components of the cellular transcription machinery acting at the TATA box. These include the TATA-binding protein (TBP) and TFIIB (Smith *et al.*, 1993). This complex formation correlates with the ability of Vmw175 to initiate transcription at minimal promoters that only contain a TATA box.

In addition to its transactivating capabilities, transient transfection assays have shown that Vmw175 is able to repress transcription from both its own and other IE promoters (DeLuca & Schaffer, 1985; O'Hare & Hayward, 1985b; Gelman & Silverstein, 1986). It has been postulated that this phenomenon may occur through binding of Vmw175 to a *cis*-acting element across the transcription initiation site of the gene (Roberts *et al.*, 1988). Vmw175 has been shown to bind to the promoters of IE genes 1, 2 and 3 (Kristie & Roizman, 1986; Muller, 1987; Faber & Wilcox, 1988), early genes UL23 (TK) and US6 (gD) (Michael *et al.*, 1988; Michael & Roizman, 1989; Flanagan *et al.*, 1991).
On the basis of functional studies and comparisons with homologues in other α-herpesviruses, five domains have been identified within Vmw175 (McGeoch *et al.*, 1986b; DeLuca & Schaffer, 1988; Paterson & Everett, 1988a, 1988b). Each of these domains has distinct functions, for example regions 1 and 2 have been implicated in the stimulation of

transcription of early genes (Dixon & Schaffer, 1980), while region 4 is believed to be involved in DNA synthesis, late gene expression and intranuclear localisation (DeLuca & Schaffer, 1988).

Vmw175 has been identified as a component of virions (Yao & Courtney, 1989), however this observation appears to be cell-type dependent. Vmw175 is readily detectable in virions produced in Vero cells and Hep-2 cells (Yang & Courtney, 1995) but studies on virus particles produced in BHK cells have indicated that it is absent in virions and only present in L-particles (see *Chapter 3; Introduction, Section 6*, McLauchlan & Rixon, 1992).

(c) Vmw110

Vmw110 is encoded by three exons from IE gene 1 and is located within R_L. Therefore in common with IE3, it is present as two copies in the genome (Perry *et al.*, 1986). As is the case for Vmw175, Vmw110 is a nuclear phosphoprotein (Ackerman *et al.*, 1984) and is a minor component of virus particles (Yao & Courtney, 1992). It non-specifically activates expression of viral and cellular genes but is not essential for virus replication as mutants are viable in tissue culture (O'Hare & Hayward, 1985a, 1985b; Everett, 1989; Cai & Schaffer, 1992; Chen & Silverstein, 1992; Stow & Stow, 1986; Sacks & Schaffer, 1987). Despite the non-essential nature of Vmw110, mutants give rise to stocks with high particle:pfu ratios and grow very poorly at low multiplicities of infection.

Five functional regions of Vmw110 have been mapped by Everett, (1988), two of which (regions 1 and 5) are of particular interest in the context of transcriptional regulation. Region 1 (amino acids 105-222) contains two zinc finger binding motifs, and mutations within this region result in either complete elimination or a significant reduction in the ability of Vmw110 to activate the HSV-1 gD promoter in the absence of Vmw175 (Everett, 1986). In the presence of Vmw175, mutations in region 5 (amino acids 633-775) markedly reduce the synergistic activation of the gD promoter by Vmw110 (Everett, 1986).

Vmw110 and Vmw175 have been shown to interact physically with each other (Mullen *et al.*, 1994, Yao & Schaffer, 1994), and a dimerization domain identified in the C-terminus of the protein appears to play an important role in this interaction (Cuifo *et al.*, 1994). Given the potent non-specific transactivating ability and DNA binding capability of Vmw110, it is possible that the interaction of Vmw110 with Vmw175 facilitates the interaction of Vmw175 with its low affinity DNA binding sites and with factors in the basic transcription complex such as TFIIB and either the TATA-binding protein (TBP) or TFIID (Smith *et al.*, 1993).

Studies on virus-cell interactions have shown that Vmw110 modifies the distribution

of cellular structures called ND10 domains. These have been identified as distinct nuclear structures that are present in low numbers in a variety of cell types. Stress in the form of heat shock induces a large increase in the number of ND10 domains present in cells (Ascoli & Maul, 1991). However, infection by HSV-1 results in the apparent disappearance of ND10 antigens from nuclear structures. This effect requires the expression of Vmw110, which co-localises with these structures (Maul *et al.*, 1993). It has been suggested that ND10 domains are specific sites of transcription, and that ND10 proteins may be involved in transcriptional regulation (Xie *et al.*, 1993). The function of ND10 domains and the significance of the interaction between Vmw110 and these structures is unclear, however it may be possible that transcriptional regulation modulated by Vmw110 is a result of its interaction with ND10 domains. One of the components of ND10 structures has been identified as PML, a cellular member of the zinc-binding domain family, and Vmw110 has been shown to be essential for the apparent release of PML from the ND10 domains (Maul & Everett, 1994). The zinc finger binding domain of PML is not functionally interchangeable with the zinc finger domain of Vmw110, and PML is not a cellular functional counterpart of Vmw110 (Everett *et al.*, 1995).

Vmw110 also plays a major role in enhancing the reactivation of HSV from latency in a mouse model system (Leib *et al.*, 1989), and of the five IE proteins, only Vmw110 is able to induce reactivation of HSV-2 from latency in an *in vitro* tissue culture system (Harris *et al.*, 1989).

(d) Vmw68

Vmw68, encoded by US1, is also known as ICP22 and is involved in the transition from early to late gene expression (Poffenberger *et al.*, 1993). Little is known about the functions of this protein but a Vmw68 deletion mutant exhibits decreased virulence during acute infection *in vivo*, and a reduced frequency of reactivation from mouse trigeminal ganglia when compared to the parental virus (Poffenberger *et al.*, 1994).

Recent studies have suggested one function of Vmw68 which may influence viral transcription (Rice *et al.*, 1995). Infection of cells with HSV-1 results in the rapid alteration of phosphorylation on the large subunit of host cell RNA polymerase II (RNAPII). *In vivo* RNAPII exists in two discrete forms, IIA and IIO, which differ in the extent of phosphorylation: IIA is unphosphorylated and IIO is hyperphosphorylated (Cadena & Dahmus, 1987; Baskaran *et al.*, 1993; Dahmus, 1993). The different forms are associated with different steps in the transcription cycle and it is thought that the phosphorylation of IIA to IIO may be coincident with transcriptional initiation (Dahmus, 1993; Zawel *et al.*, 1993) . Rice *et al.* (1995) demonstrated that HSV-1 infection results in the intermediate

phosphorylation of RNAPII. The identification of an HSV-1 mutant containing a nonsense mutation in US1 that was unable to modify RNAPII, showed that this effect was required for the normal pattern of transcription of viral genes in some cell lines.

(e) Vmw63

Vmw63, encoded by UL54, is also known as ICP27 and is an essential immediate early phosphoprotein that acts post-transcriptionally during infection to regulate gene expression by promoting the transition from the early to the late phase of infection (McCarthy *et al.*, 1989; Rice & Knipe, 1990; Smith *et al.*, 1992). It localises to the infected cell nucleus and multiple nuclear and nucleolar localisation signals (NLS, and NuLS respectively) have been identified (Mears *et al.*, 1995). These include a strong NLS mapping to residues 110-137, as well as one or more weak NLSs which map to a carboxyl terminal portion of the protein between residues 140 and 512. In addition, a short sequence mapping to residues 110-152 has been shown to function as a NuLS. Mutants with defined lesions in gene UL54 over-express IE and E gene products and greatly reduce the expression of late genes (McCarthy *et al.*, 1989; Rice & Knipe, 1990).

Functional studies have indicated that Vmw63 acts at the post-transcriptional level and influences both polyadenylation and splicing of mRNA (Sandri-Goldin & Mendoza, 1992). Both *in vitro* RNA processing analysis and studies with virus recombinants revealed that Vmw63 could specifically stimulate processing at polyadenylation sites of late viral genes (McLauchlan *et al.*, 1992; McGregor *et al.*, 1995). By contrast the presence of Vmw63 reduces splicing efficiency as assessed in an *in vitro* splicing system and in transfection studies (Sandri-Goldin & Mendoza, 1992; Phelan *et al.*, 1993; Hardy & Sandri-Goldin, 1994). This impairment in splicing is likely to contribute to the shut-off of host cell protein synthesis by decreasing levels of spliced cellular mRNAs available for translation. Such alterations in post-transcriptional processes are probably linked to the redistribution of small nuclear ribonucleoproteins (snRNPs) observed in HSV-1-infected cells (Martin *et al.*, 1987). snRNPs are key components of the cellular splicing machinery and, in infected cells, they become localised in punctate sites which are associated with RNA synthesis (Martin & Okamura, 1981; Fakan *et al.*, 1984). Vmw63 co-localises to these sites and its expression is sufficient for snRNP redistribution from a diffuse speckled pattern within the nucleus to a highly punctate distribution (Martin *et al.*, 1987; Phelan *et al.*, 1993).

In vitro transfection assays performed using plasmids containing the IE genes Vmw175, Vmw110 and Vmw63 demonstrated that in combination with Vmw175 and/or Vmw110, Vmw63 exhibited either a marked repression or stimulation of the expression of different target genes (Sekulovich *et al.*, 1988). These data suggested that Vmw63 interacts

with Vmw175 and Vmw110 and this interaction results in transcriptional activation or repression of HSV-1 genes. Mutational studies by Rice & Lam (1994) have confirmed the presence of at least two distinct essential gene regulation functions in Vmw63. They created three amino acid substitutions in the C-terminal end of the protein that retained the transcriptional repression activity of Vmw63 but were defective at transactivation in cotransfection assays. When introduced into the HSV-1 genome, recombinants containing these mutations replicated viral DNA like wild type, but showed several defects in gene expression including a failure to down-regulate immediate early and early gene expression and an inability to induce late gene expression, similar to the results seen previously with Vmw63 *ts* mutants. Vaughan *et al.* (1992) identified a putative zinc finger metal-binding domain in the carboxy terminal 105 amino acids of the protein however the importance of this domain is not clear.

Indirect immunoflourescence studies have shown that Vmw63 inhibits the normal pattern of nuclear localisation of Vmw110 and Vmw175 in infected cells and that the C-terminal half of the protein is responsible for the redistribution (Zhu *et al.*, 1994; Zhu & Schaffer , 1995). These results suggest that the regulation of HSV-1 gene expression may involve intracellular compartmental restraints for example, the inhibition of the formation of replication compartments within the nucleus.

Section 3.

Replication of HSV DNA

The replication of viral DNA will be dealt with in two parts: (i) the viral origins of replication, and (ii) the proteins required for replication.

(i) Viral origins of replication

In infected cells, viral DNA is detected from 3 hours post infection and continues to increase in abundance for at least another 9-12 hours. The viral DNA is synthesised in discrete sites located close to the nuclear membrane (Rixon *et al.*, 1983). Replication is thought to proceed through a rolling circle mechanism generating concatomers of viral DNA (Jacob & Roizman, 1977; Jacob *et al.*, 1979; Roizman, 1979b). Three origins of replication, ori_L and two copies of ori_S, were identified following studies of defective viruses generated during serial passage of HSV-1 at high multiplicities of infection (Frenkel *et al.*, 1975 & 1976). The origins of replication have been operationally defined as those HSV-1 DNA sequences which are required for amplification of plasmid DNA in cells infected with helper

virus (Vlanzy & Frenkel, 1981; Mocarski & Roizman, 1982). All three sites are not required on a single DNA molecule for efficient replication since studies with mutant viruses have shown that deletion of ori_L or one copy of ori_S has little or no effect on virus replication in cultured cells (Longnecker & Roizman, 1986; Polvino-Bodnar *et al.*, 1987). The HSV-1 strain 17 ori_S was cloned (Stow, 1982) and subsequently precisely defined by Stow and McMonagle (1983). Ori_S, situated within R_S, consists of a 90bp sequence containing a 45bp palindromic sequence featuring 18 centrally located A or T residues flanked by G + C-rich tracts. Ori_L, larger than ori_S, contains a 144bp A + T rich almost perfect palindromic sequence. Due to the size of this palindrome, plasmids containing ori_L are highly unstable in *E.coli* (Weller *et al.*, 1985), thus the functional domains of ori_L have not been investigated as thoroughly as ori_S. However, it has been suggested that ori_S allows DNA synthesis in only one direction, while ori_L, being larger than ori_S would allow bi-directional DNA synthesis. The existence of or need for bi-directional synthesis has not been established and both ori_S and ori_L appear to be functionally identical in transient expression assays (Wu *et al.*, 1988). The potential ability of the origins to form cruciform structures containing a stretch of low melting point (A + T-rich) DNA probably facilitates strand-separation in these regions of the genome (Stow, 1985).

It is interesting that both ori_S and ori_L are situated between divergent transcription initiation sites; ori_S is situated between IE3 and IE4/IE5, and ori_L is situated in the middle of U_L, between UL29 and UL30, which are both early genes. This perhaps suggests that initiation of synthesis might be activated or enhanced by the changes in the local environment of the DNA during transcription initiation events.

(ii) Herpesvirus polypeptides associated with viral DNA replication

The proteins involved in viral DNA replication include proteins that are essential for viral origin-dependent amplification of DNA, and also enzymes involved in nucleic acid metabolism which are not essential for viral growth in tissue culture. Seven HSV-1 genes have been identified as being essential for DNA replication and at least five additional genes have been shown to stimulate DNA replication (Keir & Gold, 1963; Kit & Dubbs, 1965; Cohen, 1972; Wu *et al.*, 1988).

In order to identify all of the necessary genes required during origin-dependent DNA replication, minimal fragments of the HSV-1 genome that were able to support replication of

plasmid vectors containing HSV-1 origins of replication in transient complementation assays were analysed (Challberg, 1986; Wu *et al.*, 1988). These studies identified seven genes which were directly required for DNA replication. Two of these genes, which encoded the DNA polymerase (UL30) and the major DNA binding protein (MDBP;UL29) also known as ICP8, were already known. The 140KDa DNA polymerase exhibits novel DNA polymerase activity readily distinguishable from host enzymes. For example, it is inhibited by the pyrophosphate analogue phosphonoacetic acid (PAA) (Hay & Subak-Sharpe, 1976) which in experimental systems is a useful method of preventing viral DNA replication. The enzyme has intrinsic 3' to 5' exonuclease activity serving a proofreading function (Knopf, 1979; O'Donnell *et al.*, 1987). It also possesses ribonuclease activity that specifically degrades RNA-DNA heteroduplexes as well as DNA duplexes in the 5' to 3' direction (Crute & Lehman, 1988), thus enabling removal of the RNA primers that initiate synthesis of Okazaki fragments during lagging strand synthesis. The association of ICP8 with replicating viral DNA is essential (Conoley *et al*, 1981), although the function of this protein is not well defined. It binds to single-stranded DNA and may act to reduce the melting temperature at poly(dA).poly(dT) DNA tracts (eg. at origins of DNA replication), thereby facilitating strand-separation (Powell *et al.*, 1981). After viral replication commences, indirect immunoflourescence studies have demonstrated that both Vmw175 and ICP8 localise to large globular structures within the nucleus (Randall & Dinwoodie, 1986; Knipe *et al.*, 1987). In addition, pulse labelled DNA has also been shown to accumulate in similar sites (Rixon *et al.*, 1983). These structures are known as replication compartments (Quinlan *et al.*, 1984; Kops & Knipe, 1994). The formation of replication compartments and the localisation of the HSV-1 DNA polymerase to these sites is dependent on the activity of ICP8 (de Bruyn Kops & Knipe, 1988; Bush *et al.*, 1991).

The other genes required for DNA replication were identified as UL5, UL8, UL9, UL42 and UL52. Gene UL42 encodes a DNA binding protein (Parris *et al.*, 1988), and has been shown to physically interact with the viral DNA polymerase to form a heterodimer, which acts to increase the rate of DNA synthesis (Gallo *et al.*, 1988; Gottlieb *et al.*, 1990; Hernandez & Lehman, 1990). The double-stranded DNA binding activity conferred by the UL42 gene product may act as a clamp, decreasing the probability that the polymerase will dissociate from the template after each cycle of catalysis (Gottlieb & Challberg 1994). UL9 encodes a polypeptide that binds specifically to ori sequences (Olivo *et al.*, 1988) and which also has helicase activity (Boehmer *et al.*, 1993). The UL9 gene product catalyses the unwinding of DNA in the 3' to 5' direction, and activity is specifically stimulated by ICP8, which increases the rate and extent of helicase activity. There is evidence that UL5 also encodes a helicase (Zhu & Weller, 13th International Herpesvirus Workshop abstract;

Irvine, 1988). UL5, UL8 and UL52 gene products have been shown to form a complex in which each protein is present in equimolar amounts. The complex formed acts as a primase and helicase (Crute *et al.*, 1989).

Enzymes that have accessory functions in replication include: alkaline DNase, TK, ribonucleotide reductase (RR), uracil-DNA glycosylase and dUTPase. Alkaline DNase, encoded by UL12, differs from host cell nucleases in its high pH optimum and exhibits exo- and endonuclease activity (Strobel-Fidler & Francke, 1980; Hoffman, 1981). Alkaline DNase activity does not appear to be essential for viral DNA synthesis but may play a role in the processing or packaging of viral DNA into infectious virions (Chou & Roizman, 1989; Weller *et al.*, 1990). TK, encoded by UL23, acts as a deoxypyrimidine kinase (Jamieson & Subak-Sharpe, 1974) but also phosphorylates a number of nucleoside analogues not phosphorylated efficiently by cellular kinases (Kit & Dubbs, 1965). RR functions to reduce ribonucleotides to deoxyribonucleotides, creating a pool of substrates for DNA synthesis. It consists of two non-identical subunits, R1 and R2 (Frame *et al.*, 1985), that are tightly associated in a $\alpha 2\beta 2$ complex and both subunits are required for activity (Ingermarson & Lankinen, 1987). R1 and R2 are encoded for by UL39 and UL40 respectively (McLauchlan & Clements, 1983). R1 possesses a unique N-terminal domain which is not directly involved in ribonucleotide reduction (Nikas *et al.*, 1986; Wymer *et al.*, 1989; Conner *et al.*, 1992). This N-terminal domain contains amino acid sequences which are characteristic of a protein kinase (Chung *et al.*, 1989; Paradis *et al.*, 1991), and Cooper *et al.* (1995) have characterised a novel kinase acitvity within the R1 subunit. It has been postulated that the kinase activity may play a role during immediate-early times post infection, phosphorylating cellular or viral proteins involved in subsequent stages of the replication cycle (Conner *et al.*, 1992b; Sze & Herman, 1992). The uracil DNA glycosylase, encoded by UL2 (Worrad & Caradonna, 1988), is thought to function in DNA repair and proof-reading. It acts to remove uracil residues in DNA caused by the deamination of cytosine residues. It is not essential for growth of the virus in tissue culture (Mullaney *et al.*, 1989). dUTPases act to hydrolyse dUTP to dUMP, providing both a mechanism to prevent incorporation of dUTP into DNA and a pool of dUMP for conversion to dTMP by thymidylate synthetase. dUTPase is encoded by UL50 (McGeoch *et al.*, 1988b) and is not essential for growth of the virus in tissue culture (Fisher & Preston, 1986).

Structure, Assembly and Maturation of Virus Particles

Section 1. The HSV-1 virion

The complexity of the virion has been highlighted by the fact that of the 75 genes present on the virus genome, over half are known to or suspected to encode proteins which are present in the virion or involved in virion assembly. This complexity is further emphasised by the multifunctional properties of certain structural components, for example VP16, the major immediate early transcriptional activator, is also a major structural component of the HSV-1 tegument.

The herpesvirus particle can be described as three morphologically distinct components; the nucleocapsid (consisting of the capsid containing viral DNA), tegument and envelope.

Section 2. The Capsid

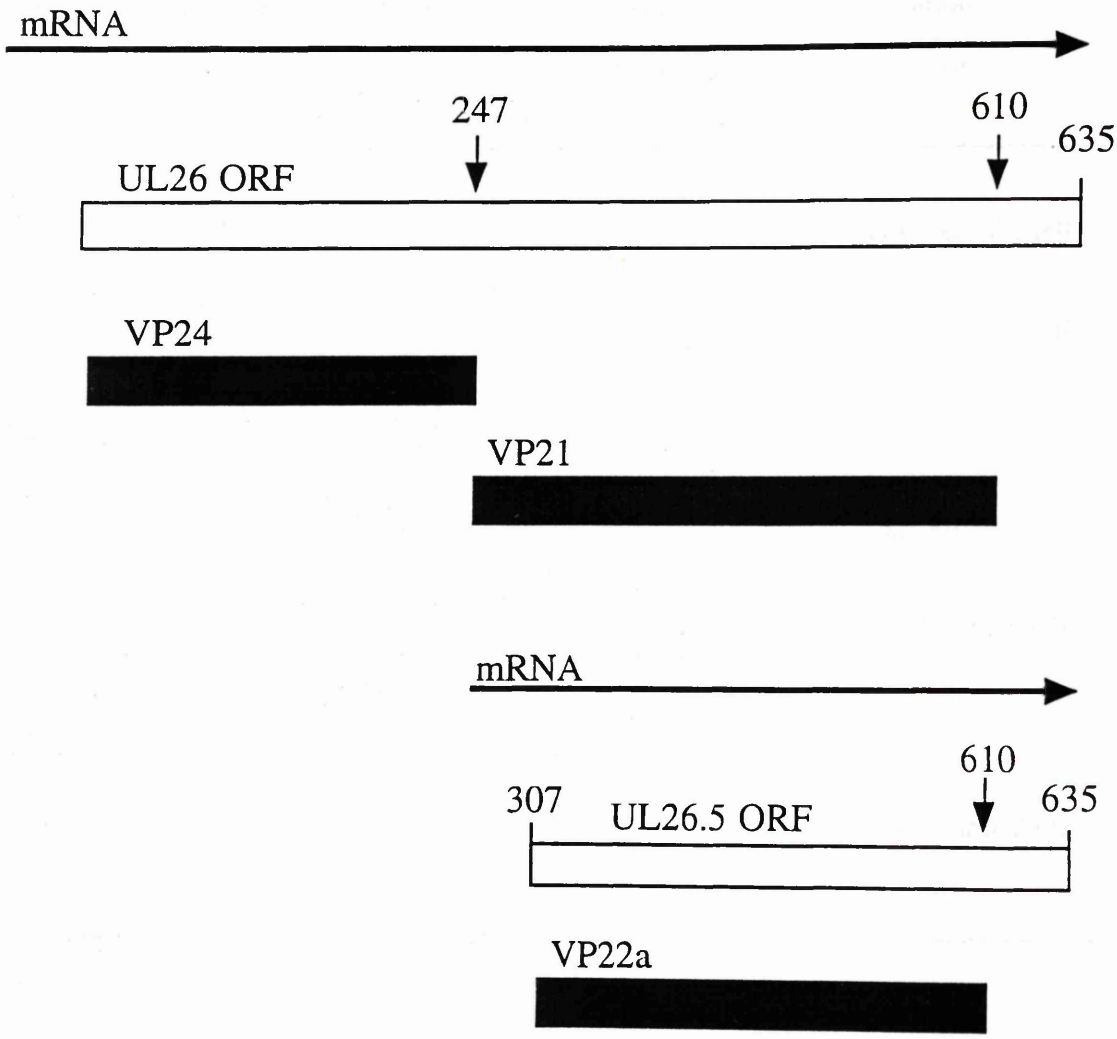
The morphology of the capsid is a characteristic feature of all herpesviruses. It is 150nm in diameter and is composed of 162 capsomers, of which 150 are hexamers and 12 are pentamers arranged in the form of an iscoahedron (Wildy *et al.*, 1960). Three separable forms of capsid can be isolated from infected tissue culture cells; empty capsids with no internal structure, intermediate capsids which lack viral DNA but possess a core in the form of a second proteinaceous layer inside the outer capsid shell, and full capsids which contain the viral genome (Gibson & Roizman, 1972; Perdue *et al.*, 1975). By convention, these are referred to as A (empty), B (intermediate) and C (full) capsids, respectively.

The polypeptides which are present in HSV-1 capsids have been determined (Gibson & Roizman, 1972; Heilman *et al.*, 1979; Cohen *et al.*, 1980, Rixon *et al.*, 1990). In addition, the contribution of individual proteins to capsid structure has been analysed by localisation studies using monospecific antibodies and immuno- and cryo-electron microscopy techniques (Gibson & Roizman, 1972; Heilman *et al.*, 1979; Cohen *et al.*, 1980; Davison *et al.*, 1992). A and B capsids have five structural proteins in common: VP5 (155KDa), VP19C (54KDa), VP23 (33KDa), VP24 (24KDa) and VP26 (12KDa). In addition B capsids also contain VP21 (42KDa) and VP22a (38KDa). With the exception of the internal scaffolding proteins VP21 and VP22a, the protein compositions of types A and C capsids are identical to that of type B capsids (reviewed by Rixon, 1993).

Table 3. Protein composition of HSV-1 B capsids

Protein	Approx. M_r ($X10^{-3}$)	Gene	Presence in A capsids	Location in capsid
VP5	155	UL19	+	icosahedral shell
VP19C	53	UL38	+	icosahedral shell
VP21	42	UL26	-	scaffold
VP22a	38	UL26.5	-	scaffold
VP23	33	UL18	+	icosahedral shell
VP24	25	UL26	+	?
VP26	12	UL35	+	icosahedral shell

Figure 4. Organisation of UL26 and UL26.5 genes and the derivation of the capsid proteins VP21, VP22a and VP24. Numbers refer to amino acid residues in UL26 and the arrows indicate positions of cleavage sites.



(i) Capsid proteins

The proteins that make up B capsids are listed in Table 3. VP5, generally referred to as the major capsid protein, is the major component of the hexons and pentons (Schrag *et al.*, 1989; Newcomb *et al.*, 1993; Zhou *et al.*, 1995), and is essential for capsid assembly (Weller *et al.*, 1987; Desai *et al.*, 1994). VP19c and VP23, also are essential for capsid assembly (Pertuiset *et al.*, 1989) and are believed to form trivalent connections which link adjacent capsomeres (Newcomb *et al.*, 1993; Desai *et al.*, 1994). Braun *et al.* (1984) have also reported DNA binding activity by VP19c. Evidence from images obtained by cryo-electron microscopy of capsids suggests that VP26, the smallest of the capsid proteins, is present at the tips of hexons (Booy *et al.*, 1994; Trus *et al.*, 1995; Zhou *et al.*, 1995). Therefore, these four proteins make up the outer capsid shell.

More recently, a 75KDa protein encoded by the UL6 open reading frame has also been identified as being associated with the capsid (Patel & Maclean, 1994). It is present in very low amounts and although its precise location has not been identified, it appears to be tightly integrated into the capsid shell. Evidence obtained from a UL6 *ts* mutant called *ts*F18 has suggested that UL6 is required for cleavage and packaging of nascent viral DNA (Sherman & Bachenheimer, 1987).

The proteinaceous core in B capsids is composed of processed forms of the UL26 and UL26.5 gene products (Lui & Roizman, 1991a). These are overlapping genes which are arranged such that the translated portion of UL26.5, termed preVP22a, also represents the C-terminal 329 residues of the 80KDa polypeptide encoded by UL26. This 80KDa polypeptide contains a serine protease activity, located in the N-terminal portion of the protein, which is responsible for cleaving itself at 2 sites. This generates 3 products: VP24 which consists of the N-terminal 247 residues, VP21 which is composed of residues 248-610 and a C-terminal fragment of 25 residues (amino acids 611-635). The C-terminal cleavage site is also present in preVP22a and cleavage by the UL26 product at this site generates VP22a. The organisation of the UL26 and UL26.5 genes and gene products are summarised in Fig. 4. The protease activity resident within the UL26 product is essential for the maturation of virions. A temperature sensitive mutant *ts*1201, with a lesion in the protease, is unable to process preVP22a and makes capsids with a larger internal core which are unable to encapsidate DNA (Preston *et al.*, 1983; Preston *et al.*, 1992). However, a mutant virus, lacking full length preVP22a, showed that although growth was severely impaired for this virus, a small number of mutant progeny were produced (Matusick-Kumar *et al.*, 1994). This unexpected observation lead to the suggestion that either the deleted form of preVP22a, produced by the mutant, was able to perform to a limited extent the functions of full length preVP22a, or more likely that the autoproteolysis of UL26 resulting in VP21

may have been able to complement to a limited extent the preVP22a deletion (Matusick-Kumar *et al.*, 1994).

(ii) B Capsid assembly

Herpesvirus capsids are assembled in the nuclei of virus-infected cells (Morgan *et al.*, 1954) and not surprisingly immunoflourescence studies of HSV-1 infected cells have found that capsid proteins localise predominantly in the nucleus (Powell & Watson, 1975; Cohen *et al.* 1980). Nicholson *et al.* (1994) showed however, that the essential proteins VP5 and VP23 are not transported to the nucleus in the absence of preVP22a and VP19C, suggesting that the formation of complexes between these proteins is an important early stage in capsid assembly. Newly synthesised VP5 has been shown to bind to the cytoskeleton and then pass from the cytoplasmic to the nuclear framework along these filaments (Quinlan & Knipe, 1983; Bibor-Hardy *et al.* 1985b). Nicholson *et al.*, (1994) therefore suggested that preVP22a may mediate the binding of VP5 to the cytoskeleton.

Significant advances in the understanding of capsid morphogenisis have resulted from the assembly of HSV-1 capsids in insect cells using a panel of recombinant baculoviruses (Tatman *et al.*, 1994; Thomsen *et al.*, 1994). The six capsid protein genes encoding the seven proteins that make up the capsid (see Table 3) were each cloned into baculovirus expression vectors. Co-expression of these six genes in insect cells resulted in the formation of capsids that were indistinguishable in appearance and protein composition from those made during HSV-1 infection in mammalian cells. This demonstrated that the proteins encoded by the known capsid genes contained all of the structural information necessary for capsid assembly and that other virus-encoded proteins are not required for this process. In addition, omission of single recombinant baculoviruses from the system allowed the role of individual HSV-1 proteins in capsid assembly to be determined. VP23, VP5 and VP19C were all shown to be essential for the assembly of capsids, while VP26 had no apparent effect on capsid formation. Omitting baculovirus recombinants that express either the UL26 or UL26.5 gene products did not prevent the formation of capsids. Moerover, omission of the UL26 gene product resulted in the appearance of large cored B capsids similar to those produced by the virus mutant *ts*1201. Removal of both UL26 and UL26.5 products precluded the formation of closed capsid shells. It had been previously documented that cleavage of UL26 and UL26.5 is not essential for the assembly of capsids, but is essential for the encapsidation of viral DNA (Preston *et al.*, 1983; Gao *et al.*, 1994). However, recent work done by Kennard *et al.*, (1995) showed that when cells were multiply infected with baculoviruses expressing truncated forms of the UL26.5 gene product

corresponding to VP22a, and the essential components of the capsid shell, no capsids were detected, whereas large numbers of capsids were observed when the full length preVP22a was used as a scaffold. These results suggested that the C-terminal 25 amino acids of preVP22a may be directly involved in the assembly of capsids. In addition, coimmunoprecipitatation of VP5 with preVP22a, requires the C-terminal 25 amino acids of the protein (Thomsen *et al.*, 1995).

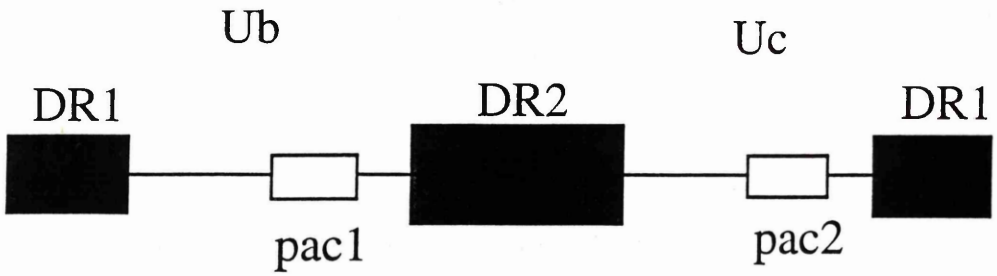
Studies by Newcomb *et al.* (1994) showed that HSV-1 capsids could be assembled spontaneously in a cell-free system consisting of extracts prepared from insect cells that had been infected with recombinant baculoviruses encoding HSV-1 capsid proteins. These capsids resembled native HSV-1 capsids in terms of morphology, sedimentation rate on sucrose density, and their ability to react with antibodies specific for VP5. However the cavity of these capsids appeared to be empty, and there was no evidence for the large mass of preVP22a. In common with the capsids produced in insect cells, capsid fromation *in vitro* required the presence of VP5, VP19, VP23, preVP22a and the mature form of the UL26 encoded protease, but not VP26.

(iii) A and C capsids

B capsids are believed to be the precursors of C capsids and probably of A capsids (Perdue *et al.*, 1976; Preston *et al.*, 1983). Formation of C capsids is the result of DNA packaging which is accompanied by the removal of the internal scaffold and an associated loss of the proteins VP21 and VP22a, while the generation of A capsids is believed to be a consequence of abortive packaging. Apart from the UL26 and UL26.5 gene products the genesis of mature DNA-containing C capsids during infection requires a range of proteins involved in the cleavage and encapsidation of viral DNA. These include the gene products of UL28 (Addison *et al.*, 1984, 1990; Tengelsen *et al.*, 1993), UL33 (Al-Kobaisi *et al.*, 1991) and UL6 (Sherman & Bachenheimer, 1987; Patel & MacLean, 1994) since *ts* mutants defective in DNA packaging have been identified with lesions in each of these genes. An alkaline nuclease deficient mutant virus, generated by inserting a *lacZ* gene cassette into the UL12 gene (Weller *et al.*, 1990) was found to be unable to produce infectious virions suggesting that the nuclease may play a role in processing or packaging of viral DNA into virions (Shao *et al.*, 1993).

Major advances have recently been made towards understanding the structure of the virion core. Due to the preparation techniques of viruses for electron microscopy it was thought that the core consisted of a cylindrical protein plug around which the viral DNA was wound (Furlong *et al.*, 1972). However, Booy *et al.*, (1991) using cryo-electron microscopy in combination with computer filtration to avoid interference from the capsid

Figure 5. Structure of the HSV-1 *a* sequence. The *a* sequence is flanked by the DR1 repeats and contains an internal series of repeated sequences (DR2). The Ub and Uc regions compose the non-repeated regions and contain the pac1 and pac2 sequences respectively.



shell, have since shown that the viral genome is packaged as a uniformly dense ball of DNA.

Section 3. Packaging of viral DNA

Following replication of the viral genome, DNA is packaged into preformed capsids via a mechanism still not fully understood. Prior to, or during the packaging process, cleavage of the concatomeric HSV-1 DNA results in unit length genomes being packaged. Although DNA molecules shorter than genome length are packaged, only capsids containing full-length genomes are enveloped (Vlazny *et al.*, 1982). Sequences responsible for packaging and the resolution of monomeric HSV-1 genomes from the concatomeric replicative intermediate (Spaete & Frenkel, 1982; Stow & McMonagle, 1983; Stow *et al.*, 1983) reside in the *a* sequences at the termini of the genome, and at the junction between the L and S segments. Multiple *a* sequences are usually evident at the L/S junction and at the terminus of the L segment, while a single copy is present at the terminus of the S segment (Wagner & Summers, 1978). The *a* sequence (illustrated in Fig. 5) is approximately 250-500bp in length, depending on the number of internal repeats (DR2) present (Roizman, 1979). The direct repeats (DR1) that flank the *a* sequence are 20bp in length. The regions flanking the internal repeats are termed Ub and Uc and contain *cis* -acting elements (pac1 and pac2) that direct cleavage and packaging (Deiss *et al.*, 1986; Varmuza & Smiley, 1985). Pac1 and pac2 elements are distinct in nucleotide sequence but both direct the cleavage of the genome at defined distances within the same positions of the flanking DR1 repeats: 40-47bp from pac1 and 30-35bp from the pac2 sequence. The *a* sequences of different HSV-1 isolates are not identical except for the pac1 and pac2 elements that are located in similar positions relative to the site of cleavage. The mechanism by which viral DNA is cleaved and packaged into capsids is not understood, however the available evidence suggests that both processes are intimately linked. The model that has emerged proposes that empty capsids scan concatomeric DNA until contact is made in a specific orientation with a pac2 sequence or a DNA/protein complex at a pac2 site. In support of the formation of a complex, two virus-induced proteins of >250KDa and 140KDa bind in a sequence specific manner to pac2 sequences (Chou & Roizman, 1989). The >250KDa species corresponds to the tegument protein VP1 but the 140KDa protein has not been identified. Following recognition, cleavage within DR1 occurs. Since cleaved DNA is nuclease resistant, it is considered that cleavage and the initiation of the packaging processes are coincident (Deiss & Frenkel, 1986). DNA is then packaged until a second *a* sequence in the same orientation and one genome length from the first *a* sequence is encountered. A second cleavage event then occurs to produce unit length DNA. Cleavage occurs at staggered sites

Table 4. HSV tegument proteins and their functions.

protein	Gene	Function	status
	UL11	2 myristilated protein products found in light particles, thought to be tegument proteins	ne
	UL13	Protein kinase	ne
VP1	UL36	Very large tegument protein, responsible for the release of viral DNA from capsids at nuclear pores	e
	UL37	Tegument phosphoprotein, function unknown, may have DNA binding role, binds to ICP8	--
vhs	UL41	Virion host shutoff (vhs) limits the expression of both viral and host cell genes by destabilising mRNAs	ne
VP11/12	UL46	major structural component of the tegument, fuction unclear	ne
VP13/14	UL47	Proteins that act to enhance VP16 mediated transactivation	ne
VP16	UL48	Major structural tegument protein and immediate-early gene transactivator	e
VP22	UL49	Major structural component, function unknown	?
	US3	Protein kinase that post-translationally modifies the UL34 gene product	ne
	US9	10K protein of unknown function	ne
	US11	RNA binding activity	ne
Vmw175	RS1	Only found in L-particles. Major transinducer of HSV genes. Mutifunctional protein that participates in the formation of complexes with the promoter regions of many early and late genes	e
Vmw110	RL2	Transactivator of early and late genes. Found in the nucleus of infected cells, strong synergy with Vmw175	ne/e

in the genome and therefore in order to maintain the integrity of the ends of the genome, it is suggested that gap repair is performed following cleavage (Deiss & Frenkel, 1986).

Section 4. The Tegument

The electron-opaque amorphous region surrounding the capsid known as the tegument accounts for approximately 65% of the volume of the virion (Schrag *et al.*, 1989). Polypeptides assigned to the tegument were traditionally those that could not be designated as being present in either the capsid or envelope. Therefore the composition of the tegument is less well defined than for the capsid or the envelope. To date there are fourteen genes known to encode tegument proteins. Table 4 summarises the polypeptides assigned to the tegument, their functions if known and whether or not they are essential components of the virus particle.

Unlike the capsid and envelope proteins which have different but widely used standard nomenclatures (VP(x), for capsid and g(x) for the envelope), no standard system exists for designating tegument proteins. Most workers in the field use the VP nomenclature where possible, but many species identified as structural components were not described in the original publication (Heine *et al.*, 1974). As a consequence, although the major and some minor structural species have been assigned a VP number, some proteins have no such assignment and for others the VP designation is obscure. For descriptive purposes therefore, the VP nomenclature system will be used wherever possible while for those which do not have a well documented VP assignment, the protein will be described according to gene number designation. The exception to this is the UL41 gene product which will be referred to as the vhs protein. Since there is no accepted systematic method for describing the tegument proteins, the following sections classify them according to their abundance in the virion. Therefore proteins which have a copy number of greater than 1000 per virion will be listed as major components while those present at less than 1000 copies will be designated minor components.

(i) Major components of the tegument

(a) VP11 and VP12

UL46 encodes two proteins, VP11 and VP12, and combined there are an estimated 1300 copies of these proteins per virion (Zhang & McKnight, 1993). VP11 and VP12 have apparent molecular weights, on denaturing polyacrylamide gels, of 93KDa and 87KDa respectively. These are thought to be two differently phosphorylated forms of the same

primary translation product since the predicted phosphorylation sites in the translated DNA sequence result in proteins of these sizes (McGeoch *et al.*, 1988b; Bairoch, 1991). The abundance of VP11/12 in the virion would perhaps imply that these proteins had important structural roles, however deletion mutants lacking the UL46 gene have shown that VP11/12 are not essential for viral growth or assembly in cell culture (Zhang *et al.*, 1991).

From transfection studies, the UL46 gene products were reported to be involved in VP16-mediated transcriptional activation, its presence acting to enhance the induction by VP16 of a TK reporter construct 2- to 3-fold in transient expression assays (McKnight *et al.*, 1987; Zhang *et al.*, 1991). In contrast to these results however, characterisation of a UL46 deletion mutant virus indicates that the UL46 gene products have no apparent effect on the ability of VP16 to induce a TK reporter gene in cells transformed with a construct which expresses TK under the control of an IE promoter (Zhang *et al.*, 1991). Despite the lack of any apparent effect on IE synthesis, a TK^+ variant of this mutant exhibits a 2 hour delay in the expression of the viral thymidine kinase gene at early times post-infection (Zhang & McKnight, 1993).

(b) VP13 and VP14

UL47 was shown to encode proteins VP13 and VP14, which have similar apparent molecular weights of 81KDa and 82KDa, using an antisera raised against a synthetic oligopeptide corresponding to the predicted protein product of the UL47 gene (McLean *et al.*, 1990). There is a combined total of approximately 1800 copies of VP13 and VP14 per virus particle (Heine *et al.*, 1974; Zhang & McKnight, 1993). Similar to VP11/12 the two products of UL47 are thought to be a result of post-translational modifications which include phosphorylation, glycosylation (McLean *et al.*, 1990; Meredith *et al.*, 1991; Whittaker *et al.*, 1991) and nucleotidylylation (Blaho *et al.*, 1994). In addition, a second in-frame initiation codon, 99bp down-stream from the predicted translational start site of UL47 could contribute to the production of the two forms (McKnight *et al.*, 1987; McLean *et al.*, 1990; Meredith *et al.*, 1991).

Similar to VP11/12, the high copy number of VP13/14 in the virion may imply that these proteins have important structural roles, however UL47 deletion mutant viruses have shown that these proteins are not essential for viral growth in cell culture and are not required for viral assembly (Zhang *et al.*, 1991). As is the case for VP11/12, a role for VP13/14 in modulating the ability of VP16 to enhance IE transcription has been suggested, however conflicting evidence exists as to whether VP13/14 stimulates or represses VP16 activity (McKnight et al., 1987; Zhang et al., 1991; Zhang & McKnight, 1993). From transfection

studies, the UL47 gene product(s) appears to reduce the levels of VP16-mediated induction by 2 to 3-fold in transient expression assays, and this effect is seen either in the presence or the absence of expression of the UL46 gene (Zhang & McKnight 1993). By contrast, TK^- virus in which UL47 is deleted induces lower levels of TK activity in cells transformed with a construct which expresses TK under the control of an IE promoter. In addition, a TK^+ variant of this mutant exhibits delayed expression (delayed by 2 hours) of viral TK activity in infected cells (Zhang & McKnight 1993). At present, the conflict in these data remain unresolved and the precise function of VP13/14 is unclear.

(c) VP16

VP16, the product of gene UL48, is one of the most abundant tegument proteins with approximately 1647 copies per virus particle (Zhang & McKnight, 1993). It functions not only as a major transactivator of IE genes (see *Introduction; Chapter 3, Section 3.3.a*) but is also essential for the assembly of the virus particle (Weinheimer *et al.*, 1992). The construction of a number of VP16 mutants containing oligonucleotide insertions along the length of the UL48 ORF showed that the structural requirements of VP16 for transcription activation and virion assembly differ (Ace *et al.*, 1989). For example, a series of seventeen mutants each containing a 12bp insertion at sites spanning the UL48 ORF identified regions of the protein which were important for virion assembly but not for transinduction. Insertions at amino acid positions 79 and 244 disrupted the assembly process of the resulting virus mutants and on superinfection the mutants containing these insertions were not able to complement the defect exhibited by *ts*2203, which has a mutation that maps to the 5' end of UL48 and is defective in DNA packaging and virus assembly. On the other hand, insertions at amino acid positions 172, 177 and 379 disrupted the ability of the protein to form the IE initiaton complex, but these mutants were able to complement *ts*2203 on superinfection.

(d) VP22

VP22 is encoded by UL49 (Elliot & Meredith, 1992) and is a major structural component, with an estimated average of 1590 copies per virion (Heine *et al.*, 1974). VP22 is extensively post-translationally modified. It is highly phosphorylated, ADP-ribosylated (Preston & Notarianni, 1983), nucleotidylylated (Blaho *et al.*, 1994), and may also be glycosylated (Meredith *et al.*, 1991). The functions of ADP-ribosylation and nucleotidylylation in HSV replication are not known but in other cases these modifications

are often seen in proteins with regulatory functions. For example, ADP-ribosylation is a characteristic feature of G proteins involved in intracellular signalling (Goldman *et al.*, 1981; Goding *et al.*, 1983). VP22 has been shown via indirect immunoflourescence studies to have a perinuclear localisation (Elliot & Meredith, 1992). An association with the nuclear matrix during the virus replication cycle in addition to the fact that the protein is able to bind to HSV DNA (Blair & Honess, 1983), might suggest that VP22 has a regulatory role in gene transcription. Although the function of VP22 and whether it is required for viral growth have not been elucidated, it has recently been shown that VP22 exhibits a strong and stable interaction with VP16. In addition, co-expression of these proteins in infected cells results in the relocalization of both proteins from their normal sites to a novel structures situated at the edge of the nucleus and the interaction was dependant on presence of the VP16 activation domain (Elliot *et al.*, 1995). These results suggest that VP22 may have a role in the regulation of VP16 function, or that the interaction is important for the condensation of tegument proteins during the assembly of the virus particle. The major part of this thesis investigates the structural properties of VP22.

(ii) Minor components of the tegument

(a) VP1

VP1 is a 270KDa protein encoded by UL36 that is present at levels of less than 150 copies per virion (Heine *et al.*, 1974). The apparent molecular weight of 270KDa is a significant underestimate of the predicted molecular weight which is 336KDa. On the basis of reactivity of oligopeptide antisera raised against the N- and C-terminal portions of the protein, the cell- and virion-associated 270KDa form of VP1 appears to represent full-length UL36 gene product that is synthesised in a mature form and is not N- or C-terminally cleaved (McNabb & Courtney, 1992a and 1992b). Studies indicate that VP1 is transcribed as a true late protein, its synthesis being dependent on viral DNA replication (McNabb & Courtney, 1992a) and it is phosphorylated by a tegument-associated protein kinase (Lemaster & Roizman, 1980). Indirect immunoflourescence has shown that VP1 is diffusely distributed throughout the infected cell with no specific compartmentalisation of the polypeptide (McNabb & Courtney, 1992a). VP1 appears to interact tightly with nucleocapsids (Campadelli-Fiume *et al.*, 1991) and a complex between VP1 and another unknown 140KDa protein (Chou & Roizman, 1989) is capable of binding to the *a* sequence of the genome (see *Chapter 4; Introduction, Section 3*; Chou & Roizman, 1989). Since *a* sequences are required for the cleavage and packaging of viral DNA into capsids VP1 may be involved in these processes, as well as the release of DNA from the capsid (Batterson *et*

al., 1983). Evidence supporting this comes from a temperature sensitive mutant, *ts*B7, which contains a disruption in UL36. Following infection with this mutant, newly-formed capsids accumulate at nuclear pores and fail to release viral DNA at the non-permissive temperature (Batterson *et al.*, 1983).

(b) UL37

Studies on the UL37 gene product have shown that it encodes a protein with an apparent molecular weight of 120KDa which is phosphorylated soon after translation (Shelton *et al.*, 1990; Albright & Jenkins, 1993). UL37 is regulated as a late gene, its expression being dependent on the synthesis of viral DNA (Shelton *et al.*, 1990). The protein co-elutes from single- and double-stranded DNA columns with the major DNA binding protein ICP8, leading to the suggestion that interaction between the UL37 protein and ICP8 accounts for the apparent DNA binding ability of the UL37 protein (Albright & Jenkins, 1993). Western blot analysis performed on detergent-treated virus particles in order to remove the envelope proteins demonstrated that the UL37 protein is a structural component of the tegument (McLauchlan *et al.*, 1995; Schmitz *et al.*, 1995). Indirect immunoflourescence studies have shown that the UL37 encoded protein is distributed throughout the infected cell, with concentrations being greater in the cytoplasm than in the nucleus (McLauchlan *et al.*, 1995).

(c) UL41

UL41 encodes a virion host shutoff function commonly referred to as the vhs protein. It is present as a minor component of the tegument and its activity within the infected cell, like the UL46, UL47 and UL48 gene products, does not require *de novo* synthesis. An HSV-1 mutant, vhs1, selected on the basis of its inability to suppress host protein synthesis was subsequently shown to be defective at inducing accelerated turnover of host mRNAs (Read & Frenkel, 1983; Kwong & Frenkel, 1987; Strom & Frenkel, 1987). Marker rescue experiments mapped the vhs1 mutation to gene UL41 (Kwong *et al.*, 1988), and the vhs protein encoded by UL41 was subsequently characterised by Smibert *et al.* (1992). vhs accumulates late in infection and is packaged into the tegument as a minor component (McLauchlan *et al.*, 1992a; Smibert *et al.*, 1992). It is not essential for virus replication in tissue culture (Fenwick & Everett, 1990b; Read & Frenkel, 1983).

vhs acts with little or no specificity and causes the degradation of cellular mRNA as well as viral mRNA (Kwong *et al.*, 1988; Oroskar & Read, 1989; Read *et al.*, 1993). The degradation of mRNAs occurs via 3'-5' exonuclease digestion, and cellular polyribosomes

are also disaggregated (Nishioka & Silverstein, 1977 and 1978; Fenwick & Walker, 1978; Fenwick & McMenamin, 1984). In order to prevent the nonspecific activity of vhs inhibiting viral protein synthesis, one or more newly synthesised viral proteins apparently reduce the vhs activity of the infecting virion, thereby allowing the accumulation of viral mRNA (Fenwick & Owen, 1988; Fenwick & Everett, 1990a and 1990b). ICP8 has been suggested to be a mRNA stabilising factor because an ICP8 ts mutant, *ts*13, accumulated significantly less Vmw175 mRNA than wild type virus (Godowski & Knipe, 1983). Recent studies which were performed in order to clarify the role of vhs in the shut-off process assayed the effect of vhs in the absence of other HSV proteins on the expression of *LacZ* reporter gene. vhs strongly suppressed the expression of the co-transfected *LacZ* reporter gene and this effect was eliminated by the vhs1 point mutation that abolishes virion-induced host shutoff during HSV infection (Jones *et al.*, 1995). Therefore the vhs protein can induce virion host shutoff in the absence of other viral proteins. Further studies by this group involved the identification of functional subdomains within the vhs polypeptide following the insertion of linker sequences along the length of UL41. These studies identified a minimum of three mutationally sensitive regions that corresponded to regions of high homology within vhs (Jones *et al.*, 1995).

Two forms of the vhs polypeptide exist in cells infected with wild type HSV-1; a 58KDa polypeptide and a less abundant 59.5KDa form of the protein (Smibert *et al.*, 1992). Both proteins are phosphorylated and the different mobilities in gels are due to the extent of phosphorylation. While both forms are found in infected cells only the 58KDa form of the protein is incorporated into virus particles (Smibert *et al.*, 1992). The post-translational processing of vhs therefore appears to affect the incorporation of the protein into the herpesvirus particle (Read *et al.*, 1993).

Smibert *et al.* (1994) demonstrated that vhs forms a complex with VP16, and the VP16 acidic domain is not required. A 21 amino acid domain spanning residues 310-330 of vhs has recently been identified as being responsible for the interaction (Schmelter *et al.*, 1996). Using gel retardation assays, vhs was shown to block the ability of VP16 to enter the multi-protein complex on the TAATGARATTC element, indicating that vhs interacts with one or more of the regions on Vmw65 required for promoter recognition (Smibert *et al.* 1994). One possible functional significance of this interaction could be that VP16 may be involved in the incorporation of vhs into the virion. In support of this, a mutant vhs protein lacking residues 149 to 344 that cannot bind to VP16 is not packaged into virus particles (Read *et al.*, 1993). It is also thought likely that VP16 modulates vhs function by sequestering newly synthesised vhs protein into the virion assembly pathway. In support of this, a VP16 null mutant initiates infection normally but the undergoes a dramatic decline in

viral protein synthesis at intermediate and late times post infection (Weinheimer *et al.*, 1992).

(d) US3

Protein kinase (PK) activity is present in HSV-1 virions and from analysis of the predicted products encoded by HSV-1, US3 and UL13 were proposed to encode protein kinases on the basis of amino acid sequence motifs (McGeoch & Davison, 1986; McGeoch *et al.*, 1988; Smith & Smith, 1989). The US3 PK has been identified as an enzyme present in the cytoplasm of infected cells (Purves *et al.*, 1986; Frame *et al.*, 1987; Zhang *et al.*, 1990). US3-defective mutants have been isolated, and it is reported that although inactivation of the gene has little effect on the growth of the virus in tissue culture, US3-defective virus is markedly impaired for growth in murine brain (Longnecker & Roizman, 1987; Meignier *et al.*, 1988). The US3 PK has been purified, and exists as a dimer consisting of two 68KDa subunits, and although its physiological role remains obscure, it has been demonstrated to phosphorylate a virion protein encoded by UL34 which may lie in the envelope (Purves *et al.*, 1986 and 1991). This may suggest that the US3 PK is present in virus particles, but to date, the protein has not been formally characterised as a structural protein.

(e) UL13

Cunningham *et al.* (1992) identified a 57KDa protein encoded by UL13 as a virion component, and correlated it with a novel kinase activity present in the nuclei of infected cells. Detergent treatment of purified virions subsequently identified the UL13 encoded polypeptide to be a component of the tegument (Coulter *et al.*, 1993). Purves & Roizman (1992) reported that the substrate of the UL13 PK is the regulatory protein Vmw68, however several other proteins including VP22 are phosphorylated by the kinase *in vitro* (Coulter *et al.*, 1993). Due to the abundance of the UL13-encoded PK, and also the presence of homologues in all the genomes of mammalian herpesviruses sequenced to date (van Zijl *et al.*, 1990) it is reasonable to suggest that it may play a significant role in herpesvirus infection. However, the disruption of the UL13 PK by the insertion of *lacZ* sequences demonstrated that it was not essential for growth of the virus in tissue culure (Coulter *et al.*, 1993). The growth of the UL13-*lacZ* mutant virus was only slightly impaired compared to wild type HSV-1 and following intracranial inoculation in mice the pathogenicity of the mutant was only slightly reduced (Robertson, Coulter & MacLean, unpublished data) Recent data also suggests that UL13 may be involved in virion-induced shutoff. A viral

mutant with an inactivating lesion in UL13 displays a vhs null phenotype, even though the mutant contains normal amounts of vhs protein (Overton *et al.*, 1994).

(f) UL11

UL11 encodes a family of myristylated polypeptides which are located in the tegument, the sizes of which range in molecular weight from 13K to 16K (MacLean *et al.*, 1992). Prelimenary pulse-labelling experiments suggest that these polypeptides are expressed at delayed early times in infection, and are phosphorylated *in vitro* (MacLean *et al.*, 1989). Following sub-cellular fractionation of infected cells, they are found predominantly associated with membranes. The gene is not essential for growth in cell culture, but the growth of the deletion mutant is impaired and plaques are smaller than wild type HSV-1, suggesting a requirement for the protein for efficient replication (MacLean *et al.*, 1992).

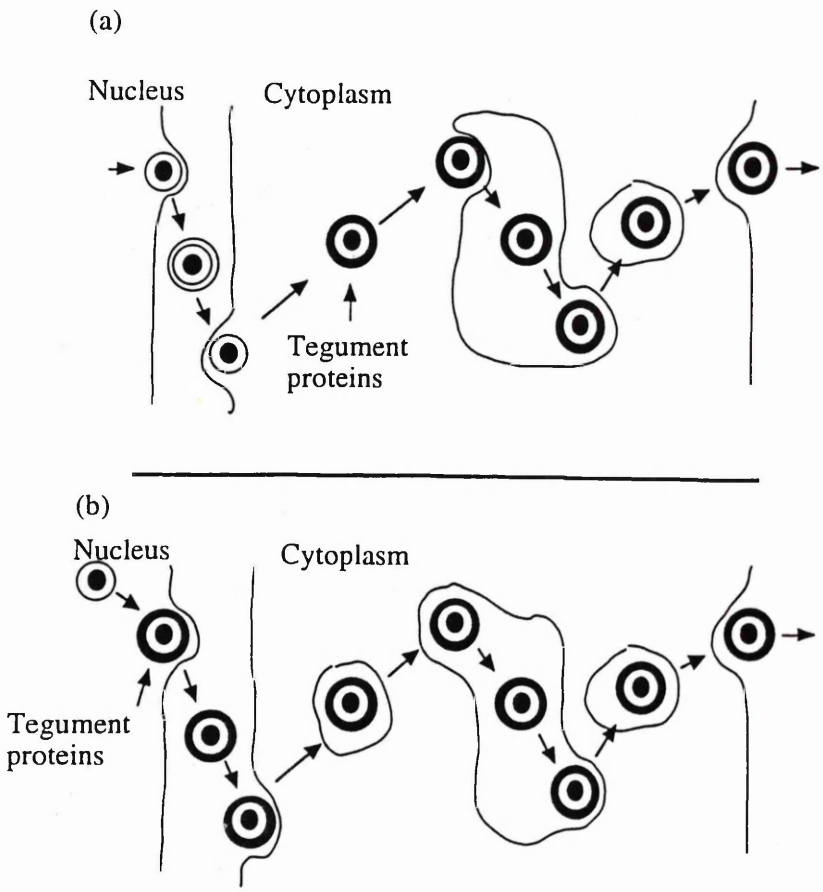
(g) US9

The 10KDa protein encoded by US9 is extensively post-translationally modified by phosphorylation, such that at least 12 electrophoretically distinct forms can be detected (Frame *et al.*, 1986b). The lower molecular weight forms are thought to be present in the tegument, due to their absence in the capsid and envelope. The phosphoprotein is not essential, and its function is unknown (Frame *et al.*, 1986b).

(h) US11

The protein product of US11 was first identified using hybrid arrest of translation by Rixon & McGeoch (1984) and was shown to be a protein of 21KDa that localizes in the nucleoli of infected cells (MacLean *et al.*, 1987). Subsequently using an antiserum raised against the US11 polypeptide sequence Johnson *et al.* (1986) showed that the US11 protein was a doublet of 21 to 22KDa. The protein is a site- and conformation-specific RNA-binding protein (Roller & Roizman, 1990), that has been shown to negatively regulate the abundance of a viral RNA called Δ34 in infected cells. US11 binds *in vitro* to a specific sequence at or near to the 3'terminus of Δ34 RNA (Roller & Roizman, 1991). It is reported that virus particles contain 600 to 1000 copies per virion and that the protein associates specifically with the 60S subunit of ribosomes (Roller & Roizman, 1992). In addition, a cell line expressing US11 has been shown to be resistant to HSV-1 infection at a step in viral entry mediated by gD (Roller & Roizman, 1994).

Figure 6. Proposed pathways for virion egress. In both of the proposed maturation pathways (see text for details), the initial step involves the capsid budding through the inner nuclear membrane (gaining an envelope in the process) and the final stages involve passage through cytoplasmic (Golgi derived) vesicles and release of mature virions from the cell by exocytosis. Differences between the models centre on whether the final virion envelope is derived from the inner nuclear membrane (b) or from a cytoplasmic membrane (a), and on whether tegumentation takes place in the cytoplasm (a) or in the nucleus (b).



(i) Other minor components of the tegument

Other minor components of the tegument include the immediate early regulatory proteins Vmw175, Vmw110, Vmw68 and Vmw63. The functions of these proteins are described in chapter 3, section 2(iii) (a) to (e). It is not known whether these proteins have any significant roles in the structure or assembly of the tegument. In addition, the products of UL21 (Baines *et al.*, 1994) and UL25 (Wood, 1993), reside in the tegument. UL21 encodes a protein of unknown function that is not essential for viral replication (Baines *et al.*, 1994). UL25 encodes an essential gene product of 67KDa. The construction of *ts* mutants with lesions in the UL25 gene, and the tight association of the protein with the capsid suggest that it may play a role in DNA packaging and virion maturation (Addison *et al.*, 1984; Wood, 1993; Ali *et al.*, 1996).

(iii) Tegument Assembly

Very little is known about the route by which nucleocapsids obtain tegument. Most of the information regarding these events has come from electron microscopy of infected cells, and the order in which these steps occur is not clear. The first stage involves the budding of the capsid through the inner nuclear membrane, which is unusual as under normal conditions the passage of materials into and out of the nucleus takes place exclusively via nuclear pores (Rixon, 1993). There is no information regarding either the mechanism by which the mature DNA-containing capsids induce membrane budding, or the trigger which initiates the journey of the capsids through the cell.

Two models have been proposed (Rixon, 1993) for the route taken by virions through the perinuclear cisternae (Fig. 6). In the first, the newly acquired envelope, derived from the inner membrane, fuses with the outer membrane or with the contiguous endoplasmic reticular membrane, releasing the capsid into the cytoplasm. Final envelopment then occurs by budding through a cytoplasmic membrane. In the second model, the envelope gained at the inner nuclear membrane is retained and virions leave the perinuclear cisternae in a vacuole formed from the outer nuclear membrane. Both models propose that the Golgi complex is involved in maturation of the virion and that the mature virion finally leaves the cell by exocytosis from Golgi-derived vesicles.

Strong evidence for the first pathway, ie. that tegumentation may take place in the cytoplasm, comes from studies on HCMV and HHV-6. In both of these viruses, the tegument is a dense structure which can be easily seen in electron micrographs. Cells infected with HCMV are seen to have capsids, present in the cytoplasm, which appear to be surrounded by tegument but which are clearly not enveloped (Duyckinck Smith & de

Harven 1973). Electron microscopic evidence suggests that these structures become enveloped by budding through cytoplasmic membranes. In addition, EM studies on HHV-6 have identified specific regions within the infected cells which appear to be invaginations of the cytoplasm into the nucleus, termed tegusomes (Roffman *et al.*, 1990). The EM data suggest that maturing capsids gain an envelope at the inner nuclear membrane, which is then lost during fusion with the outer nuclear membrane, and the naked capsids are released into the tegusome where assembly of the tegument surrounding the capsid takes place. Final envelopment then occurs by budding into cytoplasmic vacuoles.

Further evidence for a cytoplasmic envelopment step comes from the treatment of cells with the metabolic inhibitor, brefeldin A (BFA). BFA disrupts Golgi function and leads to the accumulation of unenveloped capsids in the cytoplasm of infected cells (Whealy *et al.*, 1991). Thus, BFA may block a cytoplasmic envelopment step, but other interpretations are also possible.

The evidence obtained by electron microscopy for the maturation of HSV-1 particles is not dissimilar to that for HHV-6, although a tegusome-like compartment has not been implicated. In addition, in HSV-1 the tegument cannot be resolved in micrographs. The presence of non-enveloped capsids in the cytoplasm is a consistent feature of infected cells and the apparent budding into cytoplasmic vacuoles is frequently observed (Nii *et al.*, 1968). These observations suggest a possible cytoplasmic site for tegumentation. An alternative explanation, that cytoplasmic capsids represent terminally de-enveloped virions has been suggested (Campadelli-Fiume *et al.*, 1991). This is based on the observation that the presence of gD in cell membranes appears to inhibit fusion with the virion envelope and thus prevents virus from reentering infected cells. The origin of cytoplasmic capsids was examined using an HSV-1 mutant with an alteration in gD which allows it to overcome this block (Campadelli-Fiume *et al.*, 1991). The mutant accumulates large numbers of unenveloped capsids in the cytoplasm of infected cells and this was interpreted as a result of fusion of cellular membranes with the envelopes of exiting virions passing through the cytoplasm.

In order to conclusively distinguish between whether tegumentation occcurs in the cytoplasm versus the inner nuclear membrane, the location at which one or more of the tegument proteins is added to the capsid structure would have to be directly ascertained.

Section 5. The Envelope

The envelope surrounding the tegument consists of a lipid membrane (Asher *et al.*, 1969; Ben-Porat & Kaplan, 1971) with glycoprotein spikes of 8-10nm projecting from its outer surface (Wildy *et al.*, 1960; Stannard *et al.*, 1987). The envelope is host cell derived but modified by the presence of viral proteins. The phospholipid composition of the viral envelope is similar to that of the nuclear membrane except that it contains three times the concentrations of sphingomyelin and phosphatidylserine, which are lipids that are typically enriched in the Golgi apparatus and the plasma membrane (Campadelli-Fiume *et al.*, 1993; Van Genderen *et al.*, 1994). The concentrations of these lipids within the viral envelope provide evidence that after budding through the innner nuclear membrane, the virus particle loses its envelope by fusing with the outer nuclear membrane and obtains a new membrane by budding into a compartment late in the endocytic pathway, likely to be the Golgi apparatus or membranes derived from it (Van Genderen *et al.*, 1994). The precise number of virally encoded glycoproteins present on the envelope of virions is not yet known. To date eleven membrane glycoproteins specified by HSV-1 have been identified: gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL and gM (Buckmaster *et al.*, 1984; Spear, 1985; Longnecker & Roizman, 1987; Roizman & Sears, 1990; Hutchinson *et al.*, 1991 and 1992; Baines & Roizman, 1993).

Stannard *et al.* (1987) provided evidence that the three major HSV-1 glycoproteins (gB, gC and gD) are present in three distinct structures projecting from the virion envelope. The gC-containing structures are long and slender, while those containing gB or gD are shorter. gB seems to be clustered, whereas gC is randomly scattered. gB forms heat stable oligomers, and at least some of them are homodimers (Claesson-Welsh & Spear, 1986). Two sets of heterodimers have been identified; gE and gI forming the Fc receptor, and also gH and gL (see *Chapter 3; Introduction, Section 1*). The products of genes UL10, UL20, UL34, UL53 and UL49A may also be associated with the virion envelope as they all have hydrophobic regions which could be membrane spanning domains (McGeoch *et al.*, 1988; Barnett *et al.*, 1992). In addition, UL45 encodes an 18KDa structural protein associated with the viral envelope (Visalli & Brandt, 1993) that is not essential for growth of the virus (Vissalli & Brandt, 1991).

There is evidence for both N and O-linked glycosylation on gB, gC, gD and gE (Pizer *et al.*, 1980; Serafini-Cessi & Campadelli-Fiume, 1981; Person *et al.*, 1982; Wenske *et al.*, 1982), and the differences in electrophoretic mobilities of mature and immature forms of each HSV-1 glycoprotein is due primarily to the presence of O-linked oligosaccharides on the mature forms (Johnson & Spear, 1983). All of the major HSV-1-induced glycoproteins are sulphated, gE being the most heavily labelled, and sulphation only occurs on the mature

forms of the glycoproteins (Hope *et al.*, 1982; Hope & Marsden, 1983). The biological role of sulphation is not clear but it may influence the polarity of the glycoprotein molecule which may be important for the orientation of the protein in the membrane. gE is also modified by the addition of fatty acids.

Little is known about the organisation and potential interaction of proteins in the tegument and envelope. Studies using chemical cross linking reagents were performed by Zhu & Courtney (1994) in order to determine the relationship between gB on the viral envelope and other virion proteins. Four structural proteins, thought to be tegument proteins were seen to be associated with gB, gD and gH, but not with gC. One of these four proteins was positively identified as VP16 and the others were predicted to be VP11/12, VP13/14 and VP22 due to their apparent molecular weights.

Section 6. Non-infectious Particles

During productive infection, virus-related particles known as light-particles are released form infected cells in addition to virions. These particles were identified as a diffuse band of material which migrated above virions in ficoll gradients (Szilagyi & Cunningham, 1991). Electron microscopy of negatively stained preparations revealed that light-particles consisted of an outer envelope, with surface projections, enclosing tegument-like material, but lacking the nucleocapsid of virions. Comparison of polypeptide profiles confirmed that light-particles share most, if not all, membrane and tegument proteins with virions, but lack the capsid polypeptides. Moreover, analysis of [^{3}H]thymidine-labelled particles suggested that viral DNA was absent from light-particles (Szilagyi & Cunningham, 1991). The lack of any nucleocapsid renders light-particles non-infectious. Also, light-particles contain a number of phosphopolypeptides with apparent molecular weights of 175KDa, 134KDa, 92KDa, 60KDa and 55KDa not seen in virions (Szilagyi & Cunningham, 1991). The identity of the majority of these proteins are unclear however the 175KDa polypeptide has been shown to be the IE regulatory protein Vmw175 (McLauchlan & Rixon 1992).

The processes which govern the genesis of light-particles during virus replication are not known. Studies with a *ts* mutant (*ts*1201) which has a lesion in gene UL26 and which fails to assemble infectious virions (see *Chapter 4; Introduction, Section 2.3*, Preston *et al.*, 1983, 1991), have revealed that light-particle production does not require the formation of mature virions (Rixon *et al.*, 1992). These results implied that the signals controlling the condensation of tegument and the acquisition of an envelope are intrinsic to components of light-particles. In addition, although the tegument has no apparent regular structure, removal of the envelope from light-particles by detergent treatment does not disrupt the tegument.

This suggests that the structural integrity of the tegument is independent of either capsid or envelope proteins (McLauchlan & Rixon, 1992).

To determine whether the polypeptides present in light-particles retained their function, two proteins, vhs and VP16 which are common to virions and light-particles, were assayed for biological activity (McLauchlan *et al.*, 1992a). vhs activity was determined by measuring the decrease in host cell polypeptide synthesis following inoculation with a range of virions and light-particles in the presence of the RNA synthesis inhibitor, ara C. The data revealed that the reduction in protein synthesis brought about by vhs was equivalent for both virions and light-particles. To assay the activity of VP16 in light-particles, a HSV-1 virus mutant *in*1814 which contains an insertion in the UL48 gene and as a consequence is a unable to initiate virus infection at low multiplicities of infection was used for complementation studies. The growth of *in*1814 in cells infected with the mutant can be stimulated by supplying *in trans* unmodified VP16. Co-inoculation of cells with *in*1814 and UV inactivated virions or light-particles showed that both types of particle were equally efficient at complementing growth of the mutant. Thus, the vhs and VP16 proteins had comparable activities in virions and light-particles. Since the activities of these proteins were assayed in an intracellular environment these data would indicate that the processes of binding, fusion and penetration are similar for virions and light particles. Thus, light-particles are biologically competent and may be capable of influencing the early stages of virus infection.

Recent studies have identified another virus particle that is morphologically similar to light-particles but has a slightly altered protein composition (Dargan *et al.*, 1995). These particles are produced in the presence of viral DNA replication inhibitors and have been termed pre-viral DNA replication enveloped particles (PREPs). They are deficient in gC and gD and also have reduced levels of VP1, VP13/14 and VP11/12, while the levels of VP22 are increased as compared to light-particles. While these differences are probably due to the inhibition of protein synthesis, the polypeptides within PREPs are biologically active (Dargan *et al.*, 1995).

Other alphaherpesviruses, including EHV-1 and PrV, also produce light-particles during productive infection of BHK cells (McLauchlan & Rixon 1992) The size morphology and quantity of light-partices produced by EHV-1 or PRV were largely indistinguishable from those produced by HSV-1. The betaherpesvirus HCMV has also been shown to generate two types of non-infectious particle known as dense bodies and non-infectious enveloped particles (NIEPs). Dense bodies are membrane bound electron dense particles which are considerably larger than HCMV virions. 90% of their mass is accounted for by the lower matrix protein pp65 which is encoded by gene UL83 (Irmiere &

Gibson, 1983; Eggers *et al.*, 1992). However, staining techniques have suggested that dense bodies contain DNA and RNA (Sevri *et al.*, 1991). NIEPs contain all structural components of the HCMV virion, including the capsid, but lack DNA and resemble the enveloped empty capsids seen in preparations of HSV-1 virions (Schrag *et al.*, 1989). Neither the functions of dense bodies nor of NIEPs are known, and the gene encoding pp65, which has no counterpart in HSV-1, is not essential for the growth of HCMV in tissue culture (Schmolke *et al.*, 1995).

Section 7. Maturation of Virions and Egress

Herpesviruses acquire their envelope by budding through a modified inner membrane of the nuclear envelope which forms thick and dense patches at the site of budding (Bibor-Hardy *et al.*, 1985a). This suggests that some of the viral envelope glycoproteins must be transported to the nuclear envelope in order to be incorporated into the virus. Labelling of glycoprotein gB indicated that it was uniformly distributed around the inner and outer membranes of the nuclear envelope, and that nucleocapsids were only labelled with gB after association with the nuclear envelope (Gilbert & Gosh, 1993).

Following envelopment the virus is transported via the Golgi apparatus to the extracellular space. A mutant cell line, gro29, which is defective in protein secretion, is unable to release virions when infected with HSV-1, despite normal viral gene expression. These studies suggest that the secretory pathway is critical to HSV-1 release and that specific host cell factors facilitate viral egress from infected cells (Banfield & Tufaro, 1990). The inhibition of glycosylation, following the exposure of cells to monensin (Johnson & Spear, 1982) or other late glycosylation inhibitors (Kousulas *et al.*, 1983), prevents the maturation of glycoproteins and leads to a reduction in virus egress as well as an accumulation of virions in the cytoplasm. These results suggest that specific oligosaccharide moieties contribute to efficient viral maturation and egress (Campadelli-Fiume *et al.*, 1982; Serafini-Cessi *et al.*, 1983). For example, BFA which removes Golgi structure from cells and blocks the transport of proteins into post Golgi cellular compartments (Misumi *et al.*, 1986), blocks the maturation and egress of HSV during infection (Cheung *et al.*, 1991).

Thin section fracture label elelctron microscopy was used to investigate HSV-1 envelopment and maturation (Torrisi *et al.*, 1991). Results showed that the envelope acquired at the inner nuclear membrane contained the envelope glycoprotein precursors, and that during transit to the extracellular space maturation of the virion glycoproteins occurred. Evidence of virions within and associated with the Golgi apparatus suggested that the maturation of glycoproteins is facilitated by the Golgi enzymes. In Vero cells the efficient transport of HSV-1 to the perinuclear space and plaque formation requires the UL20 gene

product (Baines *et al.*, 1991). These results suggest that viral transport from the infected cell is an active function of the virus particle rather than a passive function where the cells export any macromolecules present within the lumen of transport vesicles unless specific sequences which cause them to be retained in the cell (Pelham, 1989).

Materials

Section 1. Chemicals and Reagents

Ampicillin sodium B.P. (Penbritin)	Beecham Research
Boric acid, Butanol, Acetic acid, Chloroform, Isopropanol, Methanol	Prolabo Rhone-Poulenc Ltd., Manchester, UK.
EDTA, NaCl, SDS, $CaCl_2$, Glycine, nonidet P40, Acrylamide, N,N'-methylenebis-acrylamide, Sucrose, Formaldehyde, β-mercaptoethanol	BDH Laboratory Supplies, Poole, UK.
Tris	Boehringer Mannheim GmbH, Germany.
Gelatin, Ammonium Persulphate, TEMED,	Bio-Rad Laboratories, Richmond, California, USA.
Glycerol	May & Baker Ltd., Dagenham, UK.
Tween 20, Protein A sepharose, Agarose, Lysozyme, Ficoll 400	Sigma Chemicals Co. UK.
ECL development reagents	Amersham International plc., Buckinghamshire, UK.
Kit for large scale plasmid preparation	Qiagen plasmid kit, Qiagen GmbH, Germany.
BHK-medium, newborn calf serum, Optimem 1	Life Technologies Ltd., Scotland.

Caesium Chloride	Koch Light Ltd. (Suffolk, England)

Section 2. Solutions

Boiling mix	50mM Tris-HCl (pH6.7), 2% (w/v) SDS, 5% (w/v) β-mercaptoethanol, 10% (w/v) Glycerol, 0.004% (w/v) Bromophenol blue
Denhardt's buffer (5x)	0.1% (w/v) Ficoll, 0.1% (w/v) polyvynylpyrollidone, 0.1% (w/v) BSA
Destain	5% methanol, 7% acetic acid
Eagles medium	10x Glasgows Modified Eagle's medium (GMEM) was diluted to 1x GMEM with sterile distilled H_2O and supplemented with: 4mM L-glutamine, 0.26% sodium bicarbonate
electroelution buffer	20mM Tris-HCl pH 7.4, 10mM ammonium acetate, 2mM EDTA
ETC10	Eagles medium supplemented with: 5% tryptose phosphate broth, 100µl/ml penicillin, 100µl/ml streptomycin, 10% newborn calf serum (NBCS)
Fix	50% methanol, 7% acetic acid
Gel electrophoresis buffer	52mM Tris, 53mM Glycine, 0.1% (w/v) SDS
Gel loading buffer	5x TBE, 50% sucrose and 0.2% bromophenol blue
Gel soak I	0.6M NaCl, 0.2M NaOH

Gel soak II	0.6M NaOH, 1.0M Tris-HCl pH8.0
HBS	20mM Hepes-NaOH (pH7.4), 150mM NaCl
HEBS	137mM NaCl, 5mM KCl, 0.2mM NaH_2PO_4, 9mM D-glucose, 21mM Hepes pH 7.05
Hybridisation buffer	6xSSC, 10x Denhardt's solution 20mM Tris-HCl pH 7.5 1mM EDTA, 0.5µg/ml denatured calf thymus DNA
L-broth	170mM NaCl, 5g/l yeast extract, 10g/l Difco Bactotryptone.
Lysis solution 1	50mM glucose, 10mM EDTA, 25mM Tris-HCl pH 8 containing freshly made 10mg/ml lysozyme.
Lysis solution 2	0.2M NaOH, 1% SDS
Lysis solution 3	5M potassium acetate pH 4.8
(5x) Ligation buffer	250mM Tris-HCl pH 7.6, 50mM $MgCl_2$, 5mM DTT, 5mM ATP, 25% (w/v) PEG 6000
Na-Phosphate buffer	A: Na_2HPO_4 2.84g/l B: NaH_2PO_4 3.12g/l Add A+B until pH=7.0
(10x) NT buffer	0.5M Tris-HCl pH 7.8, 0.05M $MgCl_2$, 10mM Dithiothreitol (DTT)
Phosphate buffered saline (PBS)	170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4 and 2mM KH_2PO_4 pH 7.2
2 x PK	100mM Tris-HCl pH 8.0, 20mM EDTA,

	20mM NaCl, 0.4% SDS
Pre-hybridisation buffer	6x SSC, 5x Denhardt's solution, 0.1% SDS, 20μg/ml denatured calf thymus DNA
Regeneration Buffer	2% SDS, 50mM Tris-HCl pH 7.4, 100mM β-mercaptoethanol
Resolving gel buffer (RGB)	0.4M Tris-HCl pH8.9, 0.1% SDS
Stop solution	95% Formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% Xylene Cyanol FF
(10x) SSC	3M NaCl, 300mM tri-sodium citrate
Stacking gel buffer (SGB)	0.1M Tris-HCl pH 6.7, 0.1% SDS
STET	0.23M Sucrose, 9mM Tris-HCl pH 8.0, 50mM EDTA, 10% Triton X-100
(1x) TBS	4mM Tris-HCl pH 7.5, 0.1M NaCl
TTBS	1 x TBS, 0.05% Tween 20
TE	10mM Tris-HCl pH 7.5, 1mM EDTA
10 x TBE	0.8M Tris, 0.3M boric acid, 0.2M EDTA
Tris-saline	140mM NaCl, 30mM KCl, 28mM Na_2HPO_4, 1mg/ml glucose, 100mg/ml streptomycin. 25mM Tris-HCl pH 7.4
Trypsin	0.25% (w/v) trypsin (Difco) in tris-saline, containing 0.002% (w/v) phenol red.

Versene	0.6mM EDTA dissolved in PBS containing 0.002% (w/v) phenol red.
Western transfer buffer	25mM Tris pH 8.3, 192mM Glycine, 0.0375% SDS, 20% (v/v) methanol

Section 3. Plasmids

pGEM1 — Supplied by Promega, pGEM1 is a 2.8Kbp plasmid that contains a poly clonal linker sequence between T7 and SP6 promoter sequences. It also contains an ampicillin resistance gene.

pCAT1 — pCAT1 was made by inserting a 960bp HindIII fragment from pLW2 (Whitton, 1984) that contained the open reading frame that encodes the bacterial chloramphenicol acetyl transferase (CAT) gene into the HindIII site of pGEM1.

pFJ3 — derived from the commercially available plasmid, pCH110 (Pharmacia), pFJ3 contains the β-lactamase (*Lac Z*) gene linked to the simian virus 40 (SV40) early promoter in a 4.1Kbp sequence flanked by XbaI sites. In addition a polylinker was inserted into the BamHI site (Rixon & McLauchlan, 1990).

pTK1 — pTK1 was constructed by inserting the 3.6Kbp BamHI p fragment of HSV-1 strain17 DNA into the BamHI site of pAT153, as described by Wilkie *et al.* (1979). BamHI p contains the HSV-1 thymidine kinase gene.

pMF1 — pMF1 was constructed by inserting a SstI fragment containing the HSV-2 strain G UL41 sequences into the SstI site of pTK1 (Fenwick & Everett, 1990)

pMJ37 — pMJ37 was constructed by inserting a 750bp Sau3AI fragment containing the HCMV immediate early promoter sequence into the BamHI site of pUC18 (kindly donated by Dr. C.M. Preston).

pSAU30 Derived from pSAU3 (McLauchlan *et al.*, 1989) pSAU30 contains an oligonucleotide linker inserted at the SstI site of pSAU3. Insertion of this linker allows the removal of a 260bp DNA fragment containing the HSV-2 UL38 polyadenylation sequences by digestion with HindIII and BamHI.

pBluescript II SK(+/-) Supplied by Stratagene cloning systems, pBluescript is a 2961bp phagemid derived from pUC19. It contains a multiple cloning site between T3 and T7 promoter sequences.

Section 4. Viruses

1802 HSV-1 1802 contains a unique XbaI site located in the intergenic region between US9 and US10 (Rixon & McLauchlan, 1990). This site is located in BamHI fragment z.

vFJ10 vFJ10 is derived from HSV-1 1802 and contains the CAT gene within the U_S region of the genome under the control of the HSV-2 IE4/5 promoter and the IE5 polyadenylation site sequences (Rixon & McLauchlan,1990).

vSAU3 vSAU3 is derived from HSV-1 1802 and contains the CAT open reading frame within the U_S region of the genome under the control of the UL40 gene promoter and the HSV-2 UL38 polyadenylation site sequences (McLauchlan *et al.*, 1989).

v17G41 v17G41 was constructed by inserting the HSV-2 strain G UL41 gene into the TK locus of HSV-1 strain 17 (Fenwick & Everett, 1990a).

Section 5. Radioisotopes

All radioisotopes were supplied by Amersham International plc.. They had the following specific activities;

5'[α-^{32}P] dNTPs	~3000Ci/mmol (10μCi/μl)
5'[γ-^{32}P] adenosine triphosphate	5000Ci/mmol (10μCi/μl)
[α-^{35}S] deoxyadenosine thiotriphosphate	1000Ci/mmol (10μCi/μl)
[α-^{35}S]-L-methionine	800Ci/mmol (15μCi/μl)
[^{14}C]-chloramphenicol	50-62Ci/mmol (25μCi/ml)

Section 6. Antibodies

anti mouse IgG (whole molecule) peroxidase conjugated, anti mouse IgG (whole molecule) FITC conjugated	Sigma Chemicals Co., U.K.
CAT rabbit polyclonal antibody	5 Prime 3 Prime Inc.
CMV monoclonal antibody. Late nuclear protein (pp65)	DuPont (UK) Ltd., Hertfordshire, England.
LP-1 and CY8 monoclonal antibodies	kindly donated by Dr. H.S. Marsden
Protein A, [^{125}I] labelled with Bolton and Hunter reagent, affinity purified for blotting applications (>30mCi/mg)	Amersham International.

Section 7. Other materials

Hybond N Hybridisation transfer membranes	Amersham International
Giemsa Stain	BDH Chemicals
Polaroid-667 Film	Polaroid (U.K.)
Plasticware for tissue culture	Becton Dickinson Ltd, UK.
Restriction enzymes, T4 DNA ligase, Enzyme Buffers	Boehringer Mannheim, Lewes, E. Sussex, UK.
Nitrocellulose membrane (BA 85)	Schleicher & Schuell, Dassel, Germany.
Agarose gel electrophoresis apparatus	Hybaid, UK.
PAGE apparatus	Bio-Rad Laboratories, Richmond, California, USA.
Repelcote	BDH Cemicals
XS-1 Film	Kodak
Sequenase Version 2.0	United States Biochemical

Methods

Section 1.

Cell and Virus Methodologies

1.1. Maintenance of Tissue Culture Cells

The BHK cells used were from the BHK-21 clone 13 fibroblastic cell line derived from baby hamster kidney cells (McPherson & Stoker 1962). The cells were maintained in Glasgow minimal essential medium (GMEM) supplemented with 10% newborn calf serum (NCS), 5% tryptose phosphate broth, 100U/ml penicillin and 100U/ml streptomycin (ETC10) in an atmosphere of 95% air, 5% CO_2. To harvest, the confluent monolayers were washed with 20ml versene, followed by a trypsin wash. The detached cells were resuspended in 20ml medium and could be stored at 4°C for up to one week. Cells were routinely passaged in 2 litre sterile roller bottles. From 1 roller bottle, up to 8 roller bottles could be seeded and would reach confluence following growth at 37°C for 3 to 4 days. For long term storage, detached cells were resuspended in 5ml ETC10 supplemented with 25% calf serum and 10% glycerol. Aliquots were stored overnight at -70°C and then transferred to liquid nitrogen (-190°C). Cells were recovered from liquid nitrogen by bringing quickly to 37°C and incubating in small flasks with ETC10 medium until confluent.

1.2. Growth of Virus

For growth of virus stocks and preparation of virus particles, 5-20 roller bottles of BHK C13 cells were grown (see *Methods, Section 1.1.*) until they were confluent (assumed to be $1x10^8$ cells per roller bottle). The medium from the roller bottles was removed and each bottle was inoculated with 40ml of ETC10 medium containing $3x10^5$ plaque forming units (pfu)/bottle. The multiplicity of infection (moi) was therefore 1:300. The infected roller bottles were then incubated until all the cells had rounded and detached from the bottle which typically took 3 days at 37°C or 4 days at 31°C. The mixture of cells and medium was decanted into sterile 250ml centrifuge bottles. The cells were then separated from the medium by centrifugation at 3000rpm for 10 minutes at 4°C. Virus in the supernatant was

pelleted by centrifugation at 12,000rpm (Sorvall GS3 rotor) for 2 hours at 4°C. The pellet was resuspended in 1ml of cold 1 x Eagles medium (minus phenol red) and sonicated to disperse the virus. This material formed the cell released virus (CRV) and was stored at -70°C. Cell associated virus was not routinely prepared from infected cells.

1.3. Titration of Virus Stocks

To obtain the titre of virus, the stock was diluted in PBS + 5% NCS in step-wise dilutions up to 10^{-9}. Confluent monolayers of BHK cells on 35mm plates were infected with dilutions by removing the medium and applying 100µl of the diluted virus to the centre of each plate followed by incubation for 1 hour at 37°C. The inoculum was removed, and the plates were overlaid with carboxymethylcellulose medium (ETC10 medium containing 1.5% ($^{W}/_{V}$) carboxymethyl cellulose) and incubated at 37°C for 3-4 days. Plates were then fixed and stained with Giemsa stain for 30 minutes, after which the plates were rinsed with water and dried. Plaques were counted with a stereomicroscope.

1.4. Purification of Virions and Light particles

Purification of virions and light particles was as described by Szilagyi & Cunningham (1991). 5% and 15% ficoll solutions were prepared in 1x Eagle's medium minus phenol red. Using these solutions, 5-15% continuous gradients were formed in 1.4 x 9.5cm tubes (gradient volume was 13ml) using a gradient pouring apparatus. The gradients, rotor, centrifuge and buckets were cooled to 4°C prior to use. 1ml of virus prepared as described in *Methods, Section 1.2.* was loaded onto the top of the gradient, and centrifuged at 12,000rpm for 2 hours at 4°C in a Tst41 swing-out rotor. Two bands were visible in the gradients: a clearly defined narrow band (V, virions) and above this a wider more diffuse band (L, light-particles) (Fig. 25). The virion and light particle bands were collected by side puncture through a syringe needle, diluted with 1x Eagles medium (without phenol red) and spun for 18 hours at 12,000rpm at 4°C in a Tst41 swing-out rotor. Following centrifugation the supernatant was discarded and the pellets were resuspended in 1x Eagles medium (without phenol red) and stored at -70°C.

1.5. Particle Counts

Equal volumes of the virus stock, latex beads ($1.5x10^{11}$particles/ml) and stain (1% phosphotungstic acid, pH 7.0) were mixed and absorbed onto a parlodion-coated copper grid. Excess liquid was drained off and the number of virus particles and latex beads present in the field of view of the electron microscope counted. The concentration of the virus particles was calculated by comparison with the number of latex beads.

1.6. Detergent Treatment of Virus Particles

30µl of purified virions or light-particles (typically $2x10^8$ particles) was added to 30µl PBS + 1% NP40 and incubated on ice for 20 minutes. The samples were then centrifuged at 11,500g for 15 minutes and the supernatant which contains the envelope components was removed to a fresh tube. Pellets were washed with PBS + 2% NP40 and resuspended in 60µl of PBS. To all of the samples, 30µl of boiling mix was added and the samples were boiled for 4 minutes prior to loading on a polyacrylamide gel.

1.7. Growth Curves

To determine virus yield after one cycle of replication, growth curves were carried out at a high multiplicity of infection. The medium was removed from 35mm plates of confluent BHK cells and the virus added at a moi of 5 pfu/ml, in a final volume of 200µl. The virus was left to absorb at 37°C for 1 hour after which the monolayers were washed three times with PBS. 3ml of ETC10 medium was then added and the plates were returned to 37°C. The 0 hour sample was harvested immediately after the washes with further samples harvested at 3 hourly intervals. To harvest, the cells were scraped into the medium and subjected to ultrasonic disruption. The volume of each sample was measured and the samples were then titrated as described in *Methods, Section 1.3.* The virus yield at each time point was calculated as the titre x sample volume.

1.8. BCdR Selection of TK^- Virus

The synthetic thymidine analogue 5'bromo-deoxycytidine (BCdR) was used as a selective agent for the growth of TK^- viruses. Cells transfected with viral and plasmid DNAs, as described in *Methods, Section 1.9*, were harvested into the growth medium once

cpe was evident, (usually after 3-4 days incubation)and then this progeny virus was titrated on BHK cells. The progeny virus was then enriched for TK negative recombinants by two serial passages at low moi (0.005 pfu/cell) in the prescence of BCdR (100mg/ml) and then plaque purified as described in *Methods, Section 1.10*. Incubations were carried out at 37°C throughout.

1.9. Transfection of DNA into BHK Cells

To generate virus recombinanats, plasmid and viral DNAs were transfected into BHK cells by either lipofection or calcium phospate precipitation.

a) Lipofection

For transfection into BHK cells, on 35mm Petri dishes, two 15ml Falcon tubes were used and labelled A and B. To tube A, 70µl of HBS and 30µl of lipofectin (kindly supplied by C. Addison) were added and vortexed. To tube B was added the solution containing DNA (usually 5µg) and HBS up to a final volume of 100µl. The contents of tube A were added to tube B and the two solutions carefully mixed. This mixture was kept at room temperature for 10 minutes and then 2ml Optimem medium was added. The medium from each plate was replaced with the transfection mix and then incubated at 37°C for 5 hours. After 5 hours, 2ml of ETC10 medium containing 5% NCS was added to each plate and incubation at 37°C was continued until the virus had grown.

b) $CaPO_4$ Precipitation

This method is a modification of that described by Stow & Wilkie (1976). 1-5µg DNA was added to 1ml HEBS containing 5µg/ml calf thymus DNA and 70µl 2M $CaCl_2$. Following mixing, this solution was added to 50mm dishes containing sub-confluent monolayers of BHK cells from which medium had been removed. Following incubation at 37°C for 45-60 minutes, the cells were overlaid with 4ml of ETC10 medium containing 5% NCS. 4-8 hours later the medium was removed from the plates and they were washed twice with 1x ETC10 medium without NCS. 1ml of 20% (w/v) DMSO in 1x HEBS was added to each plate and the cells incubated for exactly 4 minutes at room temperature. The DMSO solution was removed by aspiration and the cells were washed twice with 1x ETC10 medium without NCS. Cells were finally overlaid with 4ml ETC10 medium and incubated

at 37°C until complete cpe was apparent.

Following transfection, infected cells were harvested and screened either by Western blot analysis or CAT assays in order to determine whether insertion of DNA into the HSV-1 genome had been successful.

1.10. Selection and Growth of Individual Plaques

Dilutions of virus progeny from transfections were prepared and used to infect cells as described in *Methods, Section 1.3.* After infection, cells were overlaid with agar medium (ETC10 medium containing 0.5% (w/v) agar) and incubated at 37°C for 3-4 days. Plaques which were well separated were selected by viewing under a stereomicroscope. Cells were withdrawn from the area of the plaque using 10µl disposable tips and transferred to a 1ml cryotube containing 200µl PBS + 5% NCS. To release virus from the cells, samples were frozen in dry ice and then thawed at 37°C. This was repeated a further two times. BHK cells on 24 well plates were then inoculated with a 50µl aliquot of the plaque isolate and incubated at 37°C for 1 hour. Cells were overlaid with ETC10 medium and incubated at 37°C until virus growth was apparent in all cells. After virus growth, 1ml of medium from the infected cells was removed and stored at -70°C. The infected cells were washed 3 times with 1ml PBS and then harvested into the appropriate buffer for either Western blot analysis or estimation of CAT activity.

Section 2.

Preparation of Plasmid DNA

2.1. Preparation and Transformation of Competent E.coli JM101 cells.

100ml of L-broth was inoculated with 1ml of an overnight culture of Escherichia coli (E.coli) JM101 and grown at 37°C to an approximate optical density of 0.3 at 630nm. Bacteria were then pelleted at 3000rpm* for 10 minutes at 4°C, resuspended in half their original volume with ice cold 100mM $CaCl_2$, and incubated on ice for at least 2 hours. The bacteria were again pelleted and resuspended in 1ml of ice cold 100mM $CaCl_2$, after which

*Sorvall SS34 Rotor

they were competent and ready for transformation.

For transformation, 100µl of competent bacteria were incubated on ice with 0.5-2µg DNA, or 4µl ligation mix for 40 minutes. Prior to plating onto agar plates (containing 50µg/ml of ampicillin), the bacteria were heat shocked at 42°C for 2 minutes. Plates were incubated overnight at 37°C.

2.2. Glycerol Stocks of Bacteria

50ml of L-broth, containing the appropriate antibiotic, was inoculated with bacteria and incubated overnight at 37°C in an orbital shaker. The culture was centrifuged at 3000rpm for 10 minutes (Sorvall SS34 Rotor) and the bacterial pellet resuspended in 3-4ml of 2% Bactopeptone plus an equal volume of 80% glycerol. The stock was aliquoted and stored at -20°C.

2.3. Small Scale Preparation of Plasmid DNA

For initial screening of plasmids, DNA was prepared from a 1.5ml bacterial culture which had been grown overnight at 37°C. Bacteria were pelleted by spinning at 500g for 1 minute and the pellet was resuspended in 100µl of Lysis solution 1 (50mM glucose, 10mM EDTA, 25mM Tris-HCl pH8 containing freshly prepared 10mg/ml lysozyme). 150µl of Lysis solution 2 (0.2M NaOH, 1% SDS made fresh) was then added, the samples were mixed gently by inversion and incubated at room temperature for 5 minutes. 150µl of Lysis solution 3 (5M KAc pH4.8) was added and incubated for a further 5 minutes at room temperature. After centrifugation at 11,500g for 5 minutes the supernatant was removed and the DNA was phenol/$CHCl_3$ extracted and ethanol precipitated as described in *Methods, Section 3.3.*

2.4. Large Scale Plasmid Preparation

10ml of L-broth, containing the appropriate antibiotic, was inoculated with a loop of bacteria and shaken overnight at 37°C. This culture was added to 600ml of L-broth plus antibiotic and shaken at 37°C until the OD_{630} was 0.8. In some cases, chloramphenicol was added to a final concentration of 25µg/ml and the incubation was continued overnight.

Plasmid DNA was prepared by the maxi-boiling technique of Holmes & Quigley

(1981). Cells were pelleted at 7,000rpm for 10 minutes (Sorvall GS3 rotor) and resuspended in 20ml of STET buffer. Lysozyme, was added to a final concentration of 1mg/ml and the sample was incubated at room temperature for 30 seconds prior to boiling for 45 seconds. The lysate was clarified by centrifugation at 18,000rpm at 4°C for 1 hour in a Sorvall SS34 rotor. The supernatant was then extracted with phenol/$CHCl_3$ and the DNA in the upper aqueous layer was precipitated by the addition of 0.7 volumes of isopropanol. DNA was pelleted by centrifugation at 3,000rpm for 10 minutes and the pellet washed with 70% ethanol. Once the pellet was completely dry, it was resuspended in 1ml of TE.

The plasmid DNA was then purified on a CsCl gradient. Gradients were prepared by mixing the DNA with CsCl to give a final density of 1.6mg/ml and ethidium bromide (EtBr) was added to a final concentration of 0.5mg/ml. This solution was centrifuged at 11,500g for 10 minutes to remove debris and the supernatant was then transferred to a TV865 ultracentrifuge tube. Following centrifugation at 100,000rpm for 4 hours the DNA was visualised under long wave UV light and the lower of the two bands, representing the super coiled plasmid DNA, was removed by side puncture. EtBr was removed by a series of extractions with CsCl-saturated isopropanol, and the DNA was ethanol precipitated (see *Methods, Section 3.3*). Following pelleting, DNA was resuspended in a final volume of 500µl of TE and stored at -20°C. The concentration of DNA was estimated by measuring the optical density at 260nm.

Section 3.

DNA Manipulation

3.1. Restriction Enzyme Digestion of DNA

DNA samples (0.5-2µg) were usually digested in a total volume of 20µl, containing the appropriate enzyme buffer and a suitable amount of restriction enzyme (usually 3 units/µg DNA). Larger quantities of DNA (up to 15µg) were digested in volumes of either 100µl or 200µl. Digestion was carried out at 37°C, unless otherwise stated, for 1-3 hours. For the analysis of small scale plasmid DNA preparations, samples were digested in the presence of 10µg/ml RNaseA. For partial digestion of plasmid DNA, 10µg of plasmid DNA was digested for 10 minutes with 10 units of enzyme. Following digestion, linearised molecules were purified by agarose gel electrophoresis.

3.2. Agarose Gel Electrophoresis

For analysis of DNA restriction fragments, 0.8% - 1.5% agarose gels containing 0.5mg/ml ethidium bromide were prepared in 0.5 x TBE. Before loading DNA,the appropriate volume of 5x gel loading buffer (5x TBE, 50% sucrose and 0.2% bromophenol blue) was added to the DNA samples to give a 1x concentrated solution. Gels were electrophoresed at 100V for 2-3 hours. DNA bands were visualised under short or long wave UV light, and photographed using Polaroid film (type 667).

3.3.Phenol/Chloroform Extraction and DNA Precipitation

To remove the proteins from nucleic acid solutions, phenol/chloroform extractions were performed. An equal volume of 2x PK buffer (typically 200µl) and an equal volume of phenol/$CHCl_3$ was added to the nucleic acid solution. This mixture was vortexed and centrifuged at 11,500g for 1 minute. The upper phase which contained the DNA, was transferred to a fresh tube and extraction with phenol/$CHCl_3$ was repeated a further twice. After phenol/$CHCl_3$ extraction, an equal volume of $CHCl_3$ was added to the upper phase, vortexed, and centrifuged for 1 minute. The upper phase was removed to a fresh tube and 2-3 volumes of ethanol, and 1/10th of the aqueous volume of 5M ammonium acetate was added to precipitate the DNA. The sample was left at -20°C for 30 minutes, after which the DNA was pelleted by centrifugation at 11,500g for 5 minutes and the supernatant removed. The pellet was washed with 100µl of 70% ethanol to remove salt, dried and resuspended in 30µl of distilled water or TE buffer.

3.4. Purification of DNA Fragments

Digested DNA samples were run on an agarose gel as described in section 3.2. To visualise the DNA, the gel was viewed under long wave UV light and the appropriate DNA fragments were excised from the gel. The DNA was electroeluted from the gel by placing the gel slice in dialysis tubing which had been boiled for 10 minutes in the presence of EDTA, and rinsed in deionised water. 600µl of electroelution buffer (20mM Tris-HCl pH 7.4, 10mM ammonium acetate, 0.5mM EDTA) was added to the gel slice and the tubing was sealed at both ends. The gel slice was then immersed in a shallow layer of buffer and electrophoresed at 100V for 10 - 30 minutes. The gel slice was removed and the DNA was

isolated from the buffer by phenol/$CHCl_3$ extraction and ethanol precipitation.

3.5. DNA Cloning

The vector was linearised using the appropriate restriction enzyme, and to prevent recircularisation of the vector DNA during subsequent ligations, 1 unit of Calf Intestinal Phosphatase was added after completion of digestion and incubated for 30 minutes at 37°C. The DNA was then phenol/$CHCl_3$ extracted and ethanol precipitated.

For the insertion of blunt-ended fragments into sticky ended vectors, the vector was incubated for 30 minutes at room temperature with 100nM dNTPs and 0.5 unit/µg Klenow polymerase in the presence of restriction enzyme buffer. The DNA was then phenol/$CHCl_3$ extracted and ethanol precipitated.

Ligations were carried out in a total volume of 20µl containing 2 units of T4 DNA ligase in ligation buffer (50mM Tris-HCl pH7.6, 10mM $MgCl_2$, 1mM DTT, 1mM ATP, 25% (w/v) PEG 6000). Incubation was performed for a minimum of 2 hours at 16°C.

3.6. Labelling of DNA by Nick Translation

Plasmid DNA was labelled as described by Rigby *et al.*, (1977). 500ng of DNA was mixed with 20µCi of each [α-^{32}P]dNTP, 1µl DNase I (10^{-4}units/ml), in the presence of 1x NT buffer (50mM Tris-HCl pH7.8, 5mM $MgCl_2$, 1mM DTT, 5mg/ml BSA) and 2 units of E.coli DNA polymerase I. The mixture was incubated at room temperature for 1 hour and ^{32}P-labelled DNA was then separated from unincorporated triphosphates by running through a 10ml Sephadex G50 (medium grade) column in a 10ml pipette plugged with siliconised glass wool. Columns were loaded with the sample and bromophenol blue dye, and the fastest eluting fractions taken as the peak of radio-labelled DNA.

3.7. Preparation of virion DNA

To prepare virion DNA, 100µl of 2x PK buffer and 10µl of proteinase K (20mg/ml) was added to 100µl of virions (approx $1x10^9$ particles) which had been purified as described in *Methods, Section 1.4.* After incubation at 37°C for 30 minutes, 200µl of phenol/$CHCl_3$ was added to each tube and the upper and lower phases gently mixed for 5 minutes. The two phases were separated by centrifugation at 11,500g for 1 minute and the upper layer

was removed to a fresh tube. To prevent shearing of the DNA, the ends of tips were removed to handle the solutions containing viral DNA. A total of three phenol/$CHCl_3$ extractions and one $CHCl_3$ extraction were performed and the DNA was precipitated by the addition of ethanol and ammonium acetate (see *Methods, Section 3.3*). DNA was recovered by centrifugation at 11,500g for 5 minutes after which the DNA pellet was washed, dried and resuspended as described in *Methods, Section 3.3.*

3.8. Southern Blot Analysis of Viral DNA

Purified viral DNA was digested with the appropriate restriction endonuclease, and run on a 0.8 or 1% agarose gel. After visualisation on a long wave UV transilluminator the DNA within the gel was denatured by shaking the gel in Gel Soak I for 30 minutes and this solution was neutralised by shaking the gel in Gel Soak II for 30 minutes. Capillary blotting overnight was used to transfer the DNA from the gel to nitrocellulose membrane. The DNA was fixed onto the membrane using a UV Stratalinker (120 joules), and the membrane was then incubated in 100ml prehybridisation mix (6x SSC, 5x Denhardts solution, 0.1% SDS, 20µg/ml denatured calf thymus DNA) at 68°C for 2 hours. This mix was discarded and replaced with 10ml of hybridisation mix (6xSSC, 10x Denhardt's solution, 20mM Tris-HCl, pH 7.5, 1mM EDTA, 0.5µg/ml denatured calf thymus DNA) to which 0.5ml of [^{32}P]-radiolabelled DNA had been added. For use as a probe in Southern blot analysis, 3-5µg of radiolabelled DNA in a total volume of 800µl of TE buffer was denatured by the addition of 100µl 1M NaOH followed by incubation at room temperature for 10 minutes. Just prior to adding the probe to the hybridisation mix, it was neutralised by adding 100µl of 1M HCl. Hybridisation was carried out at 68°C overnight. The radioactive solution was discarded and the blot washed twice in 2x SSC, 0.1% SDS for 1 hour at 68°C. The membrane was then rinsed in water, dried and exposed to Kodak X-Omat XS-1 film.

3.9. DNA Sequencing

Chain termination sequencing was performed using the Sequenase Version 2.0 kit (United States Biochemical). All plasmid DNA was PEG precipitated as follows prior to sequencing. Plasmid DNA, prepared as in *Methods, Section 2.3*, in a total volume of 50µl, was incubated with RNaseA at a concentration of 10µg/ml for 30 minutes at 37°C. The samples were then mixed well with 30µl of 20% PEG 6000, 2.5M NaCl and kept on ice for 1 hour. The precipitates were collected by centrifugation at 11,500g for 5 minutes and

washed with 70% ethanol. The pellet was dried and dissolved in 50µl distilled water. PEG-precipitated DNA (1-1.5µg in 18µl) was denatured by the addition of 2µl of 2N NaOH and kept at room temperature for 5 minutes. 8µl of filter sterilised 5M ammonium acetate (pH 7.4) was then added, and the denatured DNA was precipitated by addtion of 100µl ethanol at -70°C for 5 minutes. The precipitates were harvested by centrifugation at 11,500g for 5 minutes, washed with 70% ethanol and then dried under vacume. The denatured DNA pellet was kept in this form at -20°C until required. The pellet was dissolved in 10µl of distilled water immediately prior to use.

For each DNA to be sequenced a single annealing and subsequent labelling reaction was performed. To anneal primer to DNA, 1µl of primer (0.5-1.0pmol), 2µl of reaction buffer consisting of 200mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 250mM NaCl and appproximately 3µg of denatured DNA were mixed in a final volume of 10µl. The mixture was heated to 65°C for 2 minutes and allowed to cool slowly (typically over a period of 30 minutes) to room tempearature for annealing to occur. The annealed primer/template was stored on ice, and used within 4 hours. For the labelling reaction, Sequenase T7 DNA polymerase, a genetic variant of bacteriophage T7 DNA polymerase (Tabor & Richardson, 1989) was diluted 1:8 in ice-cold enzyme dilution buffer. Labelling of DNA was performed by adding to the annealed template/primer, 1µl 0.1M DTT, 2µl of (dATP) labelling mix (5x concentrate; 7.5µM dGTP, 7.5µM dCTP, 7.5µM dTTP), 50µCi/ml of [α-^{35}S]-dATP and 1µl of diluted Sequenase enzyme. For reactions in which sequences could be determined up to 500 bases from the primer, the labelling mix consisted of 1x dATP labelling mix. In order to determine sequences from 30 bases from the primer the reaction mix was composed of 0.5x (dATP) labelling mix and $MnCl_2$ buffer (15mM sodium isocitrate, 10mM $MnCl_2$) was added. In order to terminate the labelling reaction 3.5µl of the reaction mix was added to 2.5µl of ddNTP termination mix and incubated at 37°C for a further 3 to 5 minutes. 4µl of stop solution was then added to each of the termination reactions and the samples were boiled for 5 minutes prior to electrophoresis.

Electrophoresis was carried out at 70W through vertical gels 42 x 34 x 0.04 cm. Gels consisted of 6% acrylamide cross linked with 5% (w/v) N,N'-methyenelbisacrylamide and 9% urea in 1x TBE. Polymerisation was achieved by the addition of 0.05% (w/v) APS and 0.1% (w/v) TEMED. Both plates were treated with Repelcote enabling the gel to be transferred to Whatman 3mm chromatography paper following electrophoresis and dried under vacume. Dried gels were then exposed to Kodak XS-1 film.

3.10. Oligonucleotide Synthesis

Oligonucleotides were synthesised on a Cruachem PS250 Oligonucleotide Synthesiser. Following synthesis, oligonucleotides were removed from solid supports by treatment with ammonia (35% pure, specific gravity 0.88), heated to 55°C for 5 hours and then lyophilised. Oligonucleotide pellets were resuspended in 20µl deionised formamide and 5µl of 10x TBE. Purification of oligonucleotides was performed by polyacrylamide gel electrophoresis on denaturing polyacrylamide gels (15% acrylamide cross linked with 4% (w/v) N,N'-methyenelbisacrylamide in 1x TBE, 8M urea). Polymerisation was achieved by the addition of 0.05% (w/v) APS and 0.1% (w/v) TEMED. The oligonucleotides were visualised by UV shadowing, with short wave UV light, and excised from the gels. The oligonucleotides were then eluted from the gel slice by shaking in 1ml H_2O overnight at 37°C. Following phenol/$CHCl_3$ extraction and ethanol precipitation, the oligonucleotides were resuspended in 1ml of H_2O and their concentrations were estimated by spectrophotometry. The concentrations were usually in the range of 1 to 3mg/ml.

Section 4.

Polypeptide Analysis and Detection

4.1. Preparation of ^{35}S-methionine Labelled Extracts

Confluent monolayers of BHK cells in 50 mm plates were infected at a moi of 5 at 37°C. At 1 hour post infection, the cells were overlaid with 3 ml of ETC10 medium and infection continued for a further 2 to 4 hours. The media was removed and the cells were washed twice with 5 ml of Eagles medium containing one fifth the normal concentration of methionine supplemented with 2% NCS (low methionine medium). Cells were incubated at 37°C for 1 hour in low methionine medium before being replaced with fresh low methionine medium containing 100µCi/ml [^{35}S]-methionine per dish. At the required time, the infected monolayers were washed twice with PBS and drained well before the addition of 500µl of boiling mix. After 1-2 minutes, the lysed cells were transferred to a microfuge tube and boiled for 5 minutes. 15µl of the samples were then TCA precipitated to estimate the level of incorporation of ^{35}S-methionine. Samples were stored at -20°C.

4.2. Denaturing Polyacrylamide Gel Electrophoresis

The concentration of gel used to separate polypeptides depended on the resolution required which was determined by the sizes of the proteins being investigated. The ratio of acrylamide to the cross-linking agent N,N'-methylene bisacrylamide was 39:1 for single concentration gels, and 19:1 for gradient gels. Gel solutions containing the appropriate percentage of acrylamide were prepared in buffer of final concentration 375mM Tris-HCl pH 8.9 and 0.1% SDS. For gradient gels the (w/v) acrylamide solution at the higher concentration also contained 15% glycerol to stabilise the gradient. Polymerisation was initiated by the addition of ammonium persulphate and TEMED to final concentrations of 0.06% and 0.04% respectively. The gel solution was poured between 2 glass plates (22 x 24cm for full size gels or 8 x 6cm for mini-gels), separated by 1.5mm thick spacers, and sealed with rubber tubing. The gel was then left to polymerise under a thin layer of butan-2-ol. Following polymerisation a stacking gel consisting of 5% acrylamide solution in 122mM Tris-HCl pH 6.7, 0.1% SDS was added. Wells were formed by a Teflon comb.

Samples were boiled for 5 minutes in boiling mix prior to electrophoresis. This denaturing buffer contained bromophenol blue so as the dye front could be visualised. Electrophoresis was carried out in a buffer containing 52mM Tris, 53mM glycine, 0.1% SDS at 100V for approximately 3 hours.

4.3. Western Blot Analysis

Proteins were separated on a SDS-polyacrylamide gel (see *Methods, Section 4.2*) and transferred onto a nitrocellulose filter using a semi-dry blotting technique. Prior to transfer, nitrocellulose membrane and 6 sheets of 3mm blotting paper, cut to the same size as the SDS-polyacrylamide gel, were soaked in Western Transfer buffer. Three pieces of 3mm paper were placed on the anode and onto this stack was placed the nitrocellulose membrane. The gel was placed on the membrane, and to complete the transfer unit, a further three pieces of 3mm paper was stacked on top of the gel. The cathode was placed on the transfer unit and electrophoresis was carried out for one hour at 0.8mA/cm^2. After transfer, the nitrocellulose membrane was washed twice with TBS (5 minutes per wash). The membrane was then blocked with 3% gelatin in 1xTBS for 1 hour at 37°C followed by two 5 minute washes in TBS containing 0.05% Tween 20 (TTBS). The membrane was incubated with primary antibody at the appropriate dilution for 1 hour at 37°C in TTBS

containing 1% gelatin (mouse monoclonal antibodies were all used at a 1:2000 dilution). Following removal of the primary antibody, the membrane was given two 5 minute washes with TTBS and then incubated with secondary antibody diluted in TTBS containing 1% gelatin for 1 hour at 37°C. For most western blot analysis, the secondary antibody was anti-mouse IgG conjugated to horseradish peroxidase which was used at a dilution of 1:1000. The anti-mouse IgG conjugated to horesraddish peroxidase allowed the development of the blot with ECL colour development reagent. This involved washing the membrane twice (5 minutes per wash) with TTBS and then immersion in ECL colour development solution for 60 seconds. Proteins were visualised by exposing the membrane to Kodak X-Omat XS-1 film for times which varied from 5-15 seconds.

Following incubation with rabbit polyclonal antibody, anti-rabbit Protein A conjugated secondary antibody was used and development involved washing the membrane twice with TTBS (5 minutes per wash) followed by incubation for 2 hours at 37°C with ^{125}I protein A (5x 10^5 counts/strip) in protein A solution. The membrane was then washed in TTBS and bands were visualised by exposing the membrane to Kodak X-Omat XS-1 film overnight.

To reuse membranes for probing with other antibodies, the membrane was shaken in regeneration buffer for 1 hour at 55°C. The membrane was then washed twice (5 minutes per wash) in TBS. Prior to probing with another antibody the membrane was blocked with 3% gelatin in 1x TBS for one hour at 37°C. Subsequent probing with antibody was performed as described above.

4.4. Visualisation of Proteins Separated by SDS Polyacrylamide Gel Electrophoresis

a) Coomassie Brilliant Blue Staining.

Following electrophoresis, gels were fixed and stained by shaking in 0.2% Coomassie brilliant blue R250 in methanol:water:acetic acid (50:50:7) for 30 minutes. The gels were then destained with extensive washes in 5% methanol, 7% acetic acid. Coomassie brillant blue can detect protein to a limit of approx 0.5µg and was therefore suitable for visualising the major and most of the minor components of virus particles.

b) Silver Staining

Silver staining is a more sensitive method for visualising proteins than Coomassie brilliant blue, but it is not a quantitative staining technique. Gels were fixed in 30%

ethanol,10% acetic acid for at least 30 minutes followed by a 30 minute incubation in 30% ethanol, 0.5M sodium acetate, 0.5% gluteraldehyde and 0.2% sodium thiosulphate. The gels were then thoroughly rinsed in water (3x 30 minutes) and subsequently shaken for 20-60 minutes in 0.1% silver nitrate, 0.02% formaldehyde. The protein bands were visualised by placing the gels in 2.5% sodium carbonate, 0.01% formaldehyde for 5-15 minutes. Development was stopped by the addition of 0.5M EDTA for 10 minutes followed by extensive washes in water. All water used throughout this procedure was double distilled H_2O.

c) Detection of Radiolabelled Proteins

Gels were fixed for 1 hour in methanol:acetic acid:water (50:7:50) and then soaked in several changes of 5% methanol, 7% acetic acid. The gels were then dried under vacuum, onto Whatman grade 182 filter paper and exposed to Kodak X-Omat XS-1 film.

4.5. CAT Assay

CAT assays were performed on extracts of cells infected with virus expressing CAT protein as well as purified virions and light particles. For assaying activity in infected cell extracts, 35mm plates of BHK cells were infected with virus at a moi of 5 and incubated at 37°C until full cpe was visible. Cells were washed twice in PBS (3ml/wash) and resuspended in 200µl of 250mM Tris-HCl (pH 8.0). The cells were disrupted by freezing the samples on dry ice followed by thawing in a 37°C water bath and this process was performed a total of three times. The cellular debris was removed by centrifuging the samples at 11,500g for 5 minutes, and the supernatant was used as the CAT extract. For each reaction, 0.5µl ^{14}C-chloramphenicol, 1µl of Butyryl-CoA (25mM) and 18.5µl H_2O was added to 25µl of CAT extract. The mix was vortexed and incubated at 37°C for 30 minutes. The reaction was terminated by adding 200µl TMPD/Xylene (2:1) mix. The solutions were vortexed and then spun at 11,500g for 2 minutes. 150µl of the top organic phase was transferred to a scintillation vial, taking care not to disturb the lower aqueous phase. 5ml of scintillant was added to the vial and the samples counted using a Beckman Scintillation Counter.

4.6. Indirect Immunofluorescence

Cells grown on 13mm coverslips were infected with virus or transfected with plasmid DNA (*Methods, Section 1.9*). After overnight incubation the coverslips were removed, rinsed in PBS and fixed in pre-cooled acetone (-20°C) for 20 minutes, or methanol (-20°C) for 20 seconds. The coverslips were then rinsed in PBS and, in order to rehydrate the cells, 100µl of PBS/1% calf serum was added to the coverslip and incubated at room temperature for 5 minutes. The coverslips were then washed 3 times in PBS/1% calf serum. Just prior to the addition of antibody, coverslips were drained, and 100µl of pp65 antibody (diluted to 1:300 in PBS/1% calf serum) was added to each coverslip. After one hour at room temperature, the antibody was drained from the coverslips and they were washed three times in PBS/1% calf serum. The coverslips were then drained and the secondary FITC-conjugated goat anti-mouse antibody was placed on the coverslip at 1:100 dilution, and left at room temperature in the dark for one hour. The coverslips were drained and rinsed in PBS/1% calf serum, followed by rinsing in PBS and H_2O and finally mounted onto slides with PBS/glycerol (50:50). Cells were visualised using a Nikon Microphot-SA fluorescent microscope.

4.7. *In vitro* Transcription/Translation Reactions

In vitro transcription/translation reactions were performed using the Promega TNT Coupled Reticulocyte Lysate System. 1µg of plasmid DNA was added to a total volume of 50µl nuclease free H_2O containing 25µl TNT rabbit reticulocyte lysate, 1µl TNT reaction buffer, 40 units of T3 RNA polymerase, 1µl 1mM amino acid mix (minus methionine), 4µl [^{35}S]-methionine (10µCi/µl) and 1µl RNasin ribonuclease inhibitor (40 units/µl). The mixture was vortexed and incubated at 37°C for 30 minutes. Boiling mix was then added to the tubes and 5µl of the sample was run on a 12% SDS-polyacrylamide gel. The gel was then fixed in methanol:water:actetic acid (50:50:7), for 15 minutes and washed thoroughly in 5% methanol, 7% acetic acid. After drying the gel onto 3mm Whatman paper, it was exposed to Kodak X-100 film.

4.8. FPLC Purification of IgG

Anti-CAT monoclonal antibody was purified from the medium harvested following the passage of the antibody producing hybridoma cell line (5/24). In order to delipidate the hybridoma cell supernate 15mg/ml 'Cab-o-sil' was added and stirred for 30 minutes at room temperature, and then clarified by centrifugation at 2000rpm* for 20 minutes. The delipidated solution was chilled to 4°C and mixed with ammonium sulphate (312µg/ml) for 20 minutes on ice. To obtain the IgG extract from this solution it was centrifuged at 10,000g for 10 minutes and the pellet was resuspended in approx 5ml of 20mM Na-Phosphate buffer. Following dialyisis of this extract overnight in Na-Phosphate buffer, it was filtered using an 0.2µm Acrodisc and injected into a 10ml FPLC loop. FPLC purification of the sample through a Pharmacia Proten G column was then performed. Elution of the purified IgG from the column was carried out with 0.1M glycine-HCl pH 2.7. In order to neutralise the acidic elution buffer, 1.0M Tris-HCl pH 9.0 was added to each sample eluted from the column. The purified antibody was stored at -20°C in 100µl aliquiots.

*Sorvall SS34 Rotor

Part 1

Incorporation of vhs-CAT Fusion Protein into the HSV-1 Tegument

As outlined in the Introduction, acquisition of tegument is an intrinsic part of virion assembly among herpesviruses yet the pathway and factors which influence this process are poorly understood. Since only a subset of viral proteins are found in the tegument, it is reasonable to assume that targeting of proteins to this region of the virus particle is at least partly dependent on sequences resident within the tegument proteins. Identification of such sequences, and an understanding of their biological properties, would have significant impact on our knowledge of tegument assembly. A key aim of this study was to devise a manipulable experimental system that would enable characterisation of sequences that direct proteins to the tegument.

Initially, the strategy was to construct a fusion gene composed of the sequences from a tegument gene linked to a non-viral, non-structural gene. In the event that the resultant fusion product could be incorporated into virus particles, this system could then be used to manipulate the fusion gene and monitor how alterations to the sequence influenced targeting to the tegument. The tegument protein selected was the vhs polypeptide encoded by UL41 (*Chapter 4; Introduction, Section 4*) . Since this protein is not essential for virus growth, it was unlikely that alteration of its sequences would have any deleterious effect on virus viability. The non-structural component of the fusion protein was the bacterial chloramphenicol acetyl transferase (CAT) protein. This was deemed suitable since it was a polypeptide of reasonable size (25KDa), its enzymatic activity could be easily assayed and an antibody was commercially available to detect the protein. In combination, these factors would allow short stretches of amino acids from the vhs coding sequences (or sequences from other tegument genes) to be assayed for their ability to direct proteins to the tegument.

The system which was developed made use of data from previous studies where the HSV-2 strain G *vhs* gene had been inserted into the TK locus of HSV-1 strain 17 (Fenwick & Everett, 1990) These studies had shown that the HSV-2 vhs polypeptide was extremely efficient at abrogating polypeptide synthesis and was incorporated into HSV-1 virus particles.

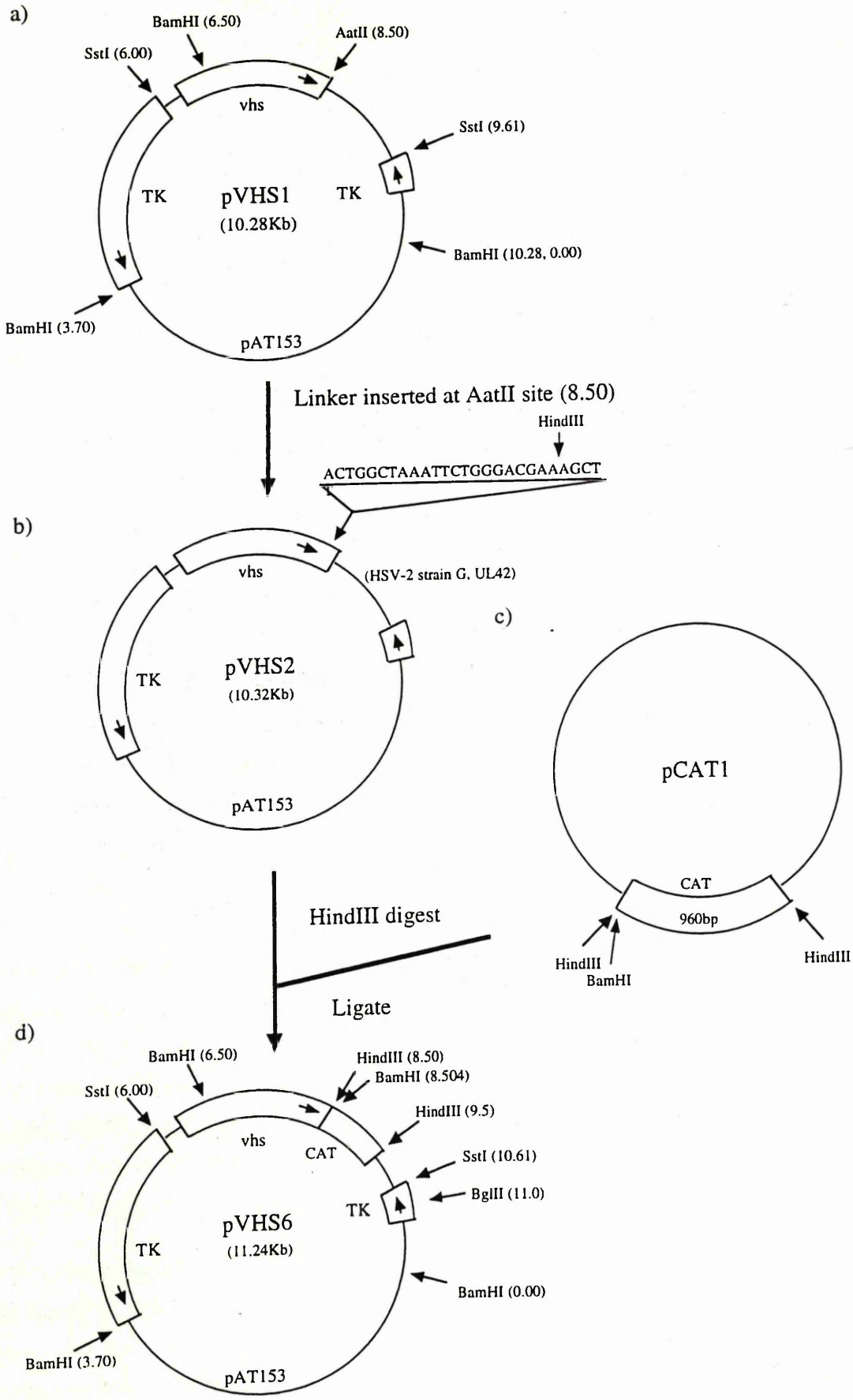

a)
BamHI (6.50)
AatII (8.50)
SstI (6.00)
vhs
SstI (9.61)
TK
pVHS1
(10.28Kb)
TK
BamHI (10.28, 0.00)
BamHI (3.70)
pAT153
Linker inserted at AatII site (8.50)
HindIII
ACTGGCTAAATTCTGGGACGAAAGCT
b)
vhs
(HSV-2 strain G, UL42)
TK
pVHS2
(10.32Kb)
pAT153
c)
pCAT1
CAT
960bp
HindIII
BamHI
HindIII
HindIII digest
Ligate
d)
BamHI (6.50)
HindIII (8.50)
BamHI (8.504)
SstI (6.00)
HindIII (9.5)
vhs
CAT
SstI (10.61)
BglII (11.0)
TK
pVHS6
(11.24Kb)
TK
BamHI (0.00)
BamHI (3.70)
pAT153

Figure 7. Details of plasmid vectors used to construct a vhs-CAT fusion gene. Plasmid pMF1 contains an SstI DNA fragment spanning the HSV-2 *vhs* sequences, inserted into the unique SstI site of pTK1 (Fenwick & Everett, 1990). The unique HindIII site within plasmid pMF1 was removed resulting in plasmid pVHS1 (7a). Partial digestion of pVHS1 with AatII and insertion at the AatII site at position 8.50 of an oligonucleotide that regenerated the C-terminal portion of the *vhs* gene resulted in plasmid pVHS2 (7b). The oligonucleotide inserted contained a HindIII site immediately following the codon specifying the C-terminal amino acid which enabled the insertion of the polypeptide coding seqences for the CAT gene as a HindIII fragment from plasmid pCAT1 (7c), This generated plasmid pVHS6 in which the CAT coding sequences are fused in frame downstream of the *vhs* coding sequences (7d).

Figure 8. Sequence read from plasmid pVHS6 across the oligonucleotide linker sequence. The oligonucleotide linker sequence and the CAT primer sequence are underlined. The CAT initiation codon and the *vhs* sequence are in bold type.

---

vhs sequence

5' **CCC CCG CCC GCG TTT TAC AAG GAC GTA**

P R P A F Y K N V

oligonucleotide linker HindIII BamHI

CTG GCT AAA TTC TGG GAC GAA AGC TTG GAT CCG

L A K F W N E S L D P

CAT initiation codon

TCG AGA TTT TCA GGA GGT AAG GAA GCT AAA **ATG**

S R F S G G K E A K M

CAT primer sequence

GAG AAA AAA ATC ACT GGA TAT ACC ACC GTT GAT

E K K I T G Y T T V N

ATA CCA CCG TTG ACC CAA TAT ATT GGC ATG 3'

I P P L T Q Y I G M

After promising initial results, the antibody which was used to detect the CAT component of the fusion protein was found to have poor avidity for the CAT protein and gave unreliable results. This necessitated the production of a new CAT antibody (*Chapter 7; Results, Part 2*) which delayed progress and prevented further use of CAT as a marker polypeptide.

Section 1

Construction of the vhs-CAT Fusion Gene

In order to construct a plasmid which would express the vhs-CAT fusion gene a series of cloning steps were performed (Fig. 7). The parent plasmid was pMF1, which had been constructed by Fenwick and Everett (1990). pMF1 contains a SstI DNA fragment, which spans the HSV-2 strain G vhs polypeptide coding sequences as well as its promoter and poly A sequences, inserted into the unique SstI site of plasmid pTK1 (Wilkie *et al.*, 1979). This site lies within the TK polypeptide coding sequences and therefore insertion at this site disrupts the gene. Plasmid pMF1 contains a unique HindIII restriction enzyme site, and to facilitate further manipulation of the plasmid this site was removed by digesting with HindIII and filling in the overhanging termini with Klenow polymerase. Ligation of the blunt ends resulted in the loss of the HindIII site and the derived plasmid was termed pVHS1 (Fig 7a). For the second stage of the cloning strategy pVHS1 was partially digested with AatII. This enzyme cleaves pVHS1 at 6 sites one of which lies 22bp upstream from the stop codon of the HSV-2 vhs polypeptide coding sequences. An oligonucleotide which regenerated the C-terminal portion of the vhs gene and contained a HindIII site immediately following the codon specifying the C-terminal amino acid was inserted at this AatII site to give plasmid pVHS2 (Fig. 7b). A HindIII DNA fragment from pCAT1 (Fig. 7c) which contained the polypeptide coding sequences of the CAT gene was inserted into the HindIII site of pVHS2; this generated plasmid pVHS6 (Fig. 7d).

In order to ensure that the oligonucleotide linker that fused the vhs sequences to the CAT sequences maintained the correct reading frame, the DNA sequence of the region spanning the oligonucleotide in plasmid pVHS6 was determined. An oligonucleotide was designed for use as a primer that annealed to sequences within the CAT open reading frame. The sequence obtained (Fig. 8) confirmed that the vhs and CAT polypeptide coding sequences would be read in the same open reading frame, generating a fusion protein with a predicted size of approximately 84KDa.

Section 2

Construction of Virus Recombinant vVHS-CAT

To insert the vhs-CAT fusion gene into HSV-1 DNA, pVHS6 was linearised by digestion with BglII (Fig. 7d) and transfected into sub-confluent BHK cells with HSV-1 strain 17 DNA. The resultant virus progeny were grown in the presence of BCdR which enables the selection of TK negative recombinants (*Chapter 6; Methods, Section 1.8*). From these progeny, 12 plaques were picked for further screening. Cells infected with each of the plaques were analysed for CAT activity. During the screening process CAT activity was recorded as being either positive or negative, and was not quantified. Extracts prepared from cells infected with all 12 plaques produced enzyme activity. One of the 12 isolates was chosen and used to infect cells again in the presence of BCdR. The process of screening for CAT activity was repeated on a further 12 isolates. After three rounds of such plaque purification one of the isolates was chosen and analysed by Southern blot analysis. This isolate was termed vVHS-CAT .

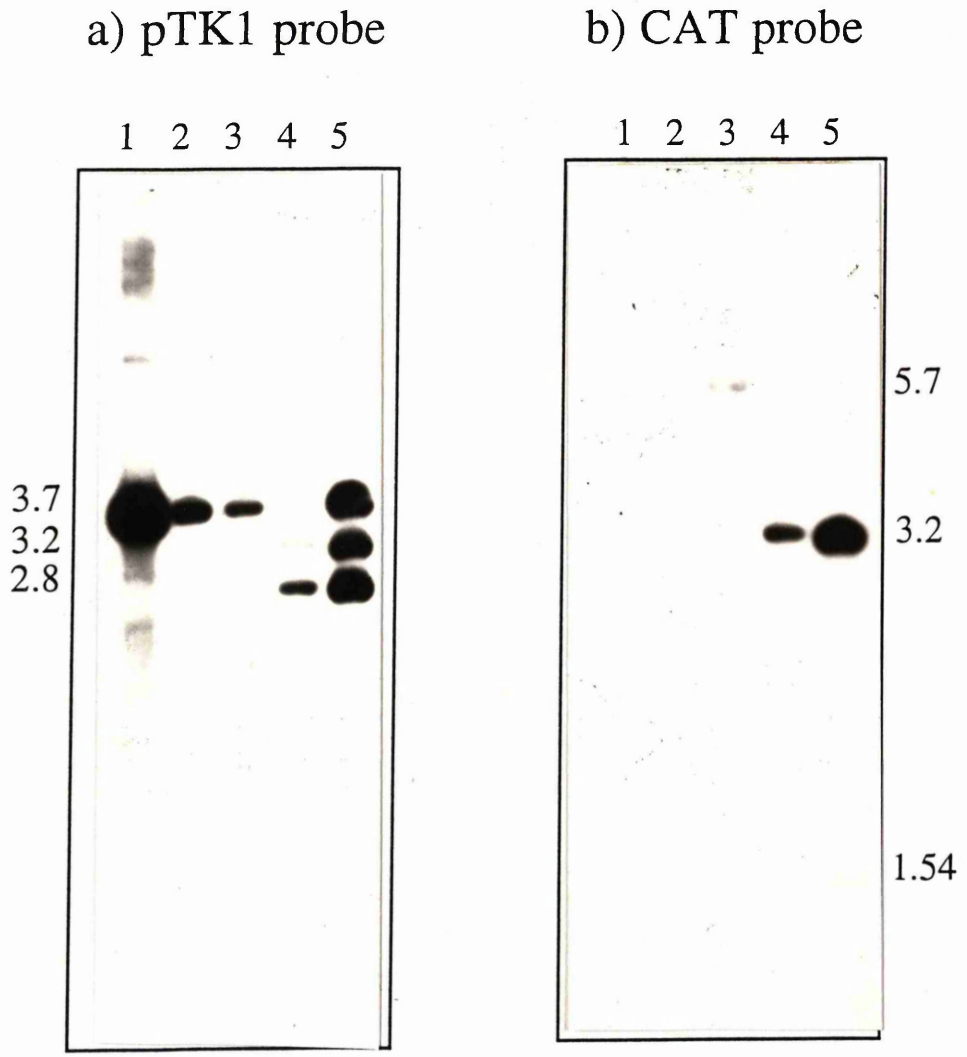
Section 3

Southern Blot Analysis of vVHS-CAT DNA

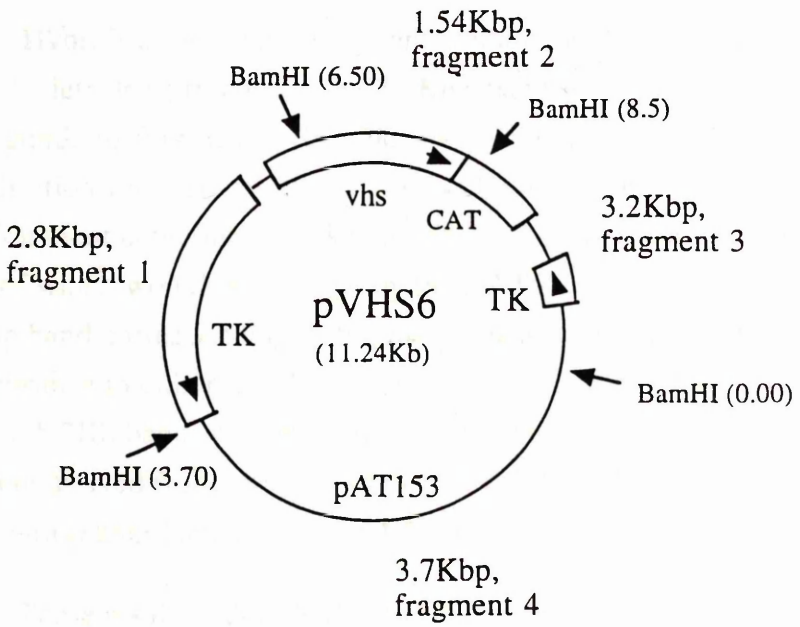
To verify that the vhs-CAT sequences had inserted at the predicted site within the TK gene, vVHS-CAT DNA was digested with BamHI along with the parent plasmid pVHS6, plasmid pTK1, wild-type HSV-1 strain 17 viral DNA, and vSau3 (*Chapter 5; Materials, Section 4*) viral DNA. Plasmid pTK1 consists of the HSV-1 strain 17 BamHI p fragment, which has a size of 3.7Kbp and contains the TK gene, inserted into the BamHI site of pAT153. Virus vSau3 contains the CAT open reading frame within the Us region of the genome under the control of the promoter sequence from HSV-2 UL40 gene. Following digestion, viral and plasmid DNAs were electrophoresed on a 1% agarose gel and transferred to Hybond membrane. Hybridisation was carried out using radiolabelled pTK1 which would recognise TK sequences and a 960bp HindIII fragment from pCAT1 (Fig. 7c) to detect CAT sequences.

Hybridisation with pTK1 detected a 3.7Kb band in pTK1 which is a doublet comprising of the pAT backbone and the BamHI p sequences (Fig. 9a, lane 1) The latter

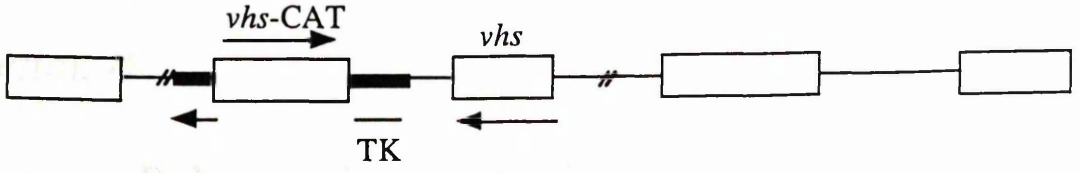
Figure 9. Southern blot analysis of vVHS-CAT viral DNA. 0.5µg of each DNA was digested with BamHI and then electrophoresed on a 1% agarose gel followed by transfer to Hybond-N membrane. Duplicate membranes were probed with radiolabelled pTK1 probe (9a) and with a 960bp HindIII fragment containing the CAT gene (9b). The DNAs in each lane were as follows: lane 1: pTK1 plasmid DNA; lane 2: HSV-1 strain 17 viral DNA; lane 3: vSau3 viral DNA; lane 4: vVHS-CAT viral DNA and lane 5: pVHS6 plasmid DNA. (9c) Plasmid pVHS6, illustrating the positions of the BamHI sites and the sizes of the fragments obtained following BamHI digestion. (9d) A diagramatic representation of the vVHS-CAT genome, containing the additional copy of *vhs* linked to the CAT sequence within the viral TK gene.



c)


1.54Kbp,
fragment 2
BamHI (6.50)
BamHI (8.5)
vhs
CAT
3.2Kbp,
fragment 3
2.8Kbp,
fragment 1
pVHS6
(11.24Kb)
TK
TK
BamHI (0.00)
BamHI (3.70)
pAT153
3.7Kbp,
fragment 4

d)


vhs-CAT
vhs
TK

fragment was also recognised in HSV-1 strain 17 and vSau3 viral DNAs (Fig. 9a, lanes 2 & 3); these virus genomes contain the intact viral TK gene. However, a band of similar size was not detected in vVHS-CAT DNA, but two bands of 3.2 and 2.8 Kbp were detected (Fig. 9a, lane 4). These bands are identical in size to two of the bands in the lane containing pVHS6 (Fig. 9a, lane 5). These correspond in size to BamHI fragments derived from pVHS6 which contain TK and vhs (2.8Kbp band; Fig. 9c, fragment 1) and CAT and TK sequences (3.2Kbp band; Fig. 9c, fragment 3). The pTK1 probe also detected a 3.7Kbp band in the lane containing pVHS6 which corresponds to the plasmid backbone (Fig. 9c, fragment 4).

Hybridisation with a fragment containing CAT sequences obtained from plasmid pCAT1, detected principally the 3.2Kbp band in pVHS6 (Fig. 9b, lane 5). This band corresponds to fragment 3 (Fig. 9c), which contains the CAT coding sequences. Weak hybridisation was also detected to a 1.54Kbp band that corresponds to fragment 2 (Fig. 9c). This fragment contains the linker sequences between the CAT and vhs coding sequences some of which would be detected by the CAT DNA probe. In vVHS-CAT viral DNA, the 3.2Kbp band corresponding to fragment 3 was detected (Fig. 9b, lane 4) but the probe did not hybridise to either pTK1 or HSV-1 strain 17 DNA (Fig. 9b, lanes 1 and 2). In vSau3 DNA, a 5.7Kb band was detected (Fig. 9b, lane 3) which corresponds in size to a BamHI fragment derived from insertion of the CAT ORF and *LacZ* sequences into the unique XbaI site of virus strain 1802 (Rixon & McLauchlan, 1990; McLauchlan *et al.*, 1989).

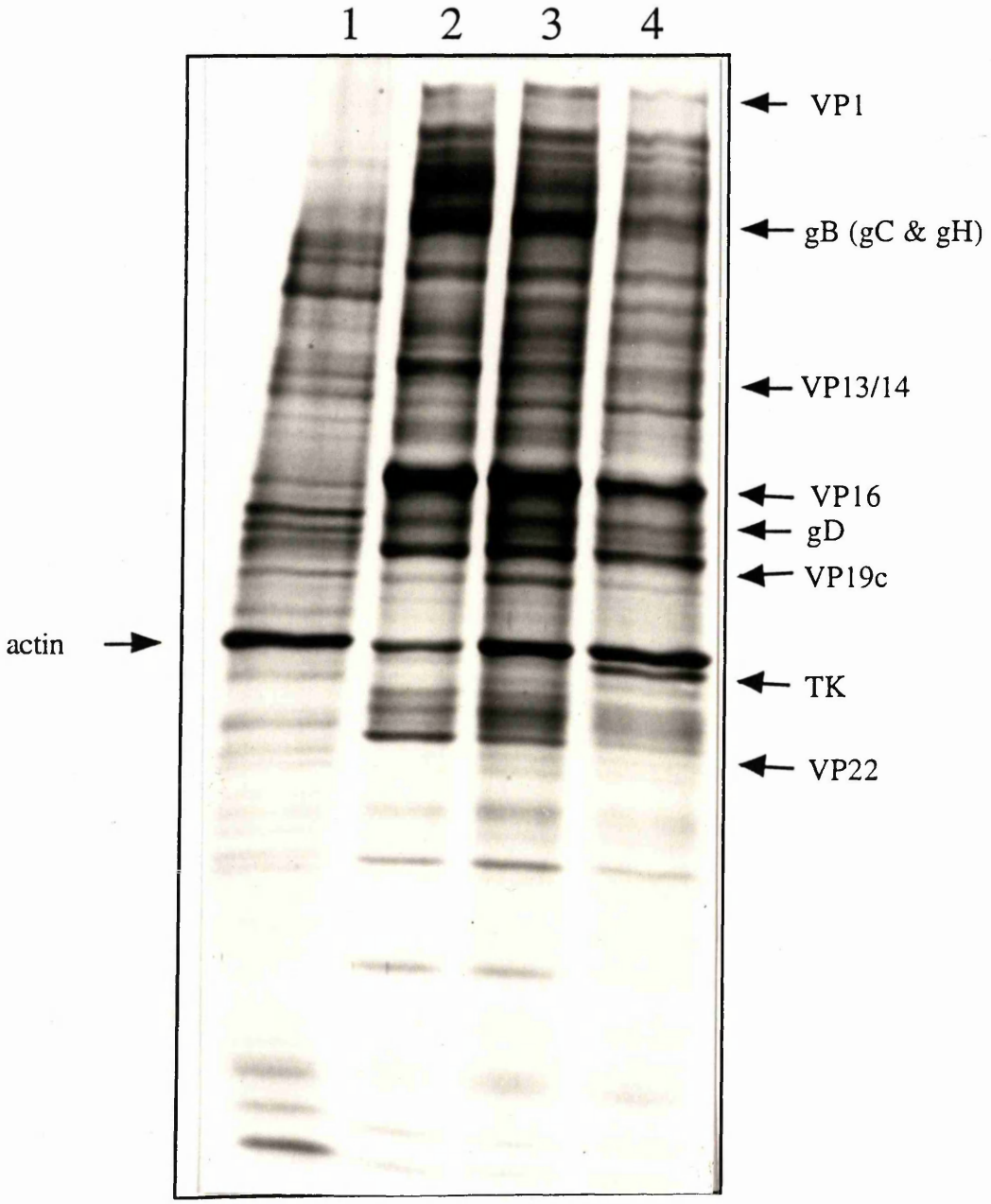
These results indicated that the fusion gene had inserted at the TK locus and that no apparent rearrangement of the inserted sequences had occurred. The genome structure of vVHS-CAT DNA shows that the recombinant virus has two copies of vhs sequences, the endogenous HSV-1 strain 17 copy and the HSV-2 strain G counterpart linked to CAT that lies within the TK gene (Fig. 9d).

Section 4

Polypeptides Synthesised by vVHS-CAT

The polypeptides synthesised by vVHS-CAT were compared to those made by virus controls. Fig. 10 compares the ^{35}S-methionine labelled polypeptide profiles of cells infected with vVHS-CAT, 17G41, and vFJ10. vFJ10 is derived from HSV-1 1802, contains an intact TK gene and expresses CAT protein under the control of the HSV-2 IE 4/5 promoter.

Figure 10. Polypeptide profile of cells infected with vVHS-CAT. Aliquots containing $5x10^5$cpm from radiolabelled extracts of cells infected with 17G41 (lane 2), vVHS-CAT (lane 3), vFJ10 (lane 4) and mock infected cells (lane 1) were electrophoresed on a 9% polyacrylamide gel. The band corresponding to TK is indicated as are some of the major viral polypeptides.



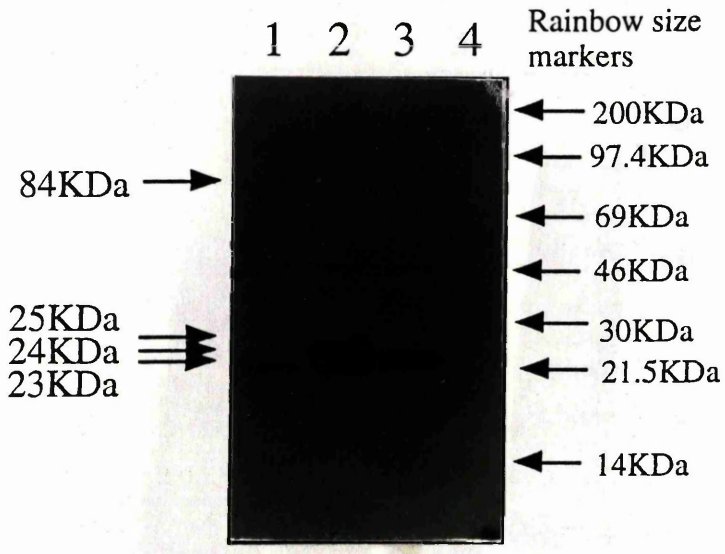


Figure 11. Detection of vhs-CAT fusion protein in infected cell extracts. Approximately $6x10^4$ infected cell equivalents were loaded onto a 9% polyacrylamide gel, and following electrophoresis were transferred to nitrocellulose membrane. The membrane was then incubated with anti-CAT monoclonal antibody 5/24. Samples were loaded as follows; lane 1: vVHS-CAT; lane 2: v65-CAT; lane 3: vSau3; lane 4: 17G41 infected cell extracts. The 84KDa vhs-CAT fusion protein is indicated, as is the 24KDa native CAT protein.

17G41 was made in a similar manner to vVHS-CAT by recombining the HSV-2 strain G vhs gene into the TK locus of HSV-1 strain 17 DNA (Fenwick & Everett, 1990a). Approximately $2x10^6$ BHK cells were infected with 5 pfu/cell of each of these viruses or mock infected and the cells were labelled with ^{35}S-methionine from 3 hours post infection. Cells were harvested after 18 hours and extracts were made by resuspending the cells in 500µl of boiling mix. Polyacrylamide gel electrophoresis showed that the polypeptide profiles for each of the infected cell extracts were very similar, the most notable difference being the absence of TK protein in the vVHS-CAT and 17G41 samples (Fig. 10, compare lanes 2 and 3 with lane 4). This is the expected result since both of these recombinants have sequences inserted into their TK genes. However a novel band of 84KDa, the predicted size of the vhs-CAT fusion protein, was not visible in the vVHS-CAT sample (Fig. 10, lane 3). Western blotting, which is a more sensitive method for detecting proteins, was therefore used to reveal whether the fusion protein was synthesised in infected cells.

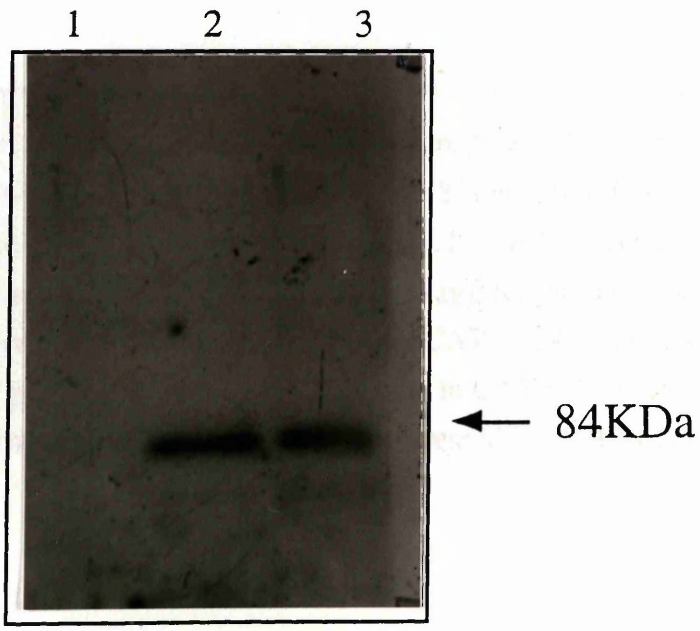
To determine whether vVHS-CAT-infected cells synthesised the fusion protein, extracts were made following an 18 hour infection of BHK cells with vVHS-CAT, 17G41, vSau3 and v65-CAT. v65-CAT is a virus containing CAT linked to a 10 amino acid epitope tag sequence under the control of the HCMV IE promoter sequences that was inserted into the unique XbaI site present in 1802 (*Chapter 8; Results, Part 1, Section 2*). Western blot analysis of these extracts was performed using an anti-CAT monoclonal antibody termed 5/25 (*Chapter 7; Results, Part 2*). The antibody detected a band of 24KDa in the vSau3-infected cell extract which corresponds to the size of CAT protein (Fig. 11, lane 3). In the v65-CAT-infected cell extract a major band of 25KDa was recognised (Fig. 11, lane 2) which represents CAT protein with the 10 amino acid epitope tag fused to the N-terminus of the protein (*Chapter 8; Results, Part 2*). A second minor band of 23KDa also was detected which presumably represents epitope-tagged CAT protein cleaved at either the N- or C-terminus by proteolysis. In the vVHS-CAT sample, two species were detected of 84KDa and 23KDa (Fig. 11, lane 1). The 84KDa protein corresponds to the predicted size of vhs-CAT polypeptide while the 23KDa species precisely coincides with the minor component in the vSau3 sample (Fig. 11, lane 2). From these data, it was concluded that the vhs-CAT protein is synthesised in vVHS-CAT infected cells. The 23KDa species was presumed to be a breakdown product of the vhs-CAT protein although it may also represent the product of internal initiation.

Preparation of vVHS-CAT had shown that CAT fused to vhs was expressed in an enzymatically active form. In order to quantify the levels of CAT activity expressed by

	vVHS-CAT-infected cells	vSAU3-infected cells	v65-CAT-infected cells
CAT activity (% conversion/μg of protein)	3.6	9.3	24.1
Relative CAT activity	1	2.6	6.7

Table 5. Relative levels of CAT activity in vVHS-CAT-, vSAU3- and v65-CAT-infected cells. 35mm dishes containing confluent BHK cells were infected with each virus at a moi. of 5 pfu/cell. The cells were harvested following an 18 hour infection, and extracts were prepared as described in *Chapter 6; Methods, Section 4.5*. CAT assays were performed on known quantities of protein(determined by Bradford assay)present in extracts prepared from cells infected with either vVHS-CAT, vSAU3 or v65-CAT. CAT activity is expressed as the % conversion/μg of protein. The calculated increase in CAT activity in vSAU3 and v65-CAT infected cells are shown relative to the activity expressed in vVHS-CAT infected cells.

Figure 12. Detection of vhs-CAT fusion protein in purified virions and light particles. Approximately 20μg gradient purified virions and light particles were run on a 9% polyacrylamide gel along with the equivalent quantity of 17G41 virions. The polypeptides were transferred to nitro-cellulose membrane and the membrane was incubated with anti-CAT polyclonal antisera. Lane 1: 17G41 virions; lane 2: vVHS-CAT virions; lane 3: vVHS-CAT light particles. The size of the band detected (84KDa) was determined by staining the membrane with imido black.



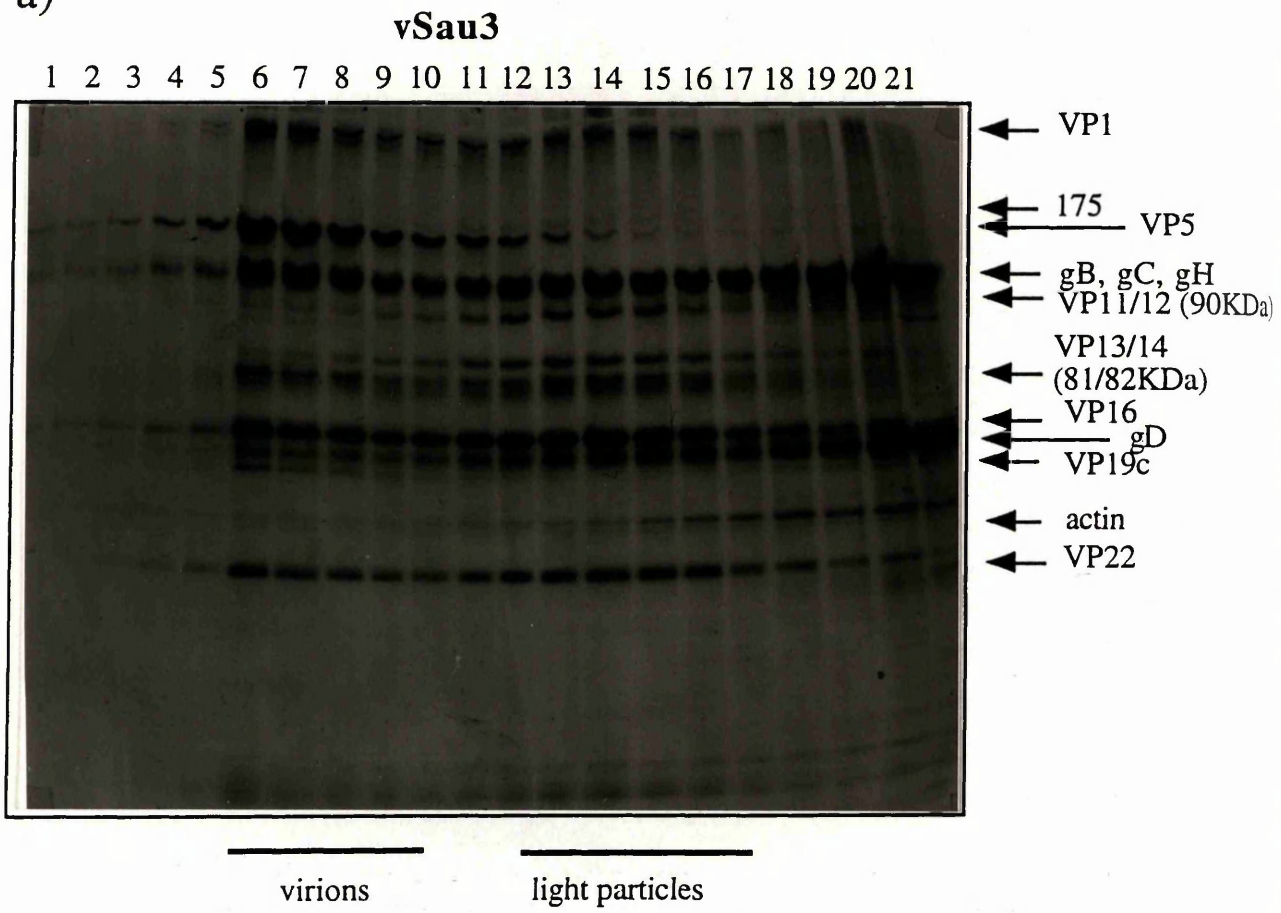
vVHS-CAT infected cells, known quantities of infected cell protein (determined by Bradford assay) were serially diluted, analysed for CAT activity and compared with levels expressed in both vSAU3- and v65-CAT- infected cells (Table 5). The results indicated that vSAU3 expressed approximatley 2.6-fold more CAT activity than vVHS-CAT but more dramatically, v65-CAT expressed approximately 6.7-fold more CAT activity than vVHS-CAT.

Section 5

The vhs-CAT Fusion Protein is Present in Purified Virions and Light Particles

In an initial attempt to determine whether the fusion protein was incorporated into vVHS-CAT virus particles, Western blot analysis was performed on gradient-purified virions and light particles. These were prepared from six roller bottles of BHK cells which were infected with vVHS-CAT at a moi of 1 pfu per 300 cells and incubated at 37°C until full cpe was visible. The virus was harvested from the culture medium and purified on 5-15% ficoll gradients. Virions and light particles were collected and pelleted for further analysis. Following resuspension approximately 20µg of the virion and light particle samples were analysed by Western blot using a rabbit polyclonal antisera raised against CAT protein. This antisera detected a polypeptide of 84KDa, the predicted size of the vhs-CAT fusion protein, in both vVHS-CAT virions and light-particles (Fig. 12, lanes 2 and 3). The antisera did not react with similar quantities of virions from a control virus, 17G41 (Fig. 12; lane 1), which contains the HSV-2 strain G vhs gene within its TK locus. This indicated the presence of the vhs-CAT fusion protein in purified virions and light particles. However from data presented in *Chapter 8; Results, Part2, Section 5*, it is possible that detection of certain proteins by antibodies in purified virus particle preparations results from the smearing through gradients of proteins which are not incorporated into virus particles. Therefore, a more thorough characterisation of the polypeptides present in vVHS-CAT virus particles was performed. For comparative purposes, 2 viruses, vSau3 and v65-CAT were used as controls to determine the behaviour of native CAT protein.

a)


vSau3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
VP1
175
VP5
gB, gC, gH
VP11/12 (90KDa)
VP13/14
(81/82KDa)
VP16
gD
VP19c
actin
VP22
virions
light particles

b)

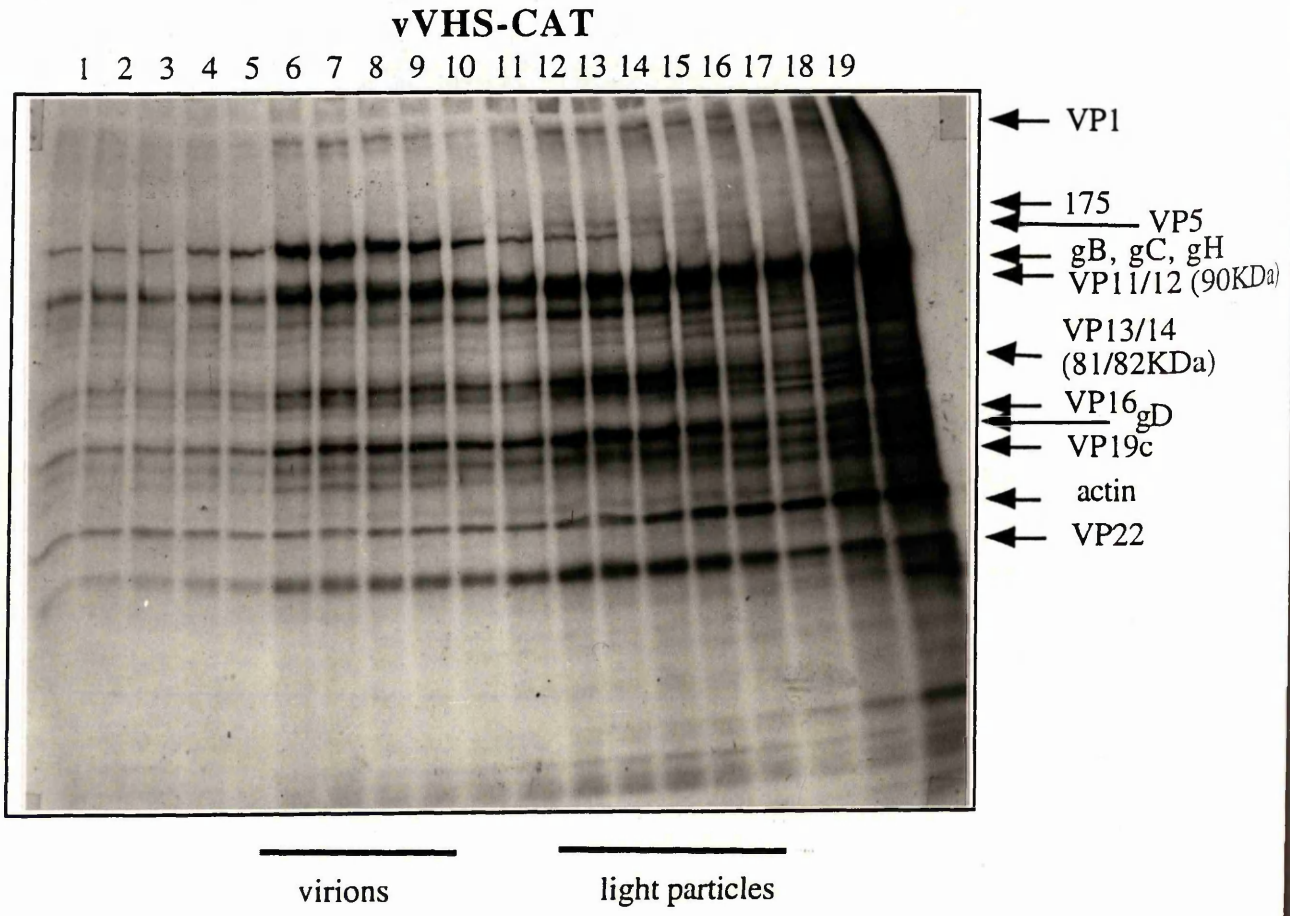

vVHS-CAT
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
VP1
175
VP5
gB, gC, gH
VP11/12 (90KDa)
VP13/14
(81/82KDa)
VP16
gD
VP19c
actin
VP22
virions
light particles

Figure 13. Comparison of polypeptide profiles of virus particles collected from across ficoll gradients. Virus was harvested 72 hours after infection and the cell released material for each virus was banded on 13ml 5-15% Ficoll gradients. Twenty, approximately 500µl aliquots were collected from about 2cm above the bottom of the tube to the top of the gradient. 40µl of radiolabelled material from each fraction across both gradients were electrophoresed on 5-12% polyacrylamide gels. a) represents the material from a vSau3 gradient, and b) is the vVHS-CAT equivalent. The bottom of the gradient is at the left of the gel and the top is to the right. The major polypeptides are indicated, as are the regions in which virions and light particles were seen to migrate. The apparent molecular weights of VP11/12 (90KDa) and VP13/14 (81/82KDa) are indicated.

* however in the vVHS-CAT gradient a band of similar molecular weight to VP19C appeared to increase in intensity in fractions 16 to 19.

Section 6

Analysis of Polypeptides Present in vVHS-CAT Virions and Light Particles

Previous studies have shown that virus particle purification on ficoll gradients provides both virions and light particles in a highly purified state (Szilaygi & Cunningham, 1991). However analysis of the changes in the patterns of polypeptides across a gradient in which virions and light particles had been separated from each other and from other cellular material had not been carried out. From such analysis, it would be predicted that structural proteins should primarily co-localise with the positions of virions and light particles in the gradient. To perform such a study and to determine whether the vhs-CAT fusion protein, which was found in preliminary analysis in virions and light particles, could be conclusively identified as a structural component, ficoll gradients containing vVHS-CAT virus particles were studied by three separate methods; i) by analysing the changes in polypeptide patterns across gradients, ii) by assaying CAT activity across gradients, iii) by performing Western blot analysis on gradient fractions.

i) Analysis of the changes in polypeptide patterns across ficoll gradients

To analyse the changes in polypeptide patterns across ficoll gradients 4 roller bottles of BHK cells were infected with either vVHS-CAT or vSau3 at a moi of 1 pfu per 300 cells at 31°C. 24 hours after infection, 250μCi of ^{35}S-methionine was added to each of the roller bottles. 4 days after infection, cell-released virus was prepared and ran on ficoll gradients. 500μl fractions across each gradient were collected and 40μl aliquots were ran on 5-15% polyacrylamide gels (Fig. 13). During collection of the fractions it was noted that virions were concentrated in fractions 6 to 10 and light particles were present in fractions 12 to 16.

Analysis of the polypeptides present in each fraction revealed that the major capsid protein, VP5 was most abundant in fractions 6 to 8 in each gradient. These fractions correspond to those containing virions. Thereafter, abundance declined and beyond fraction 14, VP5 was barely detectable. A second capsid protein, VP19c showed a similar pattern of appearance and disappearance across the gradient. The other major species identified in the virion fractions included tegument proteins, (VP1, VP11/12, VP13/14, VP16 and VP22) and glycoproteins (gB/gC/gH and gD). Beyond the virion fractions, the abundances of these

vSAU3 *

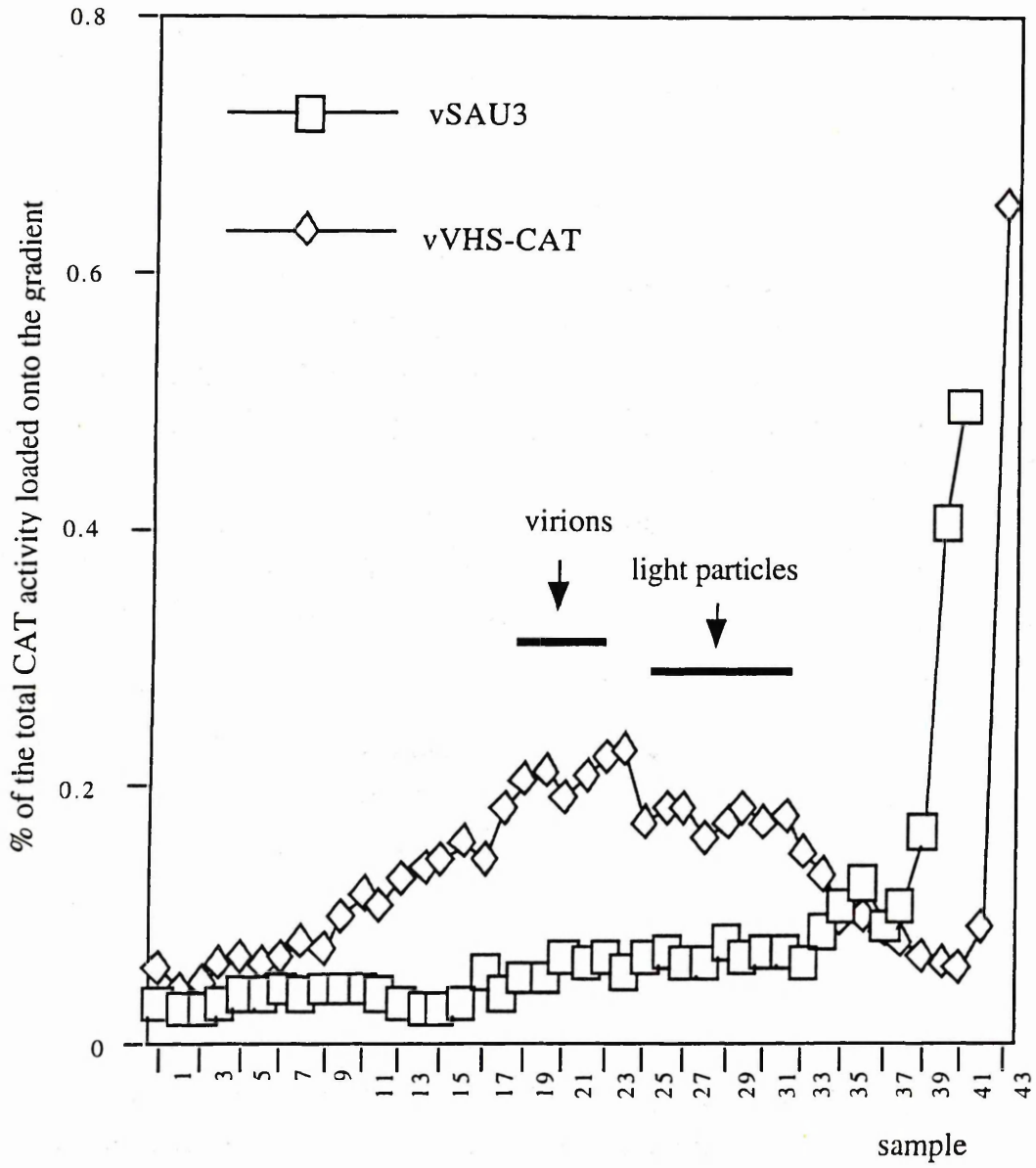


Figure 14. Detection of CAT activity in vVHS-CAT virions and light particles. Following the inoculation of 4 roller bottles of BHK cells with either vVHS-CAT or vSau3 at a moi of 1 pfu per 300 cells, virus was harvested after 72 hours incubation at 31°C, and the cell released material for each virus was banded on 13ml 5-15% ficoll gradients. 43x 250µl fractions were collected from each of the gradients from aproximately 2cm below the virion band. CAT assays were performed on 25µl of each fraction and activity is expressed as a percentage of the total CAT activity loaded onto each gradient.

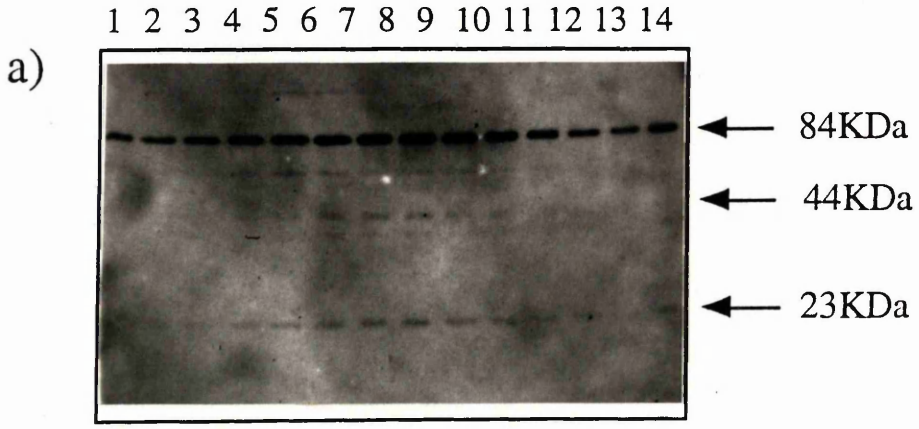
tegument and envelope proteins did not diminish. Indeed, certain proteins increased in abundance (eg. VP11/12, VP13/14, VP16, VP22) and other novel species appeared (Vmw175). These fractions (12 to 16) correspond to the region in the gradient containing light particles. Previous data has shown that Vmw175 is specific to light particles made in BHK cells (McLauchlan *et al.*, 1992a). From fractions 17 to 19 the abundance of the tegument proteins markedly decreases and the polypeptide pattern progressively resembles that of the soluble protein which has not entered the gradient (fraction 19). However at the top of the gradient there remain low but detectable amounts of tegument proteins. It is interesting to note that the glycoprotein band that contains gB, gC and gH does not specifically localise to virions and light particles but is present in high abundance towards the top of the gradient. A band of 45KDa which presumably corresponds to actin, a very abundant cellular protein, is present in low concentrations throughout the gradient but is most intense at the top. These results show that although protein is present throughout the ficoll gradients, the fractions containing virions and light particles can clearly be distinguished by the abundance of major capsid protein (for virions) and that of tegument proteins (for virions and light particles).

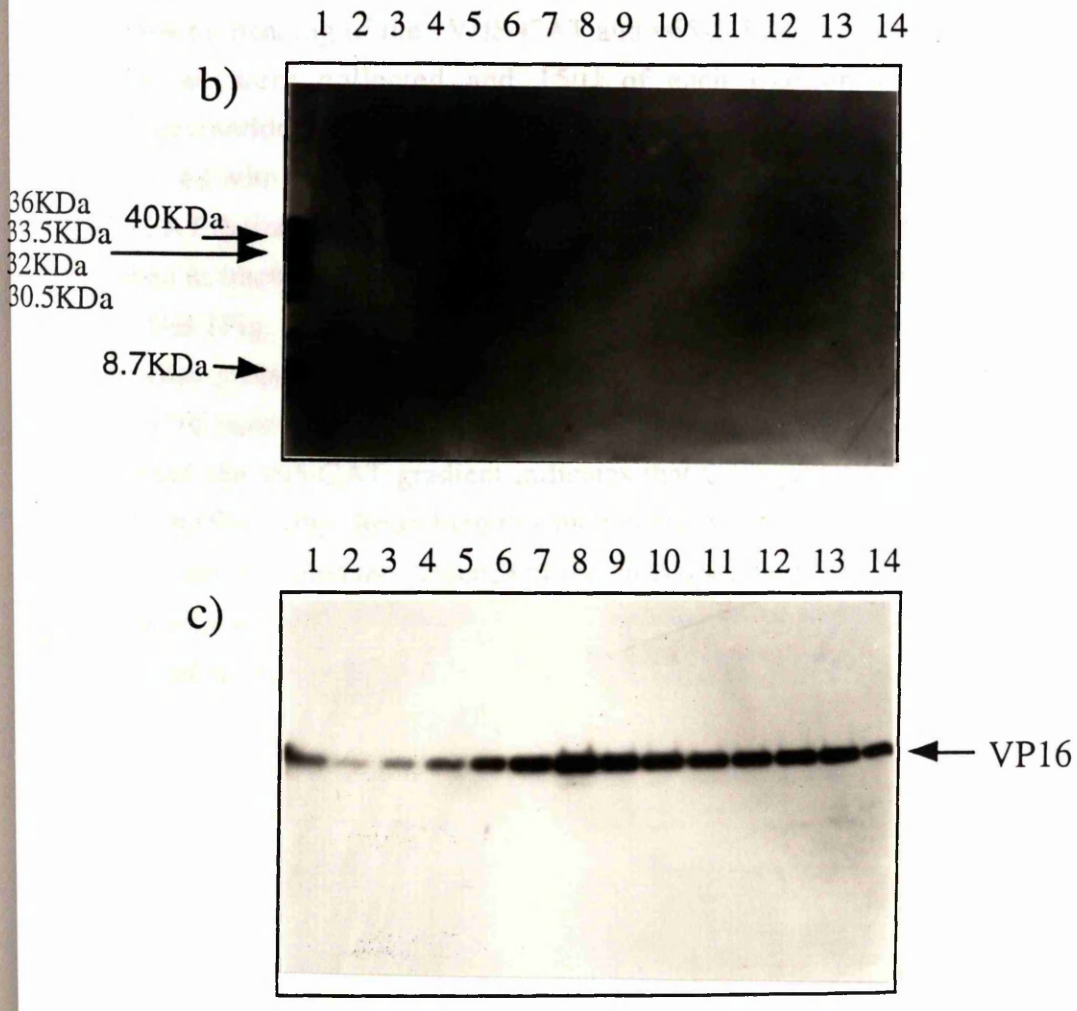
Comparison of the vVHS-CAT and the vSau3 gradients failed to identify any novel protein of 84KDa which would correspond to the fusion protein. This protein is therefore likely to be of minor abundance in vVHS-CAT virions and light particles.

ii) Analysis of CAT activity

Since the fusion protein could not be directly detected by analysing the polypeptides in virions and light particles, CAT assays were performed on fractions collected from ficoll gradients of vVHS-CAT and vSau3 virus preparations. Preliminary studies had shown that CAT activity could be directly determined without the need for biochemical disruption of the virus particle (data not shown). Gradients of vVHS-CAT and vSau3 virus particles were run and fractions collected. 25µl aliquots from each sample were analysed for CAT activity and the results are presented in Fig. 14. Since the level of CAT activity present in vSau3-infected cells was considerably higher than that in vVHS-CAT infected cells, CAT activity in each sample is expressed as a percentage of the total CAT activity loaded onto each gradient. In the vVHS-CAT gradient, a broad peak of activity was formed across the region in which virions and light particles migrated. In contrast the equivalent location within the vSau3 gradient had very low levels of CAT activity. In both gradients levels of CAT activity were seen to rise at the top where the soluble infected cell material is found.

Figure 15. Detection of vhs-CAT fusion protein in material collected from across a ficoll gradient. Following banding of the vVHS-CAT and v65-CAT virus preparations on 13ml ficoll gradients, fourteen 700µl fractions were collected from 2cm above the bottom of each tube to the top of each gradient and 15µl of each sample was loaded onto 12% SDS polyacrylamide gels. The polypeptides were then transferred to nitro-celloulose membranes. The membranes were incubated with anti-CAT monoclonal antibody, and the v65-CAT membrane was then stripped and reprobed with LP-1 monoclonal antibody. a) The vVHS-CAT membrane probed with anti-CAT monoclonal antibody 5/25. The 84KDa band representing the vhs-CAT fusion protein is indicated, as are the sizes of the major breakdown products of the vhs-CAT fusion protein. b) The v65-CAT membrane probed with pp65 monoclonal antibody. c) v65-CAT membrane reprobed with LP-1 monoclonal antibody. VP16 is indicated. In lane 1 of b) and c), vUL49ep infected cell extract was included as a positive control. This virus is described in *Chapter 8; Results, Part 2.* The sizes of the polypeptides detected by the antibody are indicated.



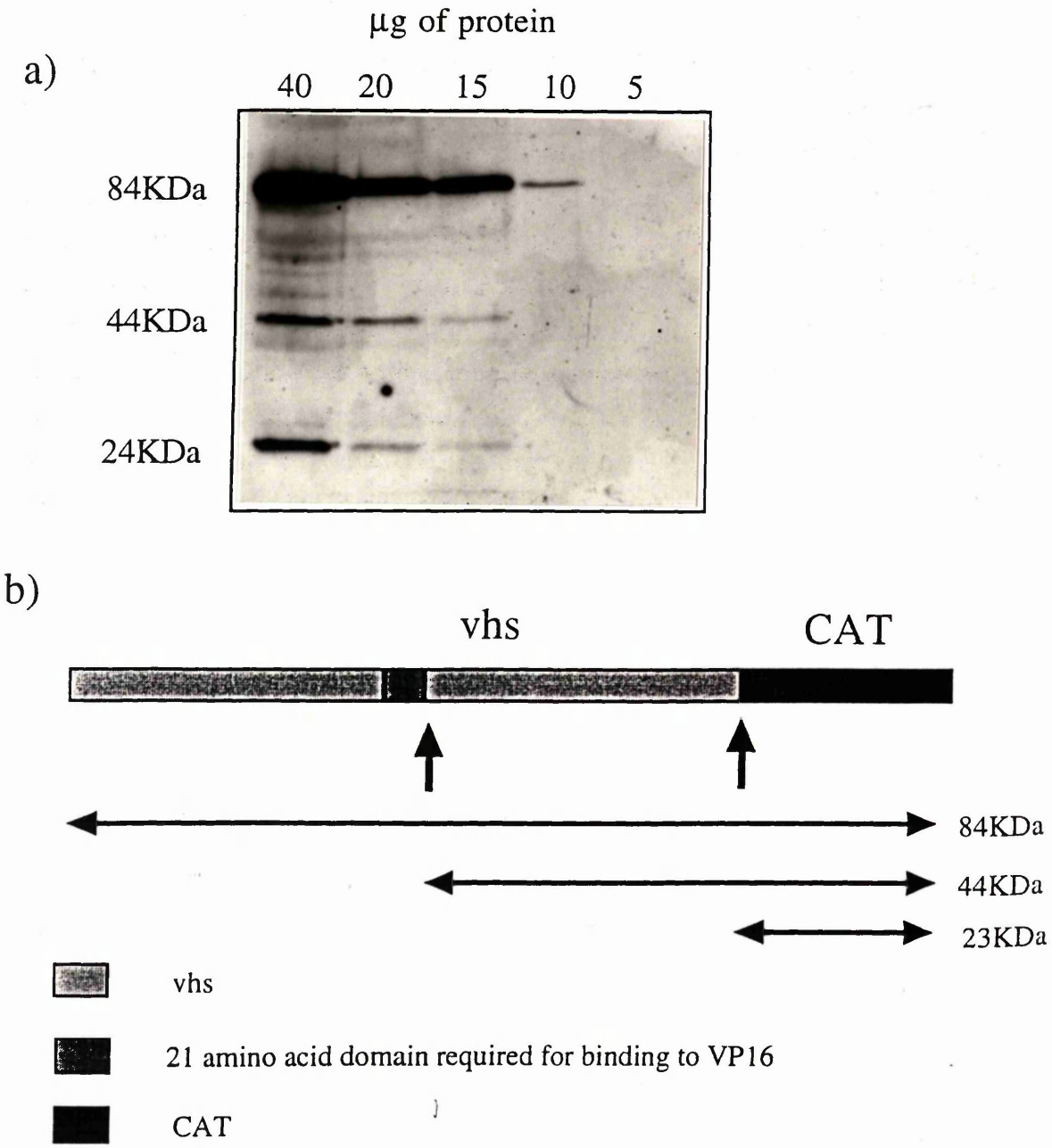

1 2 3 4 5 6 7 8 9 10 11 12 13 14
b)
36KDa
33.5KDa
32KDa
30.5KDa
40KDa
8.7KDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14
c)
VP16

The coincidence of CAT activity in fractions containing virions and light particles provide evidence that the vhs-CAT fusion protein, but not native CAT protein, is incorporated into virions and light-particles.

iii) Western Blot Analysis

For Western blot studies virions and light-particles were prepared from four roller bottles which had been infected with vVHS-CAT (as described in the previous two sections). On this occasion a parallel preparation of control virus, v65-CAT, was made. Following banding of the vVHS-CAT and v65-CAT virus preparations on ficoll gradients, fractions were collected and 15µl of each fraction was loaded onto 12% SDS polyacrylamide gels. After transfer onto nitrocellulose membranes, both membranes were incubated with anti-CAT monoclonal antibody (Fig. 15a and 15b). Results showed that the vhs-CAT fusion protein was detected in all fractions collected, and the intensity of the bands peaked in fractions 5 to 12 which is the region of the gradient that contains virions and light particles (Fig. 15a). This pattern of appearance of the vhs-CAT protein reflects that of tegument proteins in Fig. 13 where they were most abundant in fractions containing virions and light particles but could be detected even in fractions at the top of the gradient. In contrast, the v65-CAT gradient indicates that CAT protein cannot be detected within the gradient (Fig. 15b). Reprobing this membrane with LP-1 monoclonal antibody, specific for VP16, demonstrated the presence of the virus-specific VP16 polypeptide within the samples containing virions and light particles (Fig. 15c). This data strongly suggests that the vhs-CAT fusion protein is incorporated into vVHS-CAT virions and light particles.

Figure 16. a) Detection of breakdown products of the vhs-CAT fusion protein. Ficoll purified vVHS-CAT virions were loaded onto a 9% polyacrylamide gel in the quantities indicated. Following electrophoresis the polypeptides were transferred to nitrocellulose membrane and then incubated with anti-CAT polyclonal antisera (1: 3000 dilution). The sizes of the polypeptides detected are indicated. b) Illustrates the possible cleavage sites which would give rise to the breakdown products.



Section 7

Detection of Breakdown Products of the vhs-CAT Fusion Protein

From the Western blot studies shown in Figs. 11 and 15a, it was apparent that the monoclonal mouse CAT antibody detected not only the 84KDa vhs-CAT fusion protein but also lower molecular weight species in extracts from vVHS-CAT infected cells and virus particles. Further Western blot analysis with the rabbit polyclonal anti-CAT antiserum also identified breakdown products of the vhs-CAT fusion protein. Fig. 16a shows a further set of data where the breakdown products were detected in vVHS-CAT virions. The major polypeptides detected by the antiserum have sizes of 84KDa (full-length vhs-CAT fusion protein), 44KDa and 23KDa. These are consistent with the molecular weights of the smaller products found in Figs. 11 and 15a. Since these antibodies detect the C-terminal CAT portion of the fusion protein, Fig 16b illustrates the possible cleavage sites which would give rise to the breakdown products assuming that cleavage of the vhs-CAT protein does not occur in the CAT sequences. Interestingly, the 23KDa polypeptide is similar in size to native CAT protein, suggesting that cleavage of the fusion protein may occur in the region linking the vhs and CAT proteins. The 44KDa protein detected would suggest a region sensitive to proteolysis within the vhs protein.

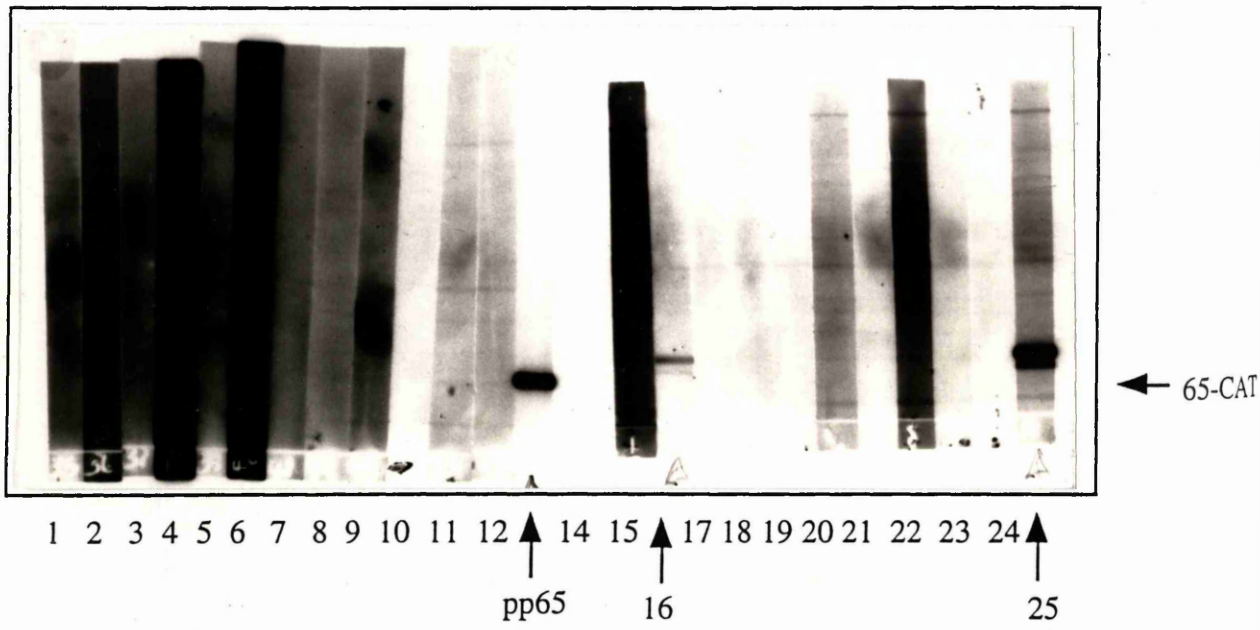


Figure 17. Western blot analysis of v65-CAT infected cell extracts using undiluted supernatant from each of the selected hybridoma cell lines. As a positive control one strip (13) was incubated with pp65 monocloanl antibody at a 1:2000 dilution. The hybridoma cell lines producing anti-CAT IgG are indicated (16 and 25).

Figure 18. Western blot analysis of v65-CAT infected cell extracts using undiluted supernatant from hybridoma cell line numbers 16 and 25. As a positive control one strip was incubated with pp65 monocloanl antibody at a 1:2000 dilution.

Part 2

Purification of Anti-CAT Monoclonal Antibody

The commercially available anti-CAT antibody was found to have poor avidity for the CAT component of the vhs-CAT fusion protein, and gave unreliable results. This necessitated the production of a new CAT antibody, which delayed progress and prevented further use of CAT as a marker polypeptide.

The production of an anti-CAT monoclonal antibody was performed by Dr. S. Graham and involved the inoculation of Balb/c mice with purified CAT protein (supplied by Professor W. Shaw, University of Leicester). Spleen cells were removed from the mice and fused with SP2/OAg-14 Balb/c myeloma cells (supplied by Flow Laboratories), and colonies were selected in Dulbecco's modified Eagles medium supplemented with 100μM-hypoxanthine, 0.42μM-aminopterin and 63μM-thymidine (HAT). The supernatants were screened for reactivity with purified CAT protein by ELISA assay. The resulting cell lines were screened for the production of anti-CAT antibody via Western blot analysis.

Section 1

Screening of Hybridoma Cell Lines for the Production of Anti-CAT IgG

Western blot analysis was performed on v65-CAT-infected cell extracts (*Chapter 8; Results, Part 2*). This virus synthesises CAT protein tagged at the N-terminus with a 10 amino acid epitope tag that is recognised by a monoclonal antibody, pp65 (*Chapter 8; Results, Part 1*). Following separation of the v65-CAT infected cell extracts, and transfer to nitrocellulose membrane, the membrane was cut into 0.5cm strips, and each strip was incubated with undiluted supernatant from hybridoma cell lines which were reactive with CAT in ELISA assays. As a positive control, one strip was incubated with pp65 monoclonal antibody. Results indicated that supernatants from two cell lines detected the CAT protein which was also recognised by the pp65 antibody (Fig. 17, strips 16 and 25).

These two cell lines were then passaged a further twice, and the Western blot analysis of v65-CAT infected cell extracts was repeated using undiluted supernatant from these cell lines (Fig. 18). The hybridoma cell line derived from clone number 25 (Fig.18),

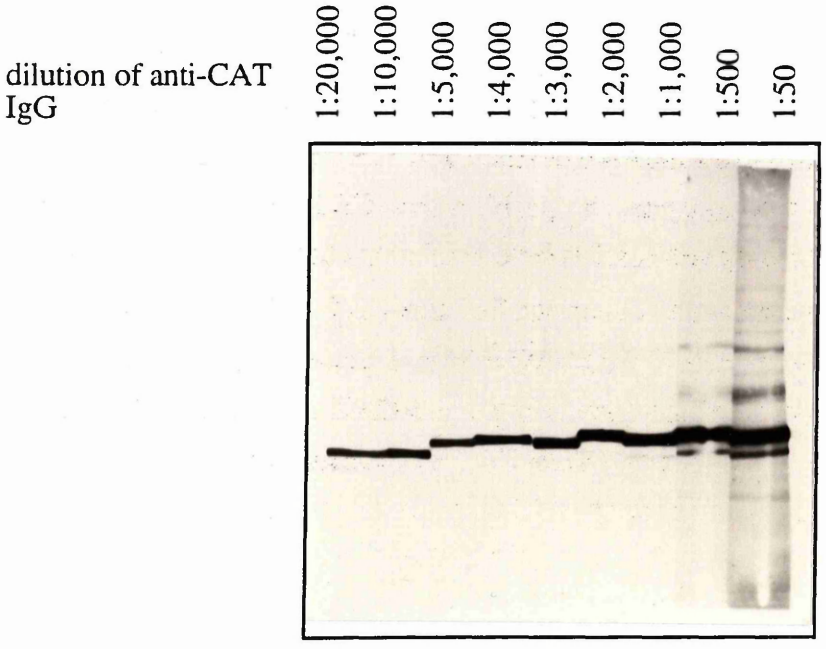


Figure 19. Titration of FPLC purified anti-CAT IgG by Western blot analysis. Following separation of v65-CAT infected cell extracts on a 14% polyacrylamide gel, the polypeptides were transferred to nitrocellulose membrane. The membrane was cut into 0.5cm strips and each strip was incubated with a different concentration of anti-CAT IgG. The dilutions of antibody are indicated.

appeared to have the greatest avidity for CAT. This hybridoma cell line was therefore selected for the production of anti-CAT IgG, and the antibody was termed 5/25. As described in *Chapter 6; Methods, Section 1.1*, cells were maintained in Dulbecco's modified medium which was harvested following each passage of the hybridoma cell line.

Section 2

FPLC Purification and titration of the anti-CAT Antibody

Anti-CAT monoclonal antibody (5/25) was FPLC purified through a Pharmacia Protein G column as decribed in *Chapter 6; Methods, Section 4.7.* In order to determine the optimal concentration at which the purified anti-CAT monoclonal antibody should be used during Western blot analysis, the purified anti-CAT antibody was titrated. 0.5cm nitrocellulose strips containg v65-CAT infected cell extracts were each incubated, for 1 hour at 37°C, with different concentrations of purified anti-CAT IgG. This showed that the CAT fusion protein could be detected by the antibody at dilutions as low as 1:20,000 (Fig. 19). The CAT protein was an abundant species in the v65-CAT infected cell extract, however the amount of CAT protein produced by vVHS-CAT was considerably less and therefore it was decided to use the antibody at a dilution of 1:2000.

Discussion

One of the key aims of this study was to devise a manipulatable experimental system that would enable the characterisation of sequences that direct proteins to the tegument. The tegument protein chosen for investigation was the vhs protein encoded by UL41. As a first step in determining which sequences were responsible for directing vhs into the tegument, it was decided to construct a fusion gene consisting of the HSV-2 UL41 gene, fused to the bacterial reporter gene chloramphenicol acetyl transferase (CAT). These sequences were recombined into the HSV-1 genome at the TK locus, and it was shown that the resulting fusion product was incorporated into virions and light particles.

This system of tagging structural polypeptides of viruses has previously been shown to be effective by two different groups (Haung *et al.*, 1988; Wu *et al.*, 1995). Haung *et al.* (1988) constructed a fusion protein composed of the 11KDa vaccinia virus structural protein linked to the bacterial *Lac Z* protein in order to develop a rapid selection procedure for vaccinia virus recombinants. Results showed that a vaccinia virus recombinant expressing the 11KDa-*Lac Z* fusion protein incorporated an enzymatically active *Lac Z* into virus particles, which could also be detected using anti-*Lac Z* antibodies in indirect immunoflourescence studies and immuno-gold labelling. More recently, in order to test whether HIV-1 and HIV-2 Vpr and Vpx proteins could be used as vehicles to target foreign proteins to HIV virions, the genes encoding these proteins were fused with the bacterial staphylococcal nuclease and CAT genes (Wu *et al.*, 1995). This study demonstrated the capability of HIV-1 Vpr and HIV-2 Vpx to direct the packaging of foreign proteins into HIV virions when expressed as heterologous fusion proteins (Wu *et al.*, 1995).

The characterisation of vVHS-CAT showed that not only was the vhs-CAT fusion protein incorporated into virus particles, but the enzymatic activity of CAT remained assayable in both purified virions and light particles. Similarly, CAT fused to HIV-1 Vpr and HIV-2 Vpx proteins was enzymatically active in recombinant virus particles (Wu *et al.*, 1995). Taken together, the results of Wu *et al.*, (1995) and those presented in this thesis indicate that CAT appears to play a passive role when fused to structural proteins but is sufficiently robust to retain enzymatic activity. Thus its use may be generally applicable to analysing the ability of short peptide sequences to be incorporated into virus particles.

The levels of CAT activity detected in vVHS-CAT infected cell extracts, as well as in purified virus particles, were low in comparison to the CAT activity detected within both vSAU3- and v65-CAT-infected cells. In vSAU3 the gene expressing CAT is under the control of the UL40 gene promoter which results in approximately 2.6-fold more activity, while in v65-CAT the strong HCMV IE gene promoter expresses approximately 6.7-fold

greater levels of CAT activity in comparison to the levels expressed in vVHS-CAT infected cells (*Section 4*). The low levels of expression of the vhs-CAT fusion protein were also evident in the ^{35}S-methionine labelled vVHS-CAT virion and light particle profiles. These did not appear to contain a novel 84KDa band corresponding to the predicted size of the fusion protein. HSV-1 and HSV-2 vhs proteins are minor components of infected cells and virus particles, and it is likely that these low levels of expression reflect the activity of the UL41 gene promoter. In an attempt to avoid this problem in subsequent experiments (*Chapters 8* and *9*), tagged tegument protein constructs were placed under the control of the strong HCMV immediate early promoter.

The detection of ^{35}S-methionine labelled virus polypeptides across gradients illustrated the different patterns of distribution of virion and light particle polypeptides compared with the abundant cellular protein, actin. This showed that cellular material did migrate through gradients. In addition, Western blot analysis across ficoll gradients of material collected from cells infected with v65-CAT (*Chapter 8; Results, Part 2*) illustrated how a non-structural protein migrated through a gradient. The 65-CAT fusion protein detected did not preferentially localise in the region of the gradient where virions and light particles banded, and the pattern was clearly distinct from that of the major virion polypeptide VP16. These results were of general interest since several other proteins have been identified as virion components solely on the basis of Western blot analysis of purified virions. For example, the 21K protein encoded by US11 was classified as a virion component on this basis (Roller & Roizman, 1992). It may also be significant that the purification of virus particles, by Roller & Roizman, was performed on sucrose gradients, and this method does not separate virions from light particles. In view of the potential for misleading results due to the high sensitivity of antibody detection, Western blotting across a ficoll gradient was adopted as a diagnostic test for incorporation of tagged proteins into the tegument of virions and light particles in all subsequent experiments.

The results presented in this section demonstrate that the vhs-CAT fusion protein was incorporated into virus particles, but sequences which were necessary for the incorporation of vhs were not identified. Following the conclusion of the investigation of vhs, Smibert *et al.* (1994) demonstrated that vhs from HSV-1 strain KOS PAAr5 (bearing a wild type *vhs* gene) forms a complex with VP16, and a 21 amino acid domain spanning residues 310-330 of vhs is responsible for the interaction (Schmelter *et al.*, 1996). It has been suggested that this interaction serves to negatively regulate vhs activity but it may also provide a route for translocation of vhs into the viral assembly pathway. There are a number of pieces of evidence supporting these conclusions. Firstly, it was recently demonstrated that infection of non-permissive cells with the VP16 null mutant 8MA (Weinheimer *et al.*,

1992) results in an abrupt decline in viral protein synthesis at early and late times, due in part to a rapid degradation of viral mRNAs (Lam *et al.*, submitted). Viral mRNA levels and protein synthesis are restored in virus expressing a transactivation defective, vhs-binding derivative of VP16 and in an 8MA derivative deleted in vhs, suggesting that rapid mRNA turnover in non permissive cells infected with 8MA is the result of uninhibited vhs activity due to the absence of VP16 (Lam *et al.*, submitted). These findings indicated that VP16 can regulate viral gene expression at thepost translational level by suppressing the activity of vhs at early and late times during infection, thereby sparing the viral mRNAs from destruction (Schmelter *et al.*, 1996; Lam *et al.*, submitted). Circumstantial evidence supporting the proposal that VP16 is involved in the incorporation of vhs into virus particles includes the fact that VP16 is essential for virion assembly (Ace *et al.*, 1988; Weinheimer *et al.*, 1992), while vhs is dispensable (Fenwick & Everett, 1990), such that within vhs negative mutant viruses levels of VP16 are normal. Thus it is reasonable to suggest that VP16 would help to package vhs not vice versa. Most significantly, a mutant virus that synthesises a vhs protein lacking residues 149-344 cannot bind to VP16, and is not packaged into virus particles (Read *et al.*, 1993). The 21 amino acid sequence that interacts with VP16 is conserved in both HSV-1 and HSV-2 vhs proteins (Berthomme *et al.*, 1993; Schmelter *et al.*, 1996). Therefore if HSV-1 vhs is packaged into the tegument via an interaction with VP16 it is reasonable to suggest that the vhs-CAT fusion protein which contains a HSV-1 vhs moiety is also packaged following an interaction with VP16.

The 44KDa band detected by Western blot analysis of purified vVHS-CAT virions (*Section 7*) suggested that breakdown of VHS-CAT was occuring. Breakdown of a vhs-protein A fusion protein was also observed by Smibert *et al.*, (1994) and electrophoretic analysis of this protein revealed that it was cleaved within its vhs moiety, two thirds from the amino terminus, in approximately the same position as the cleavage within vhs-CAT. vhs homologues from other alphaherpesviruses, such as PRV, EHV, and VZV, have regions of sequence identity with HSV-1 vhs throughout their lengths (Berthomme *et al.*, 1993). The comparison of different vhs homologues suggested that vhs is composed of four individual functional domains. Recently, mutational analysis verified this data by demonstrating that mutations which mapped to regions conserved among vhs homologues inactivated function; in contrast four out of five mutations that mapped to regions that are poorly conserved among the vhs homologs had no effect (Jones *et al.*, 1995). It is likely that cleavage of both the vhs-protein A and the vhs-CAT fusion proteins is occuring at the same position, immediately downstream of residues 310-330, which contains the 21 amino acid sequence that interacts with VP16. This region corresponds to a region of low homology and is likely to separate two different functional domains of vhs.

This chapter has demonstrated that a fusion protein between a bacterially derived reporter gene, CAT, and the gene encoding the vhs structural protein was incorporated into the tegument of both HSV-1 virions and light particles. This, together with the fact that the CAT component of the fusion protein retained its activity within virus particles, opens the possibility that such an approach could be used to construct multivalent vaccines. HLA class I-restricted cytotoxic T lymphocytes are considered to be a major mediator for the clearance of HSV infections as demonstrated by their role in limiting recurrent infections in a mouse model system. Tigges *et al.*, (1993) were able to demonstrate that the major target antigens for CTL recognition are internal virion proteins that can be effectively introduced into the cell by infection even in the absence of *de novo* gene expression. This would suggest that light particles engineered to contain foreign proteins could be effective vaccine candidates against a range of different viral infections.

Part 1

The Construction of vUL49ep

The previous chapter had shown that it is possible to modify the vhs protein without disabling its ability to enter the tegument. However, vhs is a minor component of virus particles and direct quantitation of the amount of vhs-CAT protein incorporated was not possible by standard staining methods. Since the vhs studies were hampered due to difficulties with immunological reagents, quantitative analysis of vVHS-CAT mutant proteins would have been difficult to perform. For this reason, studies on the factors that influence incorporation into the tegument were continued with a major tegument protein VP22, which is encoded by UL49. Since it was not known whether VP22 is essential for virus growth, a second copy was placed under the control of the HCMV IE promoter which gives high levels of gene expression in heterologous systems. In order that the properties of the inserted copy could be compared with those of VP22 produced by the endogenous UL49 gene, an epitope tag was linked at the C-terminus of the VP22 coding sequences. The use of the HCMV IE promoter also allowed, for the first, time, studies to be carried out on the effects of protein levels on the amount of protein incorporated into virus particles. Therefore, this section describes the basis of the system used for the mutagenesis studies in the following chapter and also addresses constraints placed on tegument assembly.

Section 1

Construction of Plasmids

To construct a plasmid that would express epitope-tagged VP22, a number of cloning steps were undertaken. The plasmids resulting from these steps are illustrated in Figs. 20a-c. The parent plasmid, pFJ22 is a derivative of pFJ3 (Rixon & McLauchlan, 1990; *Chapter 8; Results, Part 2, Section 1*) which lacks the *LacZ* gene cassette but does contain a 744bp BglII fragment containing the HCMV IE promoter sequence (Stinski & Roehr, 1985) and a 300bp Sau3AI fragment containing the HSV-2 UL38 polyadenylation sequences (McLauchlan *et al.*, 1989). A 40bp oligonucleotide which encodes the epitope tag sequence was inserted into the BamHI site of pFJ22, which resulted in plasmid pFJ22ep (Fig. 20b). Due to the design of the oligonucleotide, only the BamHI site at the 5' end of the oligonucleotide was retained, which enabled the linkage of the epitope tag directly to protein

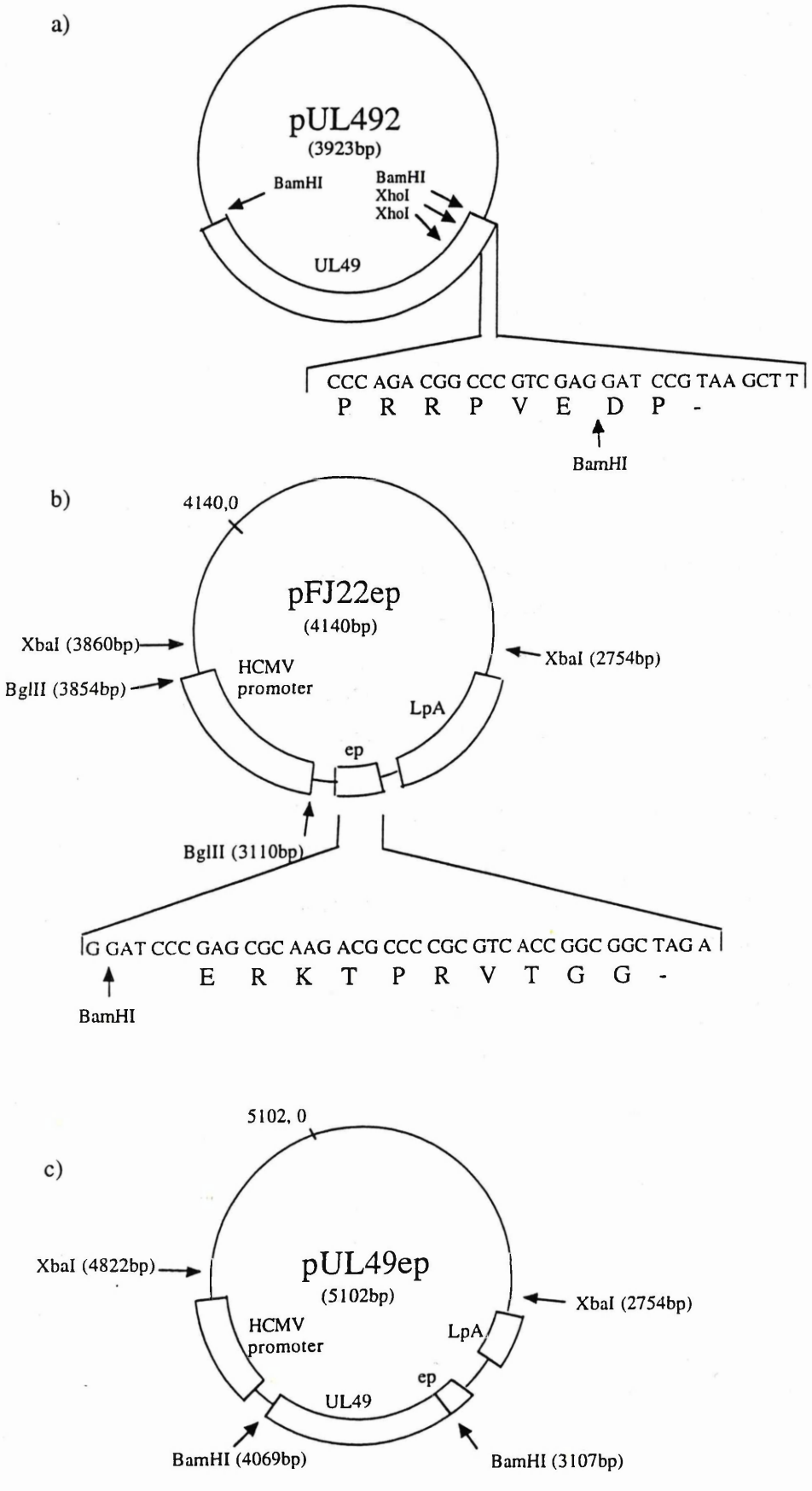

a)
pUL492
(3923bp)
BamHI
BamHI
XhoI
XhoI
UL49
CCC AGA CGG CCC GTC GAG GAT CCG TAA GCT T
P R R P V E D P -
BamHI
b)
4140,0
pFJ22ep
(4140bp)
XbaI (3860bp)
BglII (3854bp)
HCMV
promoter
XbaI (2754bp)
LpA
ep
BglII (3110bp)
G GAT CCC GAG CGC AAG ACG CCC CGC GTC ACC GGC GGC TAG A
E R K T P R V T G G -
BamHI
c)
5102, 0
pUL49ep
(5102bp)
XbaI (4822bp)
HCMV
promoter
XbaI (2754bp)
LpA
ep
UL49
BamHI (4069bp)
BamHI (3107bp)

Figure 20. Details of plasmids used to construct vUL49ep. a) Plasmid pUL492 which contains the reconstructed UL49 ORF in pBluescript KSII DNA. The nucleotide and amino acid sequences at the 3' end of the UL49 ORF are shown along with the position of the BamHI site within the oligonucleotide. b) Plasmid pFJ22ep which comprises a 744bp sequence containing the HCMV IE promoter, a 40bp oligonucleotide that encodes the epitope tag sequence (ep) and a 300bp fragment containing the polyadenylation sequences fron the HSV-2 UL38 gene (LpA). The nucleotide sequence of the oligonucleotide, the amino acid sequence of the epitope tag and the location of the unique BamHI site at the 5' end of the oligonucleotide are shown. c) Plasmid pUL49ep which contains the UL49 ORF from pUL492 inserted into the unique BamHI site of pFJ22ep. Also shown are the positions of the XbaI sites which were used for inserting epitope-tagged UL49 into 1802 viral DNA.

coding sequences (Fig. 20b).

The ORF encoding the UL49 gene product was cloned by firstly inserting a 864bp StuI/XhoI fragment from plasmid pKpnu (nucleotides 105570-106433 in HSV-1 strain 17 DNA, McGeoch *et al.*, 1988) into SmaI/XhoI digested pBluescript II KS DNA. The resultant plasmid, pUL491, contained sequences from 47bp upstream from the 5' end of the UL49 ORF to 80bp upstream from the 3' end. A 98bp oligonucleotide encoding the 3' terminal portion of the UL49 coding region was inserted into the XhoI site in pUL491 to regenerate the 3' end of the UL49 open reading frame, which resulted in plasmid pUL492 (Fig. 20a). To facilitate further manipulation of the UL49 ORF, this oligonucleotide also introduced a second BamHI site one codon before the 3' terminal codon of the UL49 ORF (Fig. 20a). Thus, cleavage of pUL492 with BamHI results in a DNA fragment containing the entire coding sequences for UL49, but lacking the stop codon at the 3' terminus of the ORF.

To construct an epitope-tagged version of VP22 which could be synthesised under the control of the HCMV IE promoter, the 962bp BamHI fragment from plasmid pUL492 was inserted into the unique BamHI site in plasmid pFJ22ep (Fig. 20b), generating plasmid pUL49ep (Fig. 20c).

Section 2

Construction of Virus vUL49ep

To construct a virus which would express the epitope-tagged UL49 ORF under the control of the HCMV IE promoter, the 2.07Kbp XbaI fragment containing the UL49 gene cassette (Fig. 20c) was purified from pUL49ep and ligated with 1802 virus DNA which had been digested with XbaI; this virus is a HSV-1 variant which contains an unique XbaI site in the intergenic region between U_S genes 9 and 10 (Rixon & McLauchlan, 1990). The products of the ligation were transfected into BHK cells and from the resultant progeny, individual plaques were selected. Monoclonal antibody, pp65, which recognises the epitope tag sequence does not cross-react to any significant extent with other HSV polypeptides (DuPont, UK; Weiner *et al.*, 1985; McLauchlan *et al.*, 1994) and thus the VP22 protein made from the inserted copy of UL49 could be readily distinguished from that made by the endogenous UL49 gene. Initially 12 plaques were picked and screened by Western blot analysis. Four of the 12 selected plaques synthesised a protein of about 40KDa which was recognised by monoclonal antibody pp65 (data not shown). One of these plaques was

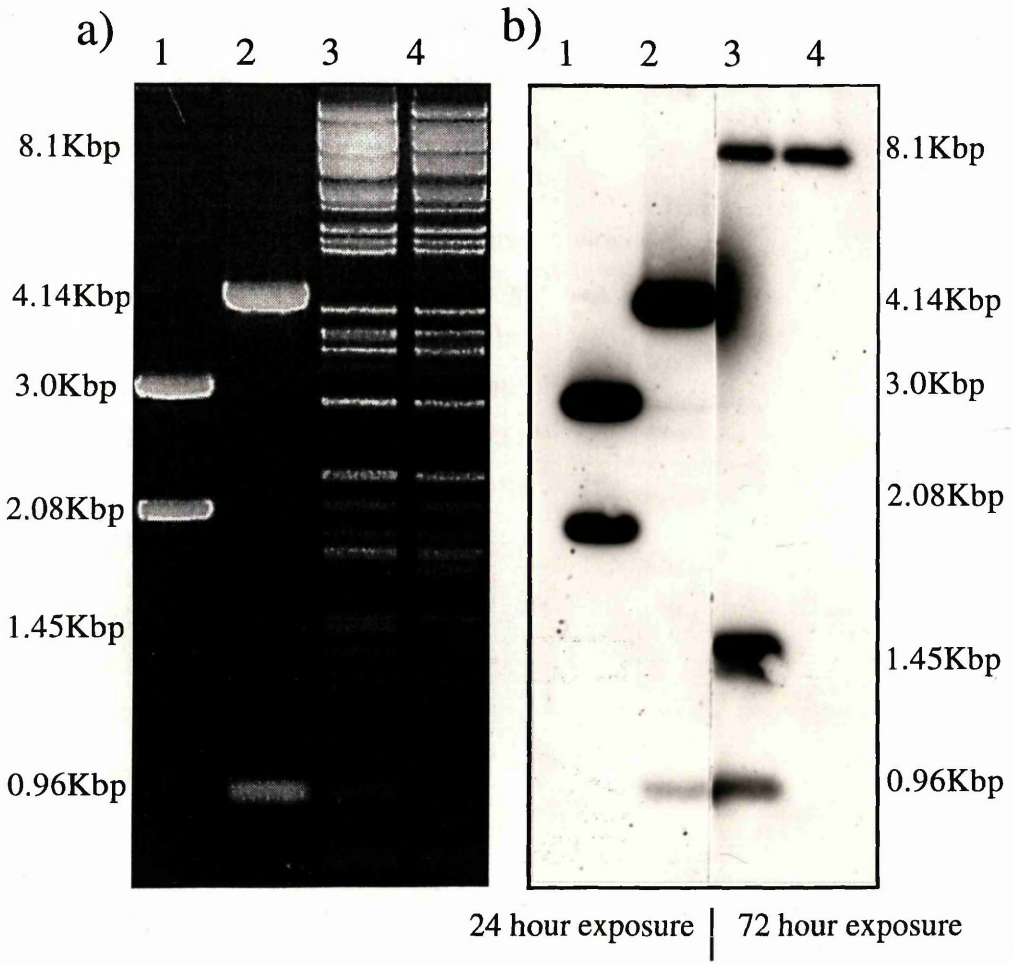
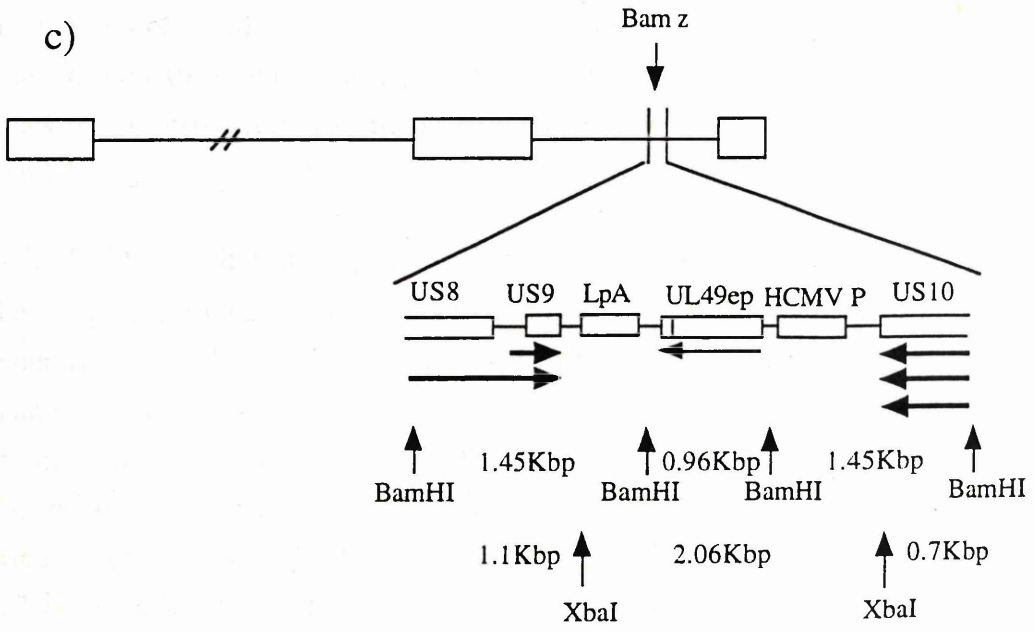


Figure 21. Analysis of vUL49ep viral DNA. a) 0.5µg of each plasmid DNA and 2µg of each viral DNA was digested with the appropriate enzyme and then electrophoresed on a 1% agarose gel. In lane 1, DNA was digested with XbaI while lanes 2 to 4 contain BamHI digestion products. The DNAs in each lane were as follows: lanes 1 and 2, plasmid pUL49ep; lane 3, vUL49 virus DNA; lane 4, 1802 virus DNA. b) Following transfer of the DNA to Hybond-N membrane, it was probed with ^{32}P-radiolabelled pUL49ep plasmid DNA. Two exposures are shown in (b). c) Organisation of the vUL49ep genome at the US9/US10 gene locus. The sizes of the fragments generated by BamHI and XbaI digestion in the region where the epitope-tagged UL49 gene was inserted are shown.


c)
Bam z
US8
US9
LpA
UL49ep
HCMV P
US10
BamHI
1.45Kbp
BamHI
0.96Kbp
BamHI
1.45Kbp
BamHI
1.1Kbp
XbaI
2.06Kbp
0.7Kbp
XbaI

chosen and further purified to homogeneity. The resulting virus was designated vUL49ep.

Section 3

Analysis of vUL49ep DNA

To verify that insertion of a second copy of UL49 did not result in rearrangement of the virus genome, virus DNA prepared from vUL49ep virions was digested with BamHI and the restriction enzyme profile was compared with that of 1802 viral DNA. For size markers, pUL49ep was digested with XbaI and BamHI. BamHI digestion of pUL49ep released the 962bp UL49 ORF, and a 4.14Kbp fragment containing the remainder of the plasmid (Fig. 21a, lane 2), while XbaI digestion of pUL49ep released two fragments of 2.08Kbp and 3.0Kbp (Fig. 21a, lane 1). The DNA profile of BamHI digested vUL49ep indicated the loss of BamHI z, and the appearance of a doublet of approximately 1.45Kbp, as well as an increase in the intensity of the 962bp band. There were no other apparent differences in the restriction enzyme patterns of the two viral DNAs (Fig. 21a, compare lanes 3 and 4).

The DNA was then transferred to Hybond-N membrane and incubated with ^{32}P-radiolabelled pUL49ep which hybridises to all of the sequences within the vUL49ep virus that are derived from pUL49ep as well as the endogenous virus-encoded UL49 sequences. Hybridisation with this probe revealed that the 8.1 Kbp BamHI f fragment which contained the unmodified copy of UL49 was identical in size for both viral DNAs (Fig. 21b, lanes 3 & 4). This probe also detected a DNA fragment of 962bp in vUL49ep DNA which corresponds to the inserted UL49 ORF derived from pUL49ep (Fig. 21b, compare lanes 2 and 3). Two other bands of approximately 1.45Kbp also were unique to vUL49ep DNA. The unique XbaI site in 1802 viral DNA lies within the BamHI z fragment and digestion of this fragment with BamHI and XbaI generates two products of 1.1Kbp and 0.7Kbp. Thus the 1.4Kbp fragments are derived from fragments containing the HCMV promoter and the HSV-2 UL38 late polyadenylation sequences linked to 1.1Kbp and 0.7Kbp portions of the BamHI z fragment. From the sizes of these fragments, it was apparent that the inserted UL49 cassette is transcribed in the same direction as genes U_S10-12 (Fig. 21c). In conclusion, the introduction of a second copy of UL49 into 1802 viral DNA did not generate any gross rearrangements in vUL49ep viral DNA.

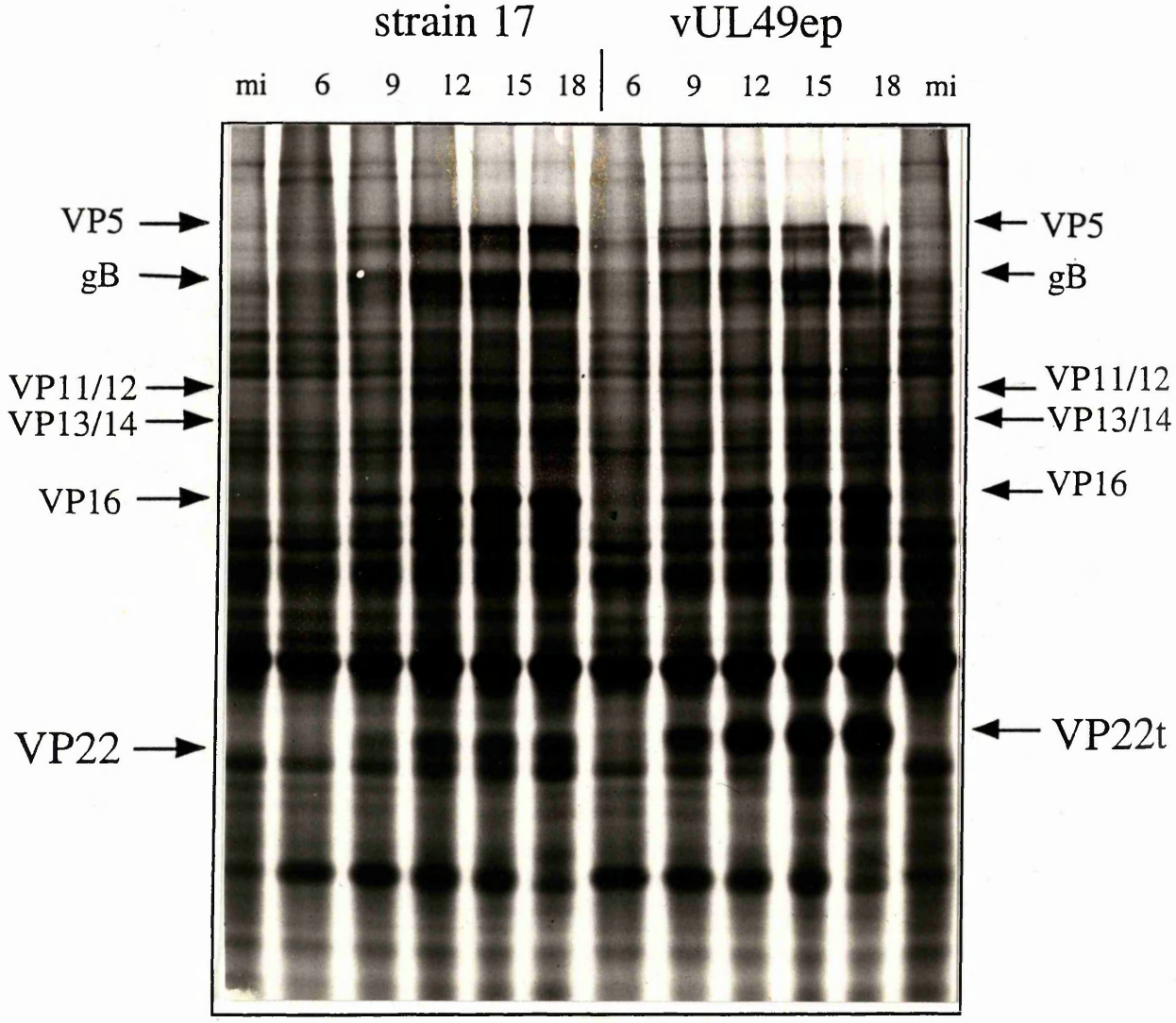


Figure 22. Comparison of the proteins produced by viruses vUL49ep and HSV-1 strain 17 at different stages of infection. Aliquots containing $5x10^5$cpm from radiolabelled extracts prepared from strain 17-, vUL49ep- and mock-infected cells were electrophoresed on a 9% polyacrylamide gel. The times, in hours post infection, at which samples were harvested are indicated. The bands corresponding to VP22 and epitope-tagged VP22 (VP22t) are shown, as are some of the major virus-specific polypeptides.

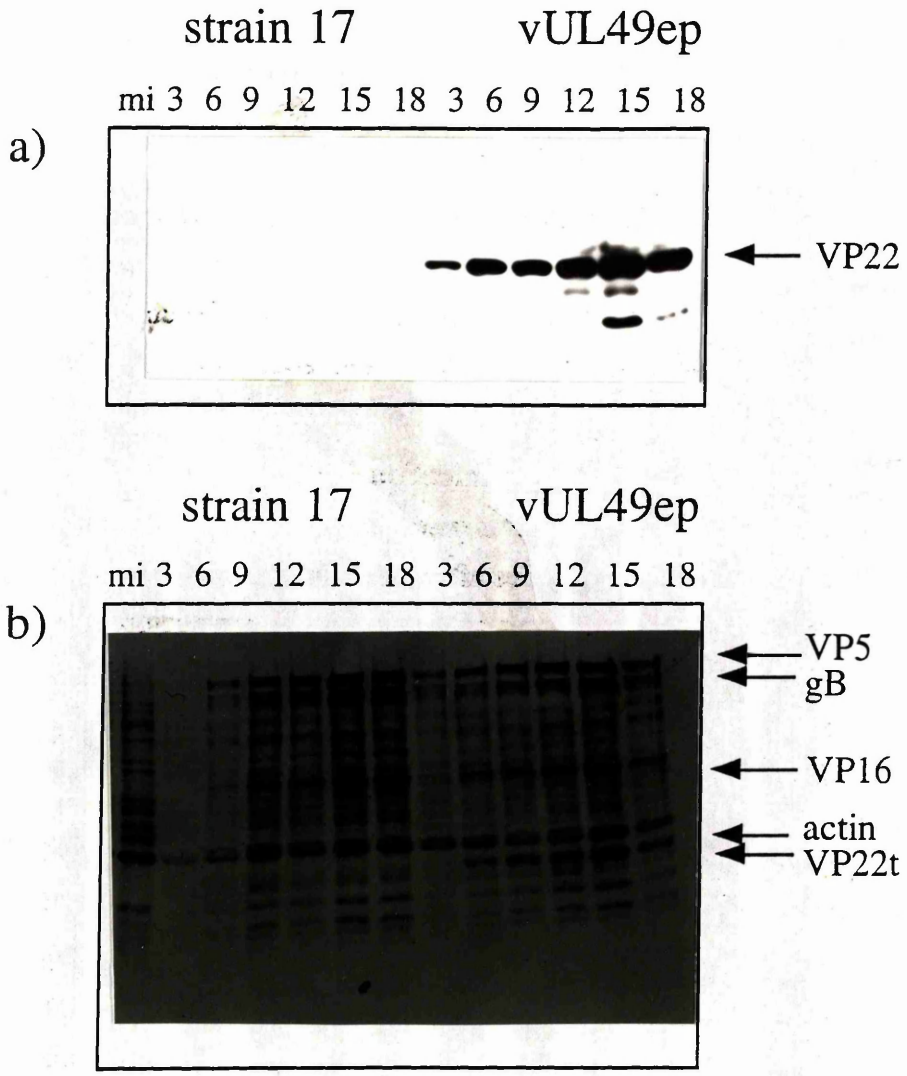


Figure 23. Western blot analysis of vUL49ep- and strain 17-infected cell extracts. Approximately $2x10^4$ cell equivalents of ^{35}S-methionine radiolabelled infected cell extracts were electrophoresed on a 9% polyacrylamide gel and then transferred to nitrocellulose membrane. a) The membrane was probed with monoclonal antibody pp65 (1:2000 dilution). b) The membrane was autoradiographed in order to determine the size of the polypeptide detected in a). The polypeptides are indicated (➔).

Section 4

Synthesis of Epitope-Tagged VP22 in vUL49ep-Infected Cells

The polypeptides made by 1802, the parental virus for vUL49ep, and HSV-1 strain 17 are indistinguishable (Rixon & McLauchlan, 1990) and therefore in the following experiment, HSV-1 strain 17 was used as the control virus. To compare the polypeptides produced by vUL49ep with those made by HSV-1 strain 17, 35mm dishes containing $2x10^6$ BHK cells were infected with either virus at a moi of 5 pfu/cell, and were labelled with ^{35}S-methionine from 3 hours post infection. Cells were harvested at various times after infection (up to 18 hours) and extracts were prepared. Examination of the radiolabelled proteins revealed that at equivalent times after infection the polypeptide profiles of the two viruses were very similar, however virus-encoded proteins did accumulate to slightly lower levels in vUL49ep-infected cells (Fig. 22). The major exception was a polypeptide of slightly lower mobility than that of VP22 produced in vUL49ep-infected cells (Fig. 22, labelled as VP22t).

To show that this protein represented expression of the tagged version of VP22, Western blot analysis was performed on the ^{35}S-methionine radiolabelled vUL49ep-infected cell extracts using monoclonal antibody pp65. Results showed that the antibody detected a protein in the vUL49ep samples which was absent in the HSV-1 strain 17 extracts (Fig. 23a). This protein could be detected by 3 hours after infection and continued to accumulate during the remainder of the infection (Fig. 23a). To verify that this protein corresponded to epitope tagged VP22, the membrane was autoradiographed (Fig. 23b). Alignment of the proteins detected with the pp65 antibody with those detected by autoradiography confirmed that the protein identified in the vUL49ep samples in Fig. 22 comigrated precisely with VP22 protein (compare Figs. 23a and 23b). Thus, it was concluded that vUL49ep produces high levels of VP22 which can be attributed to expression of the tagged version of this protein. The epitope-tagged form of VP22 shall be referred to as VP22t.

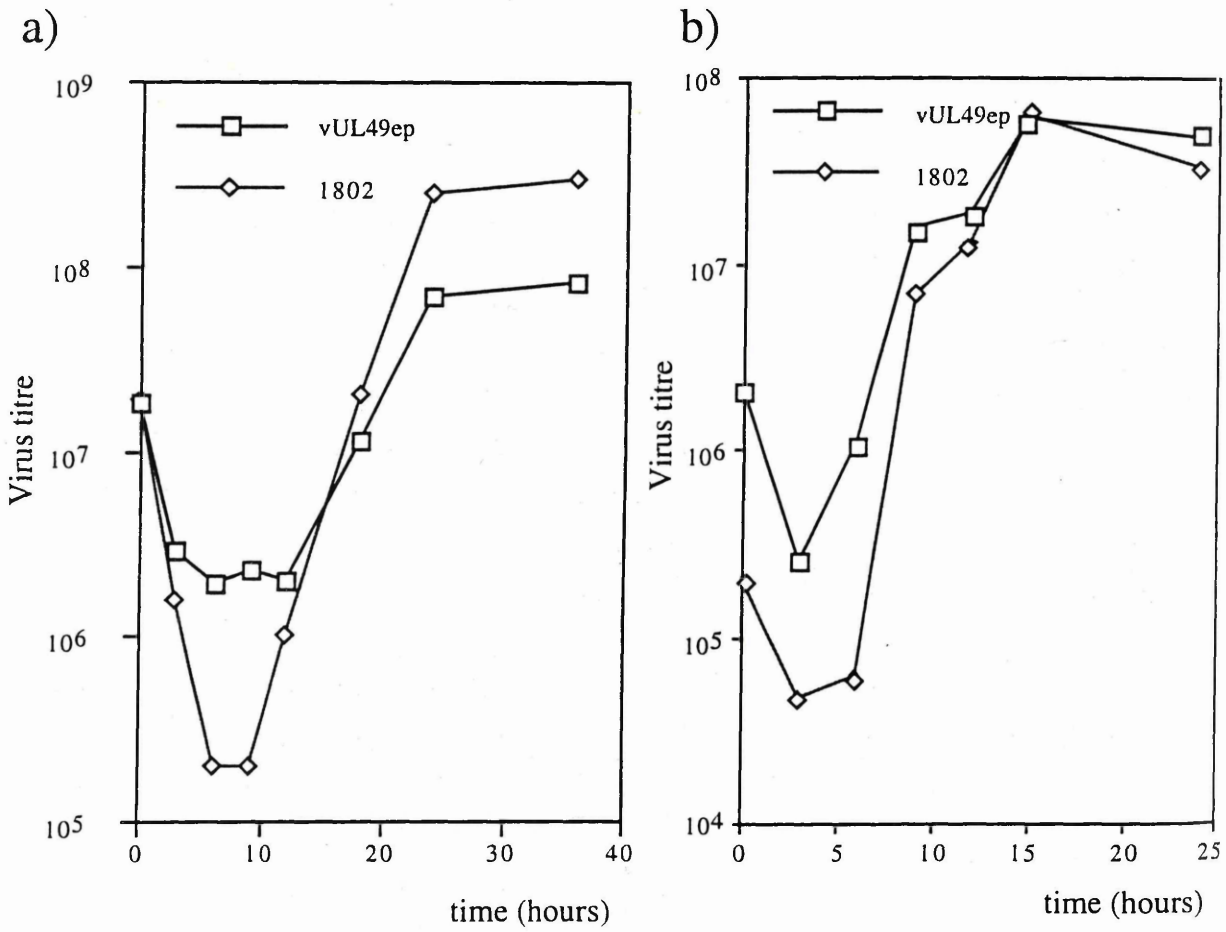


Figure 24. Growth characteristics of vUL49ep compared with the parent virus 1802. On two separate occasions, a) and b), 35mm dishes containing approximately $2x10^6$ BHK cells were infected at a moi of 5 pfu per cell and harvested at various time points ranging between 0 and 36 hours after infection. The total amount of virus present at each time point was determined by titration.

Section 5

Growth characteristics of vUL49ep

In order to determine whether the growth of vUL49ep was affected by the insertion of a second epitope tagged copy of UL49, the growth characteristics of vUL49ep were compared to those of the parent, 1802. The experimental procedure was as described in *Chapter 6; Methods, Section 1.7.* Two sets of data were obtained on separate occasions, and both are presented (Fig. 24a & b).

In order to determine the rate of growth from a growth curve, the gradient of the slope during the exponential phase of growth is calculated, and the steeper the gradient the faster the rate of growth. In addition, the final virus yields are also taken into account. In both Figs. 24a and 24b, the gradients of the slopes during the exponential growth phase (between 12 and 24 hours in Fig. 24a; between 3 and 15 hours in Fig. 24b) for vUL49ep are not as steep as that for 1802, which would suggest a slower rate of growth for vUL49ep as compared to 1802. However, in both of the vUL49ep examples the virus titre during the eclipse phase does not fall as significantly as does 1802. There is however variability between the yields of preparations of vUL49ep as shown in Figs. 24a & 24b and Table 6, and these differences suggest that yields of vUL49ep are either equivalent to or slightly less than 1802. The growth curve data therefore suggests that the insertion of the epitope tagged copy of UL49 into the U_S region of strain 1802 does not have a significant effect on the growth of the virus.

Section 6

Properties of Virus Particles Produced by vUL49ep

To analyse the migration characteristics of virus particles made by vUL49ep, four roller bottles of BHK cells were infected with either vUL49ep or 1802 at a moi of 1 pfu per 300 cells, incubated at 37°C for 3 days, and the cell released material was banded on 5-15% ficoll gradients. Examination of these gradients revealed that the vUL49ep virions and light particles each sedimented further than those of HSV-1 1802 (Fig. 25). Also the band containing the vUL49ep virions was more diffuse than the corresponding 1802 virion band. From electron microscopic analysis of the virus particles purified from these gradients, the

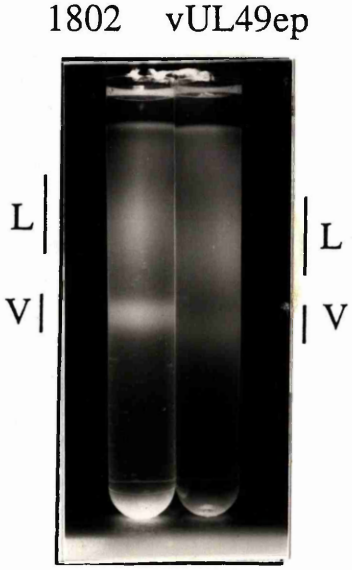


Figure 25. Comparison of virions and light particles produced from cells infected with 1802 or vUL49ep. Particulate material pelleted from cells infected with 1802 or vUL49ep was banded on 5-15% ficoll gradients. Bands corresponding to virions (V) and light particles (L) are indicated.

Virion Sample	Virions	Light particles
1802 (Prepn. 1)	$4.2x10^{11}$ (75%)	$1.4x10^{11}$ (25%)
vUL49ep (Prepn. 1)	$1.1x10^{11}$ (46%)	$1.3x10^{11}$(54%)
1802 (Prepn. 2)	$5.9x10^{11}$ (67%)	$2.9x10^{11}$ (33%)
vUL49ep (Prepn. 2)	$5.2x10^{11}$ (50%)	$5.1x10^{11}$ (50%)

Table 6. Numbers of virions and light particles in vUL49ep and 1802 virion populations. Particle counts were calculated as the number of virus particles per ml. Values in brackets are the percentage of either virions or light particles in each virion preparation.

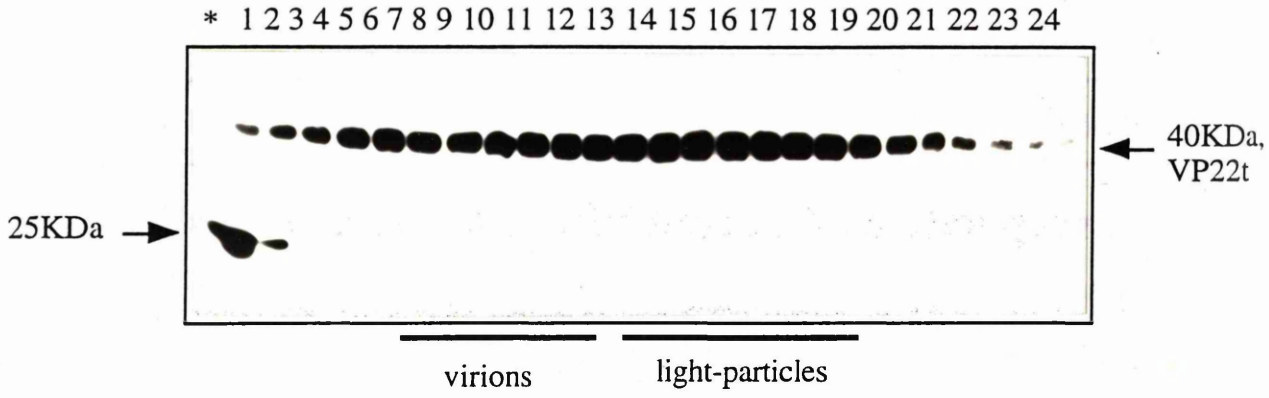


Figure 26. Western blot analysis across a vUL49ep gradient. Material was collected in 500µl aliquots from 2cm below the virion band in a 13ml, 5-15% ficoll gradient that was loaded with material from vUL49ep-infected cells. 15µl of each sample was loaded onto a 12% polyacrylamide gel and following separation the polypeptides were transferred to nitrocellulose membrane. The membrane was incubated with pp65 monoclonal antibody (1:2000 dilution), and the band detected represents epitope-tagged VP22 (VP22t) which is present in the regions where virions and light particles migrate through the gradient. The locations of visible virion and light particle bands are indicated below the blot. * represents v65-CAT-infected cell extract, and was included as a positive control for antibody binding.

population of vUL49ep virions had a high concentration of light particles while the number of light particles in the 1802 virion population corresponded to the level typically present in preparations of wild-type virus (Table 6; Szilagyi & Cunningham, 1991). Other preparations of vUL49ep and 1802 grown in parallel consistently revealed similar differences, and two examples of preparations are shown in Table 6. These results suggest that vUL49ep virus particles have a greater mass than those of 1802 and the increased abundance of light particles in vUL49ep virion preparations may suggest that there is a greater size heterogeneity in these particles as compared to 1802 light particles.

Section 7

Incorporation of Tagged VP22 into Virus Particles

In order to establish whether VP22t was incorporated into vUL49ep virions and light particles the distribution of VP22t through a gradient of vUL49ep prepared from four roller bottles of BHK cells was analysed by Western blot. Incubation of the blot with pp65 monoclonal antibody revealed that VP22t was distributed throughout the region of the gradient containing virions and light particles (Fig. 26). This distribution was consistent with that described for tegument proteins such as VP16 (*Chapter 8; Results, Part 2, Section 5*). In contrast, non-structural proteins, such as CAT (*Chapter 8; Results, Part 2, Section 5*), are detected only in the fractions towards the top of the gradient containing soluble material, if at all. This result suggests that VP22t is incorporated as a structural component of virions and light particles.

Section 8

Polypeptide Content of vUL49ep Virus Particles

To further characterise vUL49ep virions and light particles their polypeptide profiles were compared with those of the parental virus 1802 (Fig. 27). The most striking difference in the polypeptide content was a novel abundant protein in vUL49ep virions and light particles which had a slightly slower mobility than the VP22 present in 1802 virus particles (Fig. 27). This polypeptide was confirmed to be VP22t by Western blot analysis (Fig. 29a).

Examination of the gel shown in Fig. 27 revealed that vUL49ep virions and light

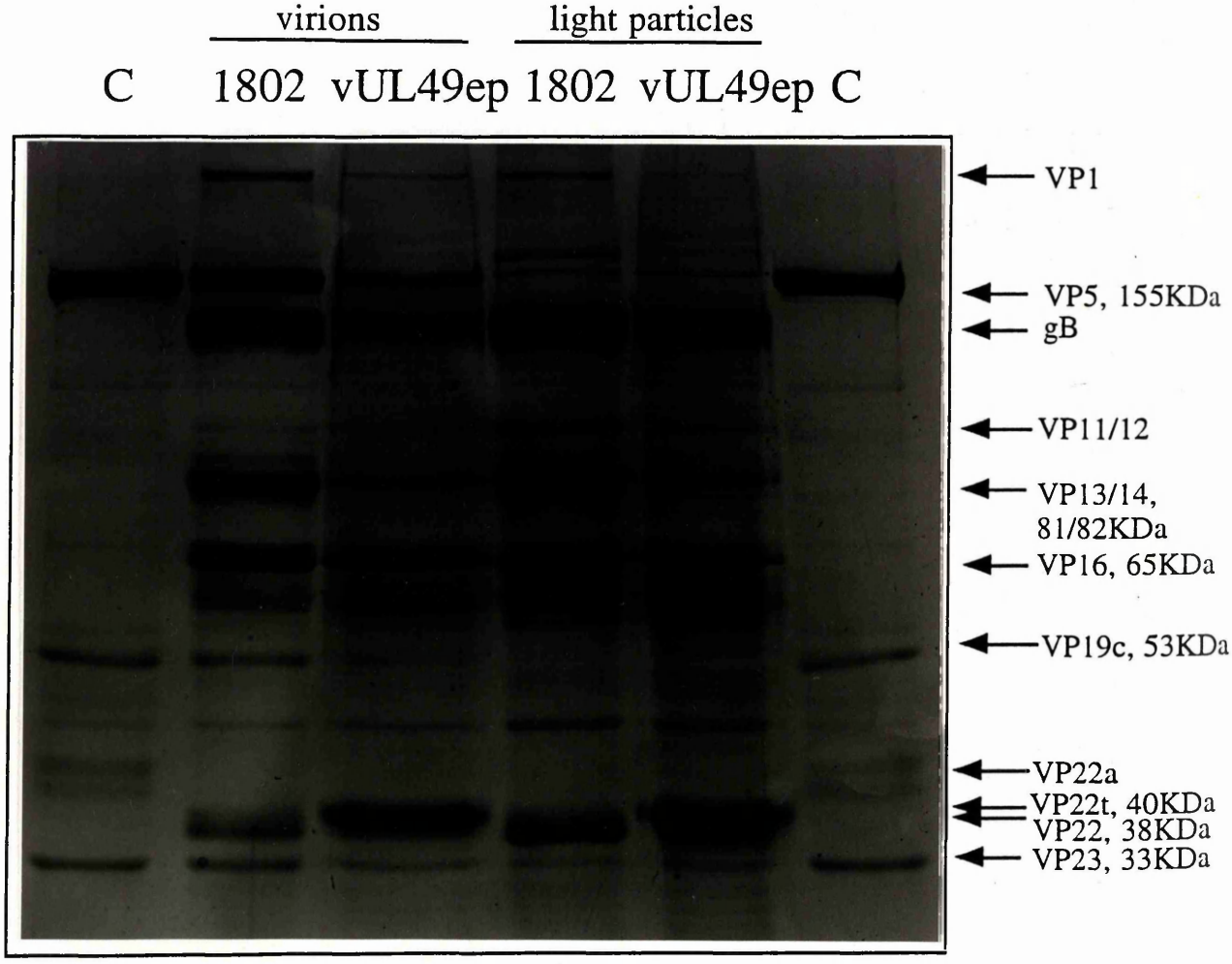


Figure 27. Comparison of polypeptide profiles of gradient-purified particles produced by 1802 and vUL49ep. Approximately $3x10^9$ virus particles were analysed on a 9% polyacrylamide gel. Following electrophoresis, the gel was stained with Coomassie Brilliant blue. Samples in each lane are as shown. C represents purified HSV-1 (B) capsid proteins, which were included as size markers. The major structural polypeptides are indicated, as is the band corresponding to epitope-tagged VP22 (VP22t).

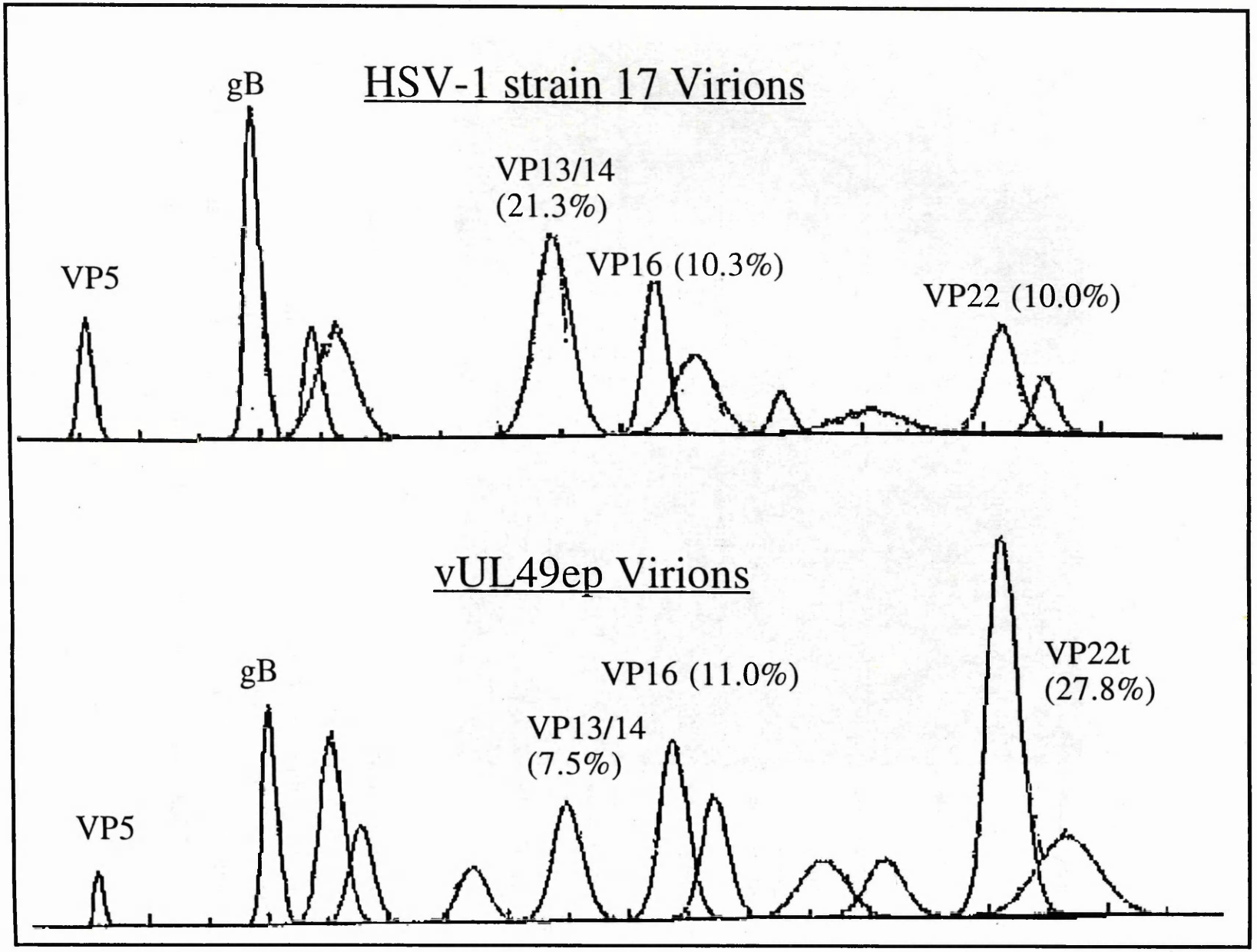

HSV-1 strain 17 Virions
gB
VP13/14
(21.3%)
VP16 (10.3%)
VP5
VP22 (10.0%)
vUL49ep Virions
gB
VP16 (11.0%)
VP22t
(27.8%)
VP13/14
(7.5%)
VP5

Figure 28. Densitometric analysis of vUL49ep virions and HSV-1 strain 17 virions. The major polypeptides are indicated, as is the area corresponding to epitope tagged VP22 (VP22t). The amount of each of the major polypeptides is expressed as a % of the total protein content within the polyeptide profile.

		Polypeptides analysed				
		VP5[d]	gB, gC, gH[d]	VP13/14[d]	VP16[c]	VP22[d]
predicted copy numbers (average)[c]		960	1750	1846	1647	ND
1802[a]	V	895	1307	1691	1647	2872
	L		1828	1730	1647	4349
vUL49ep[a]	V	477	1177	992	1647	7745
	L		1462	1076	1647	8668
1802[b]	V	1100	1288	1753	1647	2531
	L		1824	1466	1647	3570
vUL49ep[b]	V	582	1191	723	1647	6365
	L		1668	933	1647	7090

Table 7. The copy numbers of VP5, gB(gC, gH), VP13/14 and VP22 were estimated following the excision of these bands from Coomassie Brilliant blue stained gels. The protein concentration was estimated following the elution of the Coomassie Brilliant blue stain in each band in 25% piperidine solution followed by spectrophotometric analysis at 605nm. The copy number for each polypeptide was calculated assuming that the copy number of VP16 remains constant in each virus.

[a] vUL49ep and 1802 virus particles corresponding to preparation 1 in Table 6.

[b] vUL49ep and 1802 virus particles corresponding to preparation 2 in Table 6.

[c] average number of copies of VP16 present in virions was taken from Zhang & McKnight (1993).

[d] number of copies of major structural species was calculated relative to the estimated copy number of VP16.

ND = not determined

particles contain both species of VP22 expressed by the recombinant, however, the epitope-tagged version is considerably more abundant than the unmodified copy of VP22. Indeed, epitope-tagged VP22 is the most abundant polypeptide in vUL49ep virus particles. Two methods were used to quantitate the amount of VP22 present in vUL49ep virions and light particles as compared to strain 17 and/or 1802 virions and light particles. Initially, the Coomassie Brilliant blue stained polypeptide profiles of vUL49ep and strain 17 virions were scanned using a densitometer. The use of strain 17 rather than strain 1802 for comparative purposes in these analyses did not significantly affect the results since both of these viruses have indistinguishable polypeptide profiles. From densitometric analysis it was determined that there was approximately a 3-fold increase in the total amount of VP22 in vUL49ep virus particles (Fig. 28).

An alternative method of quantitation further supported the densitometric data. Following sepparation of the polypeptides on an acrylamide gel, and staining with Coomassie Brilliant blue, the amount of stain in each of the polypeptides was used to estimate the copy number of each polypeptide. Following excision of each band from the Coomassie stained gel, the stain was eluted in a 25% piperidine solution and spectrophotometrically analysed. The following bands from a gel containing two different preparations of both vUL49 and 1802 were excised: VP5, gB, VP13/14, VP16 and VP22. Due to the densitometric analysis it was clear that the abundances of two major virion bands (the 120KDa band containing gB, gC, gH and the protein encoded by UL37, and the 65KDa band containing VP16) did not significantly differ, and it was therefore assumed that the copy number for VP16 in each virus strain remained constant. With this in mind the copy numbers for each of the polypeptides excised were calculated. Both sets of data indicated that there were reductions of 40-50% in the quantities of the major capsid protein, VP5, and two related tegument proteins, VP13/14. The apparent decrease in abundance of VP5 in vUL49ep virions is likely to be a result of the high levels of light particles present in vUL49ep virion preparations (Table 6). As a result the capsid proteins will appear to be under represented in the polypeptide profiles of vUL49 virions. In the case of VP13/14, these proteins are present in similar amounts in both HSV-1 strain 17 virions and light particles. Thus, the higher quantities of light particles in vUL49ep virion preparations does not account for the reduced abundance of these proteins in vUL49ep virions and light particles. From the data presented in Table 7, there was also a 2-2.5 fold increase in the abundance of VP22 in vUL49ep virus particles.

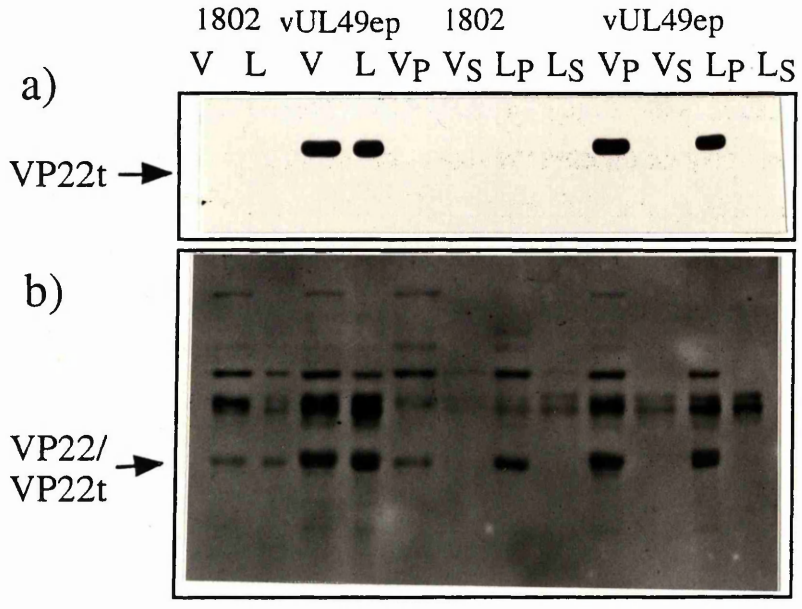


Figure 29. Proteins in 1802 and vUL49ep virions and light particles solubilised by detergent treatment. The material from approximately $3x10^9$ particles was separated on a 9% polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was first probed with pp65 monoclonal antibody (at a dilution of 1:2000), (a) then stripped and reprobed with CY8 monoclonal antibody (at a dilution of 1:2000) (b). Samples in each lane have been indicated as follows: V: virion, L: light particle, V_P: pelleted material from NP40-treated virions, V_S: supernatant material from NP40-treated virions, L_P: pelleted material from NP40-treated light particles, L_S: supernatant material from NP40-treated light particles. VP22 and epitope tagged VP22 (VP22t) are indicated (→).

Section 9.

Solubility of VP22t in vUL49ep Virus Particles

Previous studies have shown that VP22 is not removed from the tegument following solubilisation by detergent (McLauchlan & Rixon, 1992) . To determine whether epitope-tagged VP22 exhibited similar behaviour approximately $3x10^9$ virions and light particles made by 1802 and vUL49ep were treated with NP40. The resultant insoluble material which consists almost entirely of tegument/capsid proteins (from virions) and tegument proteins (from light particles) can be separated from the envelope components by centrifugation (McLauchlan & Rixon, 1992). Western blot analysis of the soluble and insoluble fractions with pp65 monoclonal antibody revealed that epitope-tagged VP22 remained in the insoluble material and could not be detected in the envelope fraction (Fig. 29a, compare V_P & L_P with V_S & L_S). Using CY8 antibody, which recognises VP22, to probe the same membrane, an identical result was obtained (Fig. 29b). Thus, although vUL49ep virus particles contain increased amounts of VP22, the protein remains tightly associated with the tegument.

Part 2

The construction of virus control v65-CAT

In parallel with the construction of vUL49ep, a control virus, v65-CAT, was also made which expressed an epitope-tagged version of the CAT protein. Since results presented in *Chapter 7; Results, Section 6* had shown that unmodified CAT protein did not incorporate into virus particles, the rationale behind the construction of v65-CAT was to ensure that the 10 amino acid epitope tag sequence, derived from the major tegument protein pp65 of HCMV, was not responsible for the incorporation of proteins into the HSV-1 tegument.

Section 1.

Plasmid Construction

To enable the expression of an epitope-tagged form of the CAT protein, a series of cloning steps were undertaken. The resulting plasmids are shown in Fig. 30a-f. The parent plasmid pFJ3 (Rixon & McLauchlan, 1990) has a multiple cloning site at position 3736. This contains XbaI, BglII, HindIII and BamHI restriction enzyme sites. A second XbaI site is present at position 6782 (Fig. 30a).

The cloning steps were as follows:

i) The polyadenylation sequences from the HSV-2 UL38 gene isolated from pSAU30 were cloned into pFJ3. Before insertion of these sequences, an XbaI site in pSAU30, was removed by digesting the plasmid with XbaI, filling in the overhanging ends with Klenow polymerase and re-ligating the blunt ends. This gave rise to plasmid pSAU30(w/o XbaI). Following digestion of pSAU30(w/o XbaI) with HindIII and BamHI a 500bp fragment was gel purified and cloned into HindIII/BamHI digested pFJ3. The resulting plasmid pFJ3/SAU30 is illustrated in Fig. 30b.

ii) An immediate early HCMV promoter sequence from pMJ37 (see *Chapter 5; Materials Section 3*) was then cloned into pFJ3/SAU30. pMJ37 was digested with Sau3AI which releases a 744bp fragment containing the promoter sequence. This was cloned into BglII digested pFJ3/SAU30 regenerating BglII sites flanking the promoter sequence. The

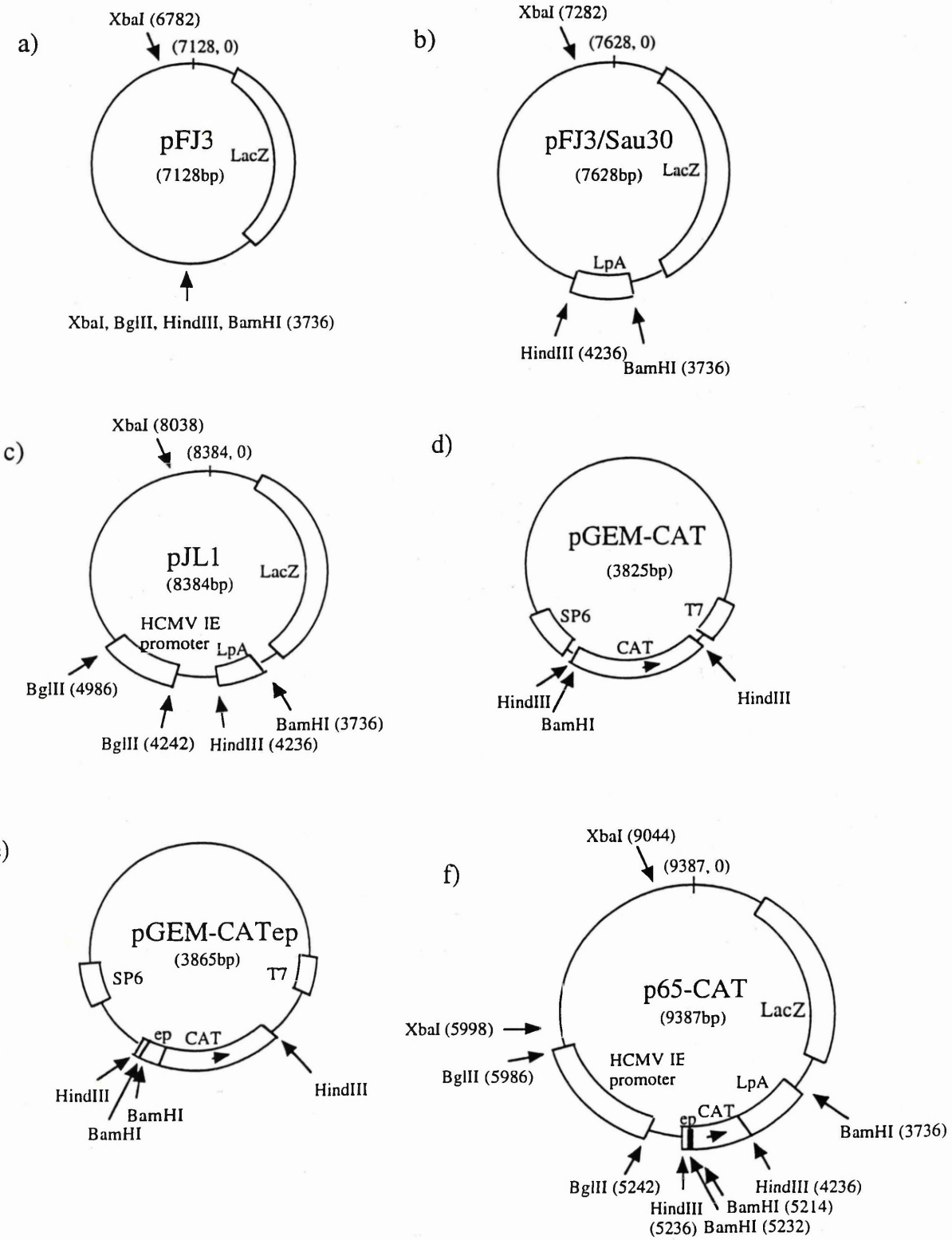

a)
XbaI (6782)
(7128, 0)
pFJ3
(7128bp)
LacZ
XbaI, BglII, HindIII, BamHI (3736)
b)
XbaI (7282)
(7628, 0)
pFJ3/Sau30
(7628bp)
LacZ
LpA
HindIII (4236)
BamHI (3736)
c)
XbaI (8038)
(8384, 0)
pJL1
(8384bp)
LacZ
HCMV IE
promoter
LpA
BglII (4986)
BglII (4242)
HindIII (4236)
BamHI (3736)
d)
pGEM-CAT
(3825bp)
SP6
T7
CAT
HindIII
BamHI
HindIII
e)
pGEM-CATep
(3865bp)
SP6
T7
ep
CAT
HindIII
BamHI
BamHI
HindIII
f)
XbaI (9044)
(9387, 0)
p65-CAT
(9387bp)
LacZ
XbaI (5998)
BglII (5986)
HCMV IE
promoter
LpA
ep
CAT
BamHI (3736)
BglII (5242)
HindIII
(5236)
BamHI (5214)
BamHI (5232)
HindIII (4236)

Figure 30. Details of plasmid vectors used to construct v65-CAT. The parent plasmid pFJ3 has a polyclonal linker sequence inserted into the BamHI site at position 3736, and the PvuII site at position 6782 was converted to an XbaI site (a). The XbaI site in the UL38 polyadenylation site from pSau30 was removed and it was then cloned into pFJ3 as a 500bp HindIII/BamHI fragment, resulting in plasmid pFJ3/Sau30 (b). A 744bp Sau3AI fragment containing the HCMV IE promoter sequence from pMJ37 was sub-cloned into the BglII site of plasmid pFJ3/Sau30, resulting in plasmid pJL1 (c). The CAT open reading frame in plasmid pCAT1 (*Chapter 7; Results,* Fig. 7c) was sub-cloned as a HindIII fragment into pGEM1 resulting in plasmid pGEM-CAT (d). Into the BamHI site close to the 5'-terminus of the CAT gene, the HCMV UL83 epitope tag sequence was inserted which resulted in plasmid pGEM-CATep (e). The epitope-tagged CAT ORF from pGEM-CATep was cloned into the unique HindIII site in plasmid pJL1 resulting in plasmid p65-CAT (f). The positons of the XbaI sites that were utilised to insert the epitope tagged CAT sequence into the HSV-1 strain 1802 genome are indicated in plasmid p65-CAT.

resulting plasmid was called pJL1 (see Fig. 30c).

iii) The CAT open reading frame was obtained from pCAT1 and was initially subcloned as a 960bp HindIII fragment into pGEM1 (see *Chapter 5; Materials, Section 3*) resulting in plasmid pGEM-CAT (Fig. 30d). In order to construct an epitope-tagged CAT fusion protein, the unique BamHI site upstream of the N terminus of CAT was utilised. A 40bp oligonucleotide which includes sequences encoding the 10 amino acid epitope used to tag the VP22 protein that is recognised by a monoclonal antibody, pp65 (DuPont, UK; Weiner *et al.*, 1985; McLauchlan *et al.*, 1994) was cloned into this unique BamHI site. Due to the design of the oligonucleotide, insertion of the linker regenerated a BamHI site at the 5' end of the oligonucleotide. The oligonucleotide linker contained sequences for a further BamHI site and therefore insertion of the linker created two BamHI sites, which flanked the initiation codon for the epitope-tagged CAT gene (Fig. 31). Insertion of the epitope tag sequence into pGEM-CAT resulted in plasmid pGEM-CATep (Fig. 30e). Commercially available primers that annealed to sequences within pGEM1 were used to sequence the 5' portion of the CAT fragment containing the oligonucleotide insert. The sequence data obtained confirmed that the HCMV UL83 epitope tag sequence lay in the correct orientation at the 5'-terminus of CAT (Fig. 31). Also, the initiation codon within the oligonucleotide was shown to be in-frame with the CAT sequences.

iv) Finally the CAT coding sequences containing the epitope tag sequence from pGEM-CATep were cloned as a 1000bp HindIII fragment into the unique HindIII site in plasmid pJL1 (Fig. 30c) resulting in plasmid p65-CAT. (Fig. 30f).

Section 2.

Construction of Virus Recombinant v65-CAT

To insert the epitope-tagged CAT gene into HSV-1 DNA, p65-CAT was digested with XbaI, and the products of the digestion which contained the 6.3Kbp fragment containing the CAT and *LacZ* gene cassettes were ligated into the unique XbaI site engineered into the U_S region of HSV-1 1802. The products of the ligation were transfected into sub-confluent BHK cells. Once full cpe was visible the medium was harvested and extracts were prepared from the infected cells. These were then screened by Western blot and CAT enzyme assay which showed that virus synthesising the epitope-tagged CAT

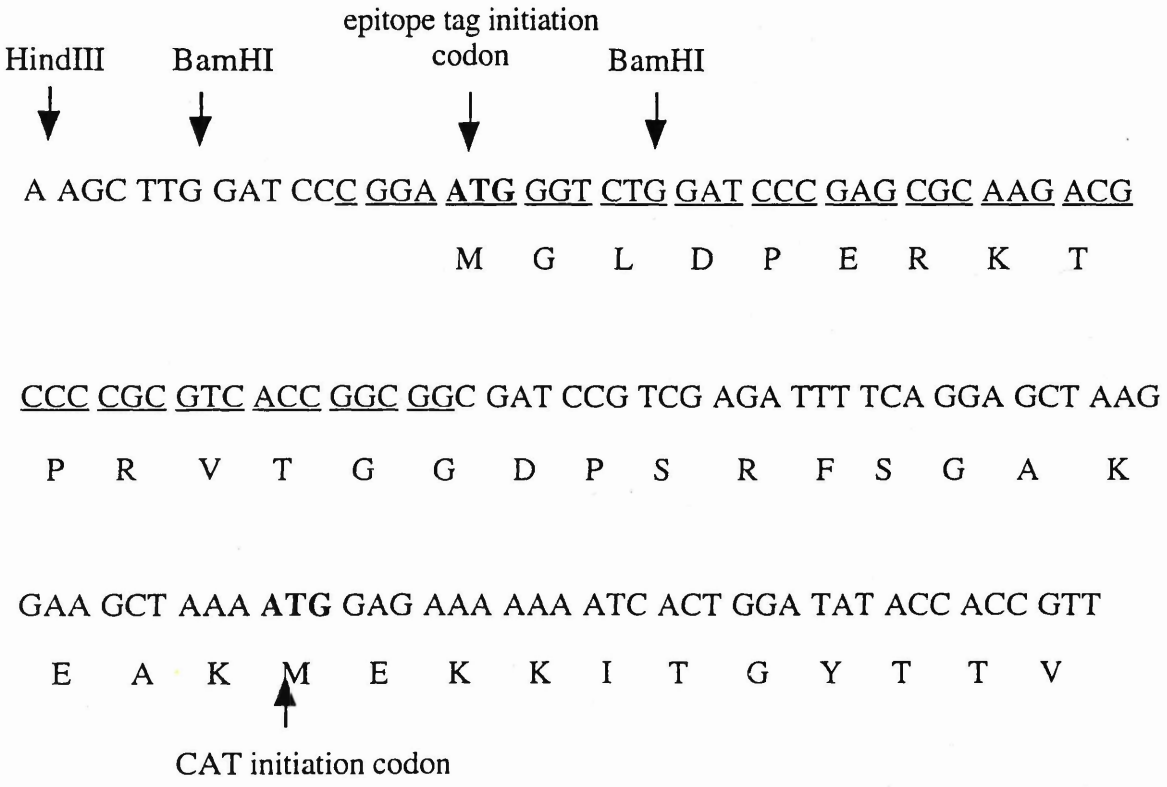


Figure 31. Sequence across the HCMV UL83 epitope tag sequence. The oligonucleotide sequence encoding the HCMV UL83 epitope tag sequence is underlined. The initiation codons for epitope-tagged CAT and native CAT proteins, and the HindIII and BamHI restriction enzyme sites are indicated ( ).

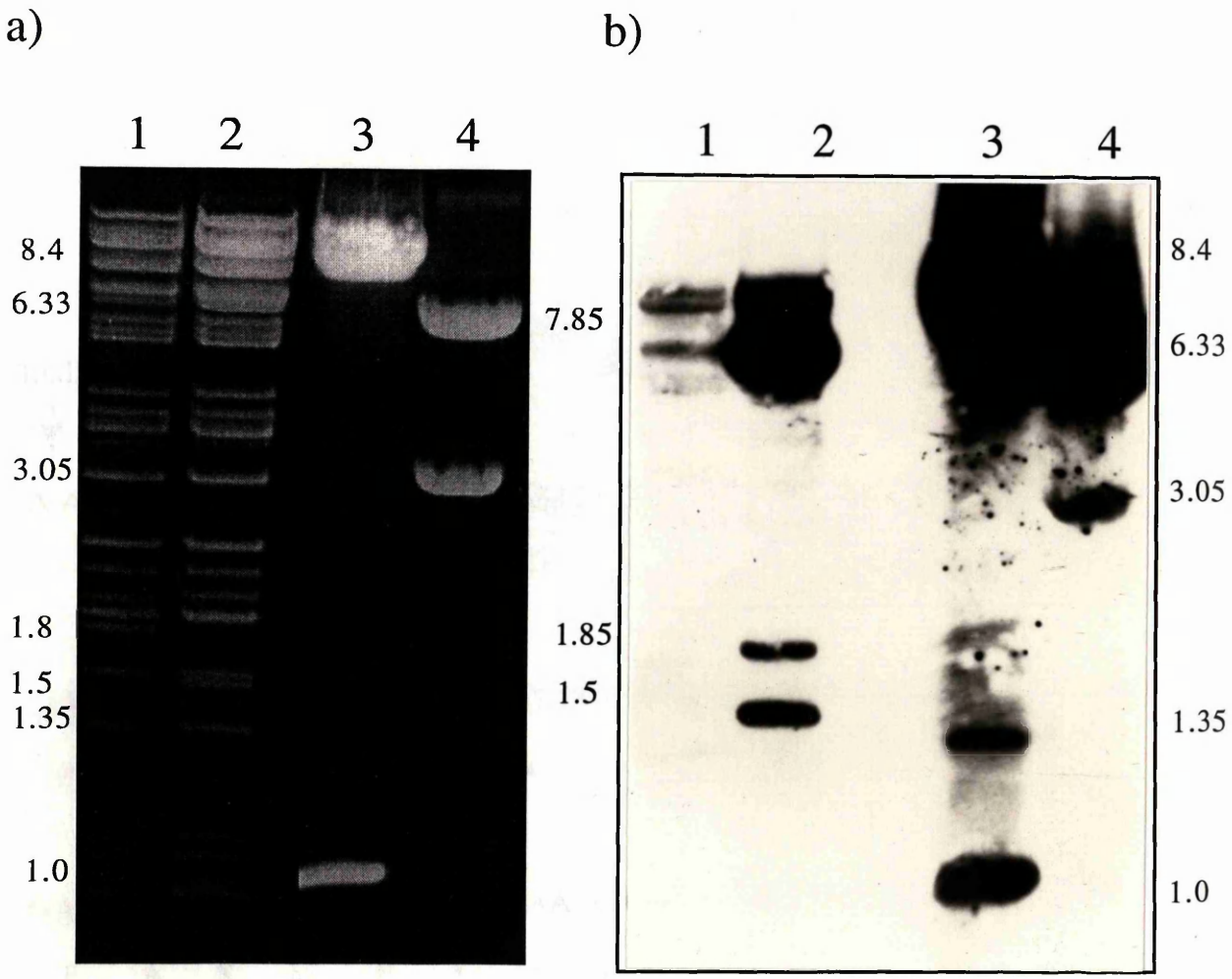


Figure 32. Analysis of v65-CAT DNA. a) Following digestion of 2µg of HSV-1 strain 1802 (lane 1) and v65-CAT (lane 2) viral DNA with BamHI, the resulting fragments were electrophoresed on a 1% agarose gel. As size markers, 0.5µg of plasmid p65-CAT was digested with HindIII (lane 3) and XbaI (lane 4). The sizes of the fragments released are indicated. b) Southern Blot analysis of v65-CAT viral DNA. Following electrophoresis, the DNA samples on the gel were transferred to Hybond-N membrane and hybridised with ^{32}P-radiolabelled p65-CAT DNA. The DNAs in each lane were as in (a). c) Diagramatic representation of the fragments released following BamHI and XbaI digestion of p65-CAT. d) Diagram illustrating the v65-CAT viral genome. The orientation of the inserted epitope CAT cassette, the positions of the BamHI sites and the sizes of fragments released following BamHI digestion are shown.

protein was present (data not shown).

After having established that epitope-tagged CAT protein was synthesised in infected cells, the virus was plaque purified. 12 individual plaques were picked and cells infected with each of the 12 plaques were analysed for CAT activity. Extracts prepared from cells infected with three of the 12 plaques produced CAT activity. One of the three isolates was chosen and used to infect cells, and the process of screening for CAT activity was repeated on a further 12 isolates. After three rounds of such plaque purification one of the isolates was chosen and its DNA structure was analysed. This isolate was termed v65-CAT.

Section 3.

Analysis of v65-CAT DNA.

To verify that the epitope-tagged CAT sequences had inserted at the predicted site within the HSV-1 genome, the BamHI digestion pattern of v65-CAT was compared to that of the parent virus HSV-1 strain 1802. For size markers, p65-CAT was digested with HindIII and XbaI. HindIII digestion of p65-CAT releases a 1.0Kbp fragment containing the CAT sequences, and a 8.4Kbp fragment containing the remainder of the plasmid (Fig. 32a, lane 3). Preparations of HindIII have been shown to exhibit star activity (Nasri & Thomas, 1986), and it is this activity that is thought to have been responsible for the appearance of another minor band of approximately 1.35Kbp in the digested sample (Fig. 32a, lane 3). XbaI digestion of p65-CAT released two fragments of 3.05Kbp and 6.33Kbp (Fig. 32a, lane 4). Examination of the profiles of the viral DNAs revealed that the 1.8Kbp BamHI z fragment is absent in the v65-CAT DNA sample (Fig. 32a, lanes 1 and 2); this viral DNA fragment contains the XbaI site into which the XbaI fragment containing the CAT and *LacZ* gene cassettes were inserted. In addition to the loss of BamHI z, a novel fragment of 1.5Kbp was present in the v65-CAT DNA sample but no other significant differences in the profiles of the two viral DNAs were apparent.

The DNA was then transferred to Hybond-N membrane and incubated with radiolabelled p65-CAT as a probe which hybridises to all of the sequences within the v65-CAT virus that are derived from the plasmid p65-CAT. Hybridisation with this probe detected three bands that are absent in HSV-1 strain 1802. The unique XbaI site in 1802 viral DNA lies within the BamHI z fragment and digestion of this fragment with BamHI and XbaI generates two fragments of 1.1Kbp and 0.7Kbp. Into this XbaI site the 6.33Kbp

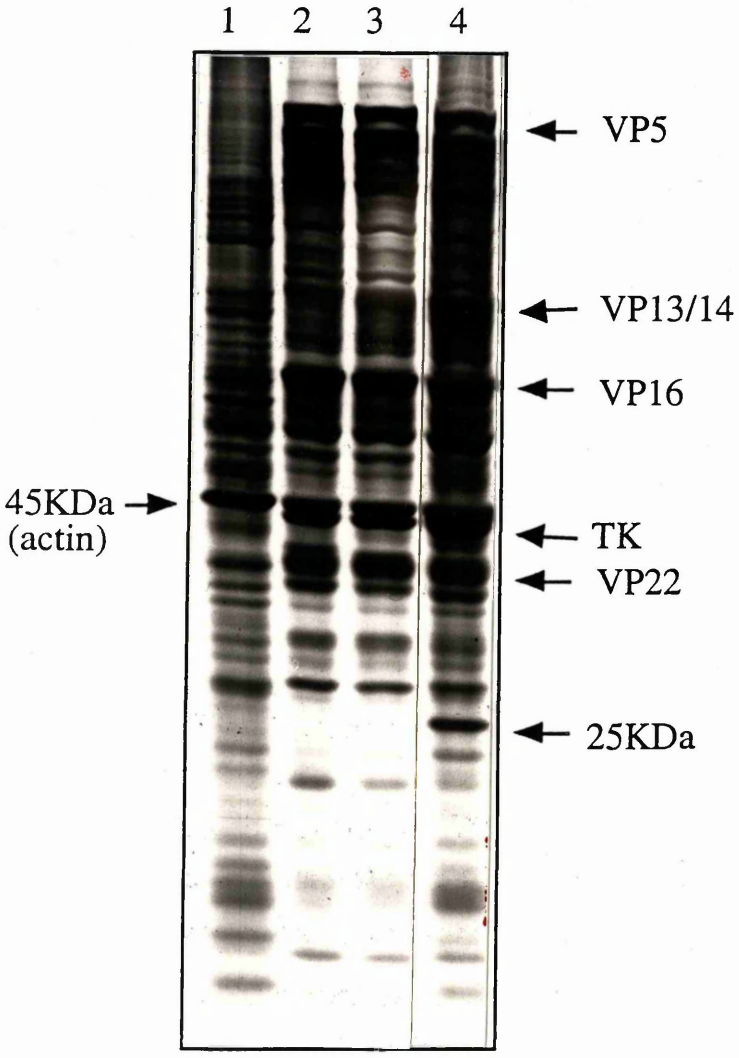


Figure 33. Polypeptide profile of cells infected with v65-CAT. Aliquots containing $5x10^5$cpm from radiolabelled extracts prepared from HSV-1 strain 17 (lane 2), 1802 (lane 3), v65-CAT (lane 4) and mock infected cells (lane 1) were electrophoresed on a 12% polyacrylamide gel. The 25KDa band in lane 4, corresponding to CAT is indicated (◄—) as are some of the other major infected cell polypeptides, and the cellular polypeptide, actin.

fragment consisting of the 65-CAT expression cassette and the *LacZ* ORF from p65-CAT was inserted, however because the XbaI digested p65-CAT DNA was not gel purified before being inserted into the 1802 virus genome, the p65-CAT backbone sequences (base pairs 5998-9044) were also inserted alongside the 6.33Kbp 65-CAT expression cassette (Fig. 32c, fragments 1+2+3). Within this 6.33Kbp fragment there are three BamHI sites, two in very close proximity, within the epitope tag sequence at the N-terminus of CAT and the other following the LpA site (Fig. 32c, fragment 2). BamHI digestion of v65-CAT DNA therefore resulted in a fragment of 1.5Kbp which contained the CAT sequences and the LpA site (Fig. 32c, fragment 2). The IE promoter sequence of 0.76Kbp linked to the 1.1Kbp BamHI z fragment accounts for the 1.85Kbp fragment detected (Fig. 32b, lane 2). Finally the 7.85Kbp fragment detected was accounted for by the 4.1Kbp *LacZ* fragment and the 3.05Kbp p65-CAT plasmid sequences (Fig. 32c, fragment 3) linked to the 0.7Kbp BamHI z fragment (Fig. 32b, lane 2). From the sizes of the fragments detected, the inserted 65-CAT cassette is transcribed in the same direction as genes U_S8 and U_S9. The orientation of the CAT insert is shown in Fig. 32d.

Section 4.

i) Polypeptides synthesised by v65-CAT.

The polypeptides synthesised by v65-CAT were compared with those made by the parent virus 1802 and wild type virus. BHK cells were infected with each virus at a moi of 5 pfu/cell, radiolabelled with ^{35}S-methionine from 3 hours post infection and extracts were prepared at 18 hours post infection. The volume equivalent to $5x10^5$ cpm for each sample was run on a 12% SDS polyacrylamide gel (Fig. 33).

The gel shows the presence of an additional band of approximately 25KDa, the predicted size of epitope-tagged CAT, within the v65-CAT sample as compared to the 1802 and HSV-1 strain 17 samples (Fig. 33, compare lanes 2 and 3 with lane 4). No other significant differences to the infected cell polypeptide profiles were apparent.

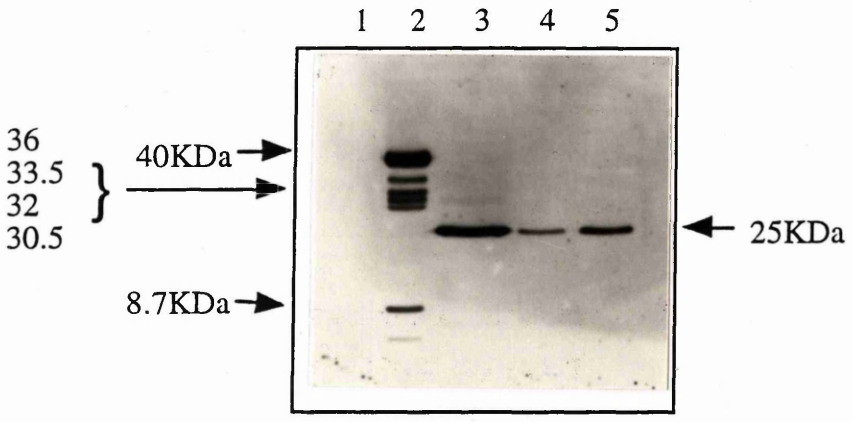


Figure 34. Detection of epitope-tagged CAT fusion protein in v65-CAT infected cell extracts and in v65-CAT virion and light particle preparations. Approximately $2x10^8$ gradient-purified virions and light particles were run on a 12% polyacrylamide gel alongside v65-CAT-, vUL49ep- and 1802-infected cell extracts. The polypeptides were transferred to nitro-cellulose membrane and the membrane was incubated with pp65 monoclonal antibody. Samples were as follows: lane 1: 1802-infected cell extract, lane 2: vUL49ep-infected cell extract, lane 3: v65-CAT-infected cell extract, lane 4: v65-CAT virions, lane 5: v65-CAT light particles. The 25KDa band indicated is of the size predicted for the 65-CAT fusion protein. Also indicated is the 40KDa epitope tagged VP22 polypeptide, and the sizes of each of the vUL49ep breakdown products which were used as size markers.

ii) Detection of 65-CAT Fusion in Infected Cell Extracts by Western Blot.

In order to confirm that the novel protein seen in the polypeptide profiles was the epitope-tagged form of CAT, Western blots were carried out using the pp65 monoclonal antibody that specifically recognises the epitope tag sequence. (Fig. 34). As before, extracts were prepared from 35mm dishes of BHK cells infected for 18 hours at a moi of 5 pfu/cell with v65-CAT. As a positive control, and size marker, an extract from vUL49ep-infected cells was used and as a negative control an extract was made from HSV-1 strain 1802-infected cells. Approximately $6x10^4$ cell equivalents were separated on a 12% polyacrylamide gel, transferred to nitrocellulose and probed with pp65 monoclonal antibody. The v65-CAT extract contained a band of approximately 25KDa (Fig. 34, lane 3). This was the size predicted for native CAT linked to the 10 amino acid epitope tag sequence and confirmed that the 65-CAT fusion protein was synthesised in infected cells. Data presented in *Chapter 7; Results,* Fig. 11 also shows the comparison of the size of epitope-tagged and unmodified CAT protein.

Section 5.

Analysis of v65-CAT Virions and Light Particles.

To determine whether the fusion protein was incorporated into v65-CAT virus particles, Western blot analysis was performed on gradient-purified virions and light particles. These were prepared from four roller bottles of BHK cells which were infected with v65-CAT at a moi of 1 pfu per 300 cells and incubated at 37°C until full cpe was visible. The cell released virus was harvested and purified on 5-15% ficoll gradients. Bands containing virions and light particles were collected and pelleted for further analysis. Approximately $2 x10^8$ virions and light particles were separated on a 12% polyacrylamide gel, transferred to nitrocellulose membrane and probed with pp65 monoclonal antibody. The 25KDa epitope tagged CAT protein was detected in both virions and light particles (Fig. 34, lanes 4 and 5). This result was unexpected and initially indicated that fusion of the 10 amino acid epitope tag sequence to CAT could lead to incorporation of CAT protein into virus particles.

In order to determine the origin of the 65-CAT fusion protein in v65-CAT virions and light particles, the polypeptides present throughout a gradient of v65-CAT cell released material were analysed. A preparation of v65-CAT virus was made as described above and

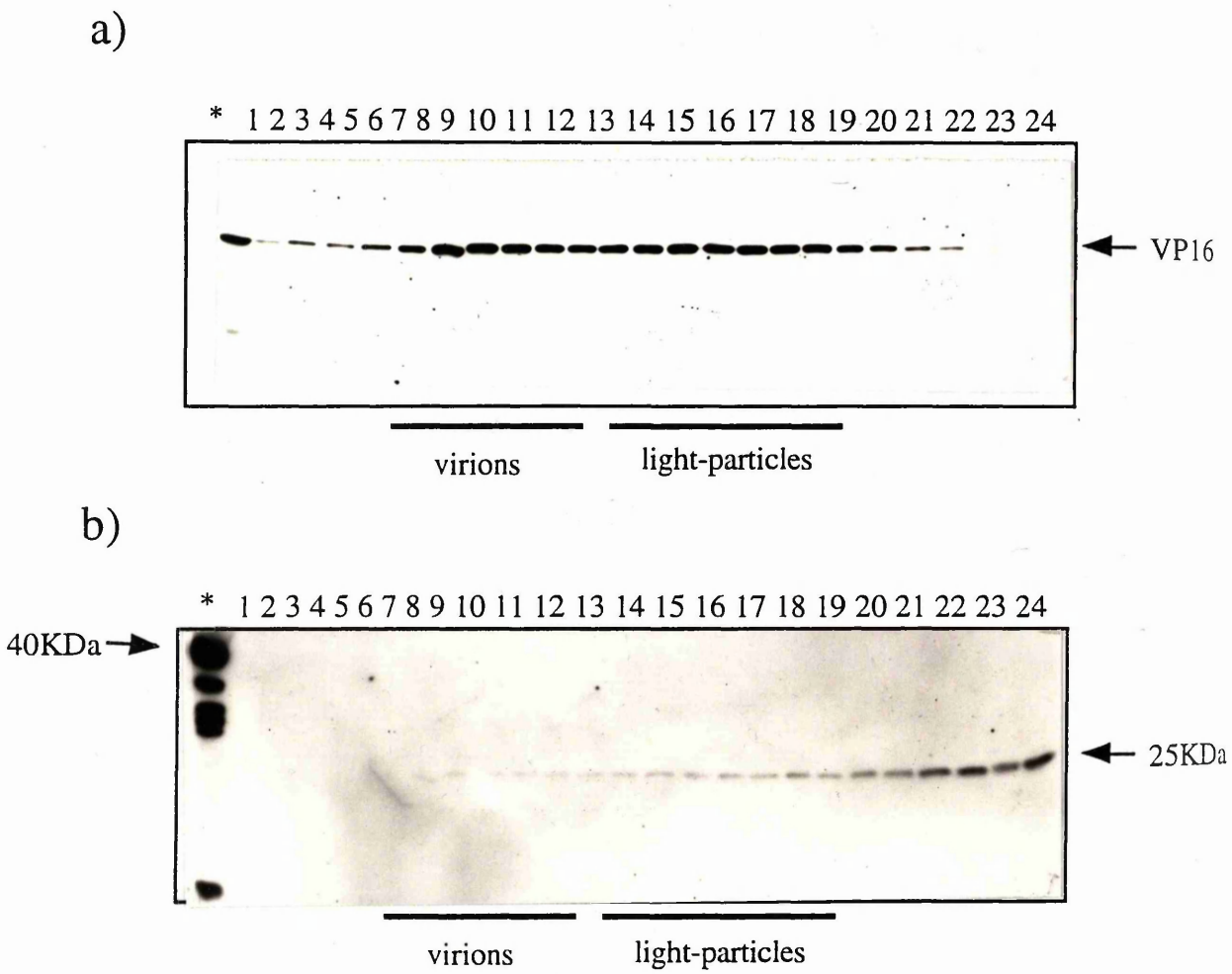
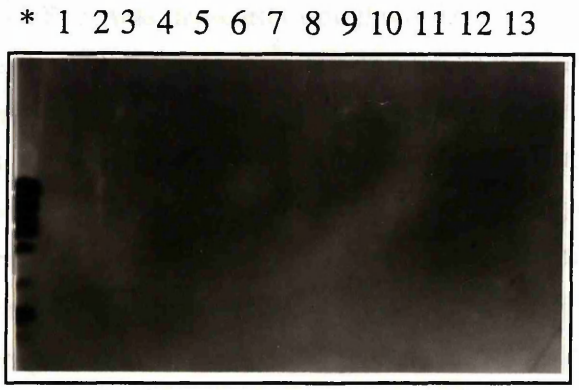
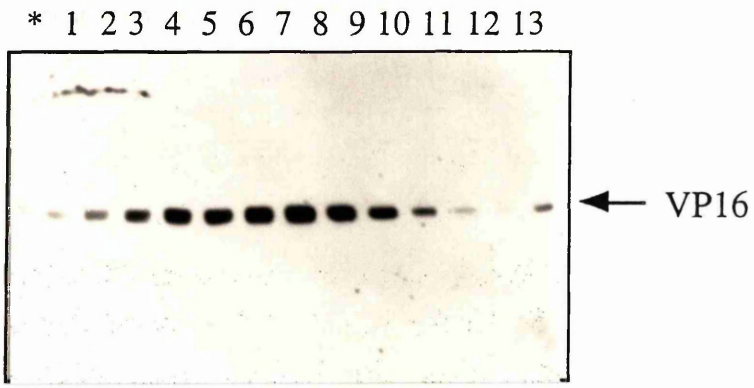


Figure 35. Detection of 65-CAT fusion protein in material collected from across a ficoll gradient. From a 13ml 5-15% ficoll gradient, 24x 450µl aliquots were collected from about 2cm above the bottom of the tube. 15µl from each sample was loaded onto a 14% polyacrylamide gel, and the polypeptides were then transferred to nitrocellulose membrane. The membrane was incubated with two antibodies: (a) The membrane was probed with LP-1 monoclonal antibody specific for VP16; (b) the same membrane was stripped and re-probed with pp65 monoclonal antibody, specific for the epitope tag sequence. The above experiment was repeated on another preparation of v65-CAT virus. 13x 700µl aliquots were collected from about 2cm above the bottom of a 13ml ficoll gradient loaded with v65-CAT CRV. All other parameters are the same as described above. (c) The membrane was probed with pp65 monoclonal antibody. (d) The membrane was stripped and reprobed with LP-1 monoclonal antibody. * represents vUL49ep-infected cell extract.

c)



d)



following banding on 13ml ficoll gradients, 24x 450µl fractions were collected from 2cm below the virion band to the top of the gradient. 15µl of each sample was analysed by Western blot using two different monoclonal antibodies pp65 and LP-1. Incubation of the blot with LP-1 (Fig. 35a) revealed that most of the VP16 was distributed throughout the region of the gradient containing virions and light particles (fractions 6 to 20). Reprobing the membrane with pp65 monoclonal antibody (Fig. 35b), revealed that the 65-CAT fusion protein, in contrast to VP16, was detected throughout the gradient but was most abundant at the top of the gradient. Significantly, this distribution is markedly different from that for VP16 which is a structural protein but similar to that for actin on ficoll gradients, a cellular protein which is not a component of virus particles (see *Chapter 7; Results,* Fig.13a and 13b). This experiment was repeated on several occasions using other preparations of v65-CAT virus and following successive incubation of these membranes with pp65 and LP-1 monoclonal antibodies, the pp65 antibody failed to detect any 65-CAT fusion protein within the gradients; an example of such a preparation is shown in Fig. 35c and 35d. These results strongly suggest that the 65-CAT fusion protein is not incorporated into v65-CAT virions and light particles, and it was concluded that the result obtained in Fig. 34 was due to contaminating soluble protein present in the cell released material loaded onto the top of the ficoll gradient.

Discussion

This chapter describes the introduction into the HSV-1 genome of a second epitope-tagged copy of the UL49 gene, encoding VP22, under the control of the strong HCMV IE promoter. The resulting recombinant virus synthesises approximately 5-fold higher levels of VP22 than wild type virus. The elevated expression of VP22 results in an increase in the amount of VP22 present in the tegument of virions and light particles, thereby demonstrating for the first time a direct positive correlation between the abundance of a structural component within a herpesvirus particle and its level of expression.

The epitope tag sequence was derived from the HCMV UL83 gene, which encodes a structural protein, pp65 (DuPont, UK; Weiner *et al.*, 1985; McLauchlan *et al.*, 1994). To ensure that this sequence was itself not capable of directing the proteins to which it was tagged into the tegument, a control virus, v65-CAT was also constructed. This virus had incorporated into its genome a copy of the bacterial CAT reporter gene, which was tagged at the N-terminus. Results presented in *Results; Chapter 7, Section6* with virus vSAU3 had shown that CAT was not incorporated into virus particles. Western blot analysis across gradients containing purified v65-CAT virions and light particles indicated that CAT protein could only be detected in the fractions towards the top of the gradient, having a different pattern of distribution from virions and light particles across the gradients. The detection of CAT protein in the fractions at the top of gradients was thought to have been due to contaminating cellular protein smearing into the gradient. It was therefore concluded that the pp65 epitope tag sequence was not capable of directing CAT into virus particles, and was not resonsible for the increased efficiency of tagged VP22 to enter the tegument of vUL49ep virions and light particles.

Much of the understanding of the tegument has evolved from studies on deletion mutants which fail to synthesise tegument proteins and on the consequent effects on virus particle formation. Most strikingly, it has been shown recently that the UL46 and UL47 genes which encode the major tegument components VP11/12 and VP13/14 respectively are dispensable for production of infectious virus particles (Zhang and McKnight, 1993). Several other less abundant tegument proteins are also not required for virus particle production (McLean *et al.*, 1989; Fenwick & Everett, 1990; Coulter *et al.*, 1993). It is clear therefore that the process of tegument assembly and the structural integrity of the tegument are sufficiently flexible to to accommodate major alterations in composition without loss of virus viability. The data in this chapter demonstrates that it is also possible to increase the absolute amount of a particular protein component of the tegument. Based on analysis of Coomassie stained gels, it is estimated that vUL49ep virus particles contain approximately

between 6000 and 8500 copies of VP22, making it by far the most abundant structural protein. Despite this major alteration in tegument composition, there is no dramatic reduction in either the viability or growth of the virus. There was however, a change in the migration of the virus particles through ficoll gradients (*Chapter 8; Results, Section 6,* Fig. 25) which suggested that vUL49ep virus particles have a greater mass and also size heterogeneity than those of 1802. Examination of the fine strucure of HSV-1 virus particles by cryo-electron microscopy suggested that light particles had a significant variation in size, and on average they were smaller in diameter than virions (140nm compared to 180nm) (Szilagyi & Berriman, 1994). Preliminary examination of vUL49ep virus particles by electron microscopy however, failed to detect any appreciable increase in the size of either vUL49ep virions or light particles. Therefore the change in migration of vUL49ep virus particles through ficoll gradients may have been a result of an increase in the density of these particles in comparison to virus 1802. The flexibility in tegument composition contrasts markedly with the strict stoichiometry of the proteins present in the capsid (Newcomb *et al.*, 1993).

In addition to the dramatic changes in VP22 abundance, there is a consistent reduction in the abundance of VP13/14 in vUL49ep virus particles. It is not clear whether these lower levels reflect reduced synthesis of VP13/14 in vUL49ep infected cells although levels of VP13/14 in infected cells do not appear to be reduced in comparison to virus 1802. It is possible that the mechanisms for incorporating VP22 and VP13/14 into the tegument are similar and the high concentration of tagged VP22 at the site of assembly results in the displacement of VP13/14. By contrast the lack of any effect on the abundance of VP16, the other major tegument protein common to virions and light particles, suggests that its mode of insertion into the tegument is distinct from that of VP22.

The rise in the abundance of VP22 in virus particles was not accompanied by any detectable increase in other tegument components. This suggests that incorporation of VP22 into virus particles is not solely dependent upon interaction with other tegument proteins and also that VP22 is not a limiting determinant for the incorporation of other structural components. Recent studies have shown that insect cells infected with a baculovirus recombinant which expresses high levels of VP22 contain large regular aggregates of the protein (J. McLauchlan, unpublished data) presumably reflecting the ability of VP22 to interact with itself. In addition, the inability to remove VP22 from the tegument following detergent treatment of virions and light particles may have beeen due to interactions between VP22. The formation of VP22 multimers may be enhanced in vUL49ep infected cells thereby resulting in a higher level of incorporation into virus particles. The characterisation of viruses containing mutated forms of epitope-tagged VP22 (*Results; Chapter 3*) support

the suggestion that the ability of VP22 to interact with itself may be the principal determinant of VP22 abundance in the tegument, and increased levels of VP22 could be accommodated without requiring increases in the abundance of any other structural components. This does not preclude the possibility that VP22 is ultimately assembled into the tegument through an interaction with other structural proteins. Indeed, recent evidence indicates that VP22 interacts with VP16 (Elliot *et al.*, 1995) and this interaction may be important for incorporation of VP22 into virus particles.

These results clearly demonstrate that the abundance of VP22 in virus particles can be modulated by altering its level of expression. However, increasing the level of synthesis of another tegument protein, encoded by UL37, by as much as 20-fold does not result in a measurable increase in its abundance in either virions or light particles (J. McLauchlan, personal communication). Thus, in this case there appears to be strict stoichiometric control over the abundance of UL37 protein within virus particles. The reasons behind these differences are not clear. Some constraints can be suggested, for example, in virions there may be an invariant numerical requirement for certain tegument proteins which interact with the capsid. However, the behaviour of VP22 indicates that beyond such constraints, the composition of the tegument may in part reflect the concentration of proteins present in the correct location at the time of assembly and the ability of these proteins to compete for sites in the tegument.

In conclusion, this chapter demonstrates that the composition of the tegument can be dramatically altered by changing the levels of transcription of a tegument component. This suggests that while there may be a process which selects proteins for inclusion into the tegument, at least for certain structural components, the relative amounts of protein incorporated may be a reflection of their abundance in the infected cell. Thus, the relative stoichiometries of certain tegument components may be modulated through transcriptional regulation.

Mutagenesis of UL49ep

The key aim of this study, as described previously, was the development of a manipulatable experimental system that would enable the characterisation of sequences that direct proteins to the tegument. The preceding chapter has described the development of such a system, and this chapter shows how this system was put to use in an attempt to identify a sequence or motif within the UL49 sequence that was responsible for directing VP22 into the tegument.

The procedure involved the development of a set of insertion and deletion mutants derived from the epitope-tagged UL49 gene. Oligonucleotides encoding four amino acids were inserted into chosen sites along the gene, thereby making small changes in the coding sequence of the protein. The design of the oligonucleotides also allowed a series of UL49 mutants containing deletions within the coding sequence to be derived from the insertion mutants. Viruses expressing mutant forms of the epitope-tagged UL49 gene were generated in an identical manner to vUL49ep. Thus, the virus recombinants express not only the mutated form of VP22 but also the unmodified endogenous version. This approach to create both insertion and deletion mutants in UL49 was adopted for two principal reasons. Firstly, no mutants within this gene have previously been described. Hence, the sensitivity of the protein to mutagenesis could not be predicted. Secondly, it was not known whether UL49 is an essential gene. By creating virus recombinants which contained two copies of the UL49 gene, it was anticipated that the unmodified version would permit virus assembly and growth despite the presence of a mutated variant whose function might be disabled.

1. CCA GAT CTG GGC

2. GGT CTA GAC CCG

3. TAG ATC TGA **TGT**

4. ATC TAG ACT **ACA**

Figure 36. Oligonucleotides for the insertional mutagenesis of UL49. The BglII sites are underlined, and the ATG initiation codon incorporated into oligonucleotides 3+4 is indicated in bold type.

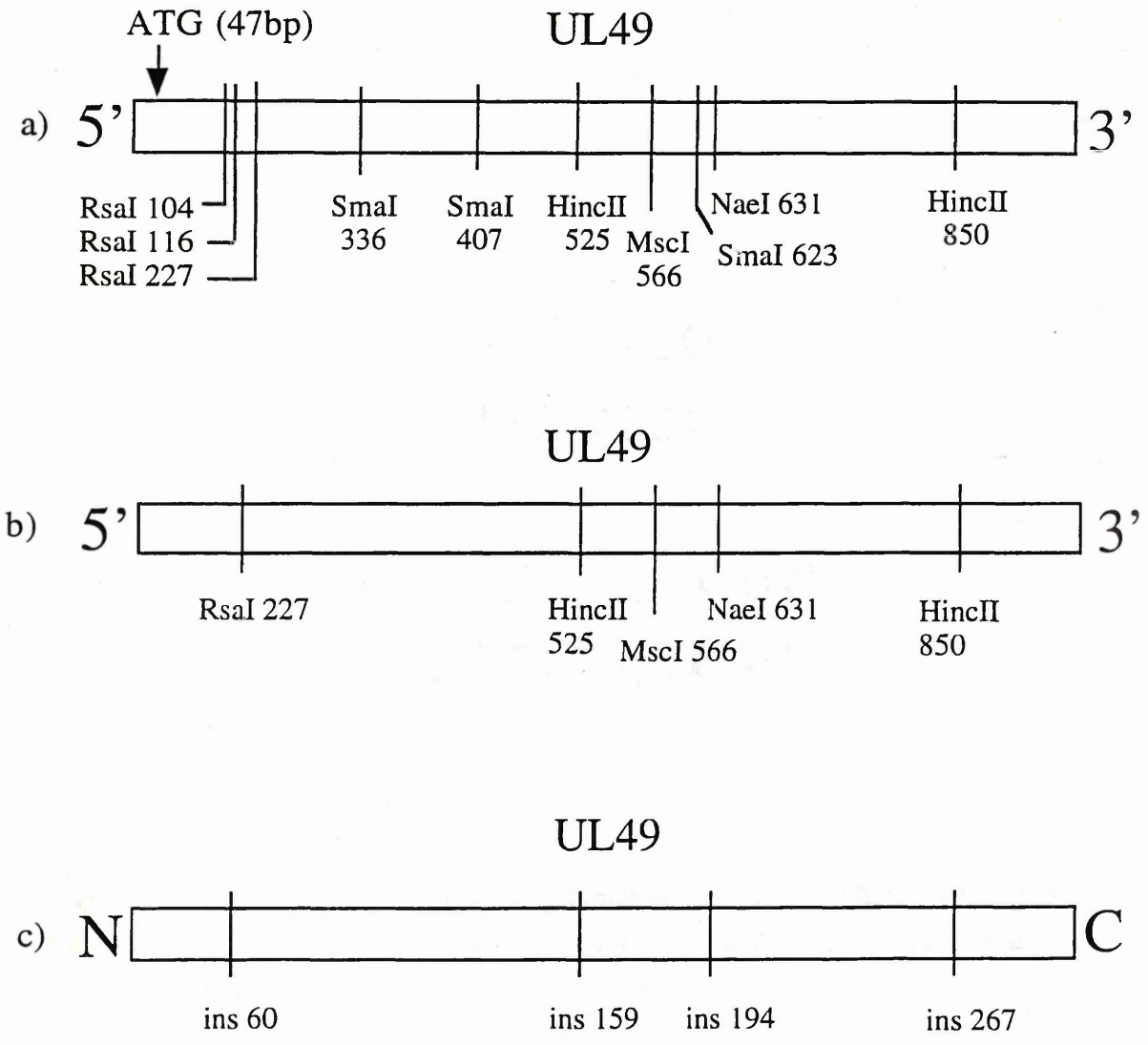


Figure 37. Overview of the restriction enzyme sites used in the construction of the UL49 insertion mutants. a) The restriction enzyme sites selected for the construction of the UL49 insertion mutants. The base pair positions relative to the 5' terminus of the fragment containing the UL49 coding sequence are indicated. b) The restriction enzyme sites and their base pair positions relative to the 5' terminus of the UL49 coding sequence into which oligonucleotide insertions were made. Plasmids containing the UL49 ORF with insertions at each of these restriction enzyme sites were constructed, and are referred to as the pUL494ins plasmid series. c) The amino acid positions of the oligonucleotide insertions relative to the UL49 initiation codon are illustrated. vUL49ins virus mutants containing the UL49 insertions shown in c) were constructed.

Site[a]	oligo inserted[b]	Nucleotide and amino acid sequences at insertion sites[c]	plasmid derived (from pUL492)
RsaI (227)	(3+4)	GTC CAG TAC ATC AGA TCT AAC GAC GAG V Q **Y I R S** N D E	pUL494ins(60)
HincII (525)	(1+2)	CCC GCG TCC CAG ATC TGG GCG ACG GCG P A **S Q I W** A T A	pUL494ins(159)
MscI (566)	(1+2)	CTG GCC AGA TCT GGG CCC AGA AAG CTG L A **R S G P** R K L	pUL494ins(172)
NaeI (631)	(1+2)	CGG GTG GCC CCA GAT CTG GGC GGC TTT R V A **P D L G** G F	pUL494ins(194)
HincII (850)	(1+2)	CAG GAC GTC GCC CAG ATC TGG GAC GCG Q D V **A Q I W** D A	pUL494ins(267)

Table 8. Sequence analysis of pUL494ins plasmids across each oligonucleotide insert.

[a] The positions (in brackets) of the restriction enzyme sites at which oligonucleotides were inserted are shown. Positions are given relative to the 5'end of the fragment containing the UL49 sequences.

[b] The sequences of the oligonucleotides are shown in Fig. 36.

[c]. The nucleotides inserted into the UL49 coding sequences and the predicted changes to the amino acid sequence are in bold type.

Part 1

Section 1

Construction of UL49 Insertion Mutants

For mutagenesis studies, 2 pairs of oligonucleotides of 12bp (Fig. 36) were designed that could be inserted into specific sites along the UL49 gene thereby creating four amino acid inserts in the VP22 polypeptide sequence. To enable rapid screening of plasmids for the presence and location of the oligonucleotides, the recognition site for BglII was incorporated into the sequence of the oligonucleotides. One set of the oligonucleotides contained an ATG transcriptional start site which could enable the construction of N-terminal deletion mutants (Fig. 36, oligos 3+4) (see *Chapter 9; Results, Part 2, Section 1,* describing the construction of pUL494del1-192).

Insertion of the oligonucleotides into the UL49 coding sequences was performed by partially digesting pUL492, used in the construction of epitope tagged UL49 (Fig. 20a) with selected enzymes. Enzymes were chosen on the basis that 1) they cleaved the UL49 sequences at no more than three sites 2) cleavage generated blunt-ended termini and 3) the enzymes used would give rise to a range of mutants spanning the gene. The enzymes which were chosen and the locations of their cleavage sites within the UL49 sequence are shown in Fig. 37a. Partially-digested DNA (prepared as described in *Chapter 6; Methods, Section 3.1*) was ligated with the appropriate oligonucleotide pair (Table 8) and competent bacteria were transformed with the ligation products.

Plasmids from up to 36 of the colonies from each transformation were analysed by digestion with BglII and XbaI to screen for the presence and location of oligonucleotides. This screening procedure gave rise to a panel of plasmids predicted to contain oligonucleotides inserted at the restriction enzyme sites shown in Fig. 37b. To verify the location of inserted oligonucleotides and determine their orientation, the nucleotide sequence at insertion sites for a range of plasmids was determined. Nucleotide sequence data were obtained using primers derived from the plasmid sequences flanking the UL49 DNA in pUL492 (for insertion sites close to the 5' and 3' termini of the UL49 gene), and from residues 430-449 and 354-377 (for insertion sites that lay more than 300 nucleotides from the 3'terminus of the UL49 gene). Of the 10 sites that were initially selected (Fig. 37a),

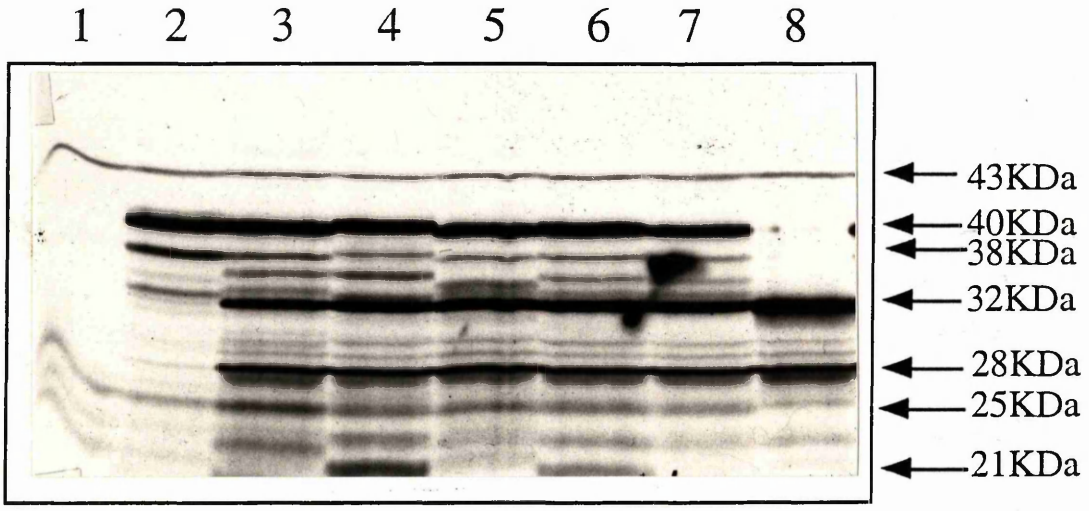


Figure 38. *In vitro* transcription/translation analysis of the polypeptides made by pUL494ins plasmids. 5µl (approximately $5x10^2$ cpm) of the reaction sample was electrophoresed on a 14% polyacrylamide gel. The sizes of the reaction products were estimated by comparing their sizes with the sizes of the bands detected in lane 1. The samples in each lane were as follows; lane 1: no DNA added, lane 2: pUL492, lane 3: pUL494ins60, lane 4: pUL494ins159, lane 5: pUL49ins172, lane 6: pUL494ins194, lane 7: pUL494ins267 and lane 8 pUL49del121-192.

insertion mutants were obtained which contained oligonucleotides at 5 of the sites (Fig. 37b and Table 8); these plasmids were termed the pUL494ins series.

Section 2

In vitro Transcription/Translation Products of VP22 Insertion Mutants

To verify that the insertion of oligonucletides into the UL49 ORF did not alter the frame in which the UL49 coding sequence was read, the ability of each of the UL49 insertion mutants to direct translation of full-length VP22 was investigated *in vitro* using a coupled transcription/translation reticulocyte lysate system. In these experiments, transcription was mediated through the T3 promoter sequence present in plasmid pBluescript KS II, the parental construct for the pUL494ins plasmid series. As a positive control and size marker, plasmid pUL492, containing the unmutated UL49 ORF was used and no DNA was added to the negative control sample. Fig. 38 shows the results of the *in vitro* transcription/translation experiments.

In the reaction containing no plasmid DNA, only minor bands are synthesised (Fig. 38, lane 1) which represent polypeptide species generated through the presence of endogenous RNA in the reticulocyte lysate extract. Addition of pUL492 to the system results in the synthesis of a major product, assumed to be VP22, which has a molecular weight of 40KDa (Fig. 38, lane 2). Major species of similar size were produced in each of the reactions containing the UL49 insertion mutant plasmids (Fig. 38, lanes 3-7). It was concluded therefore that insertion of the oligonucleotides had not prevented synthesis of intact VP22 protein. In addition to the major species of 40KDa, smaller, less abundant products also were generated. These varied in size between the different plasmids, however within each of the reactions containing a pUL494ins plasmid (Fig. 38, lanes 3-7), two bands of approximately 32KDa and 28KDa were most prominent. In the positive control sample containing pUL492 DNA (Fig. 38, lane 2) apart from the major 40KDa band, there was only one major product of approximately 38KDa, and this band was also seen in each of the pUL494ins samples, although it was not as intense. It was assumed that these represented breakdown products of full-length VP22 and their characteristics were not studied further.

Section 3

The Construction of Viruses Containing Epitope-Tagged UL49 Insertion Mutants

Based on both the nucleotide sequence and the *in vitro* transcription/translation results, it was concluded that the plasmids containing the UL49 insertion mutants were capable of producing full-length VP22. Before inserting the UL49 insertion mutants into 1802 viral DNA, each of the mutated ORFs was subcloned into the plasmid vector pFJ22ep, which contains the oligonucleotide sequence encoding the 10 amino acid epitope tag sequence between the HCMV IE promoter and the UL38 late polyadenylation site (Fig. 20b). Following BamHI digestion of each of the pUL494ins plasmids, 0.97Kbp fragments containing the mutated UL49 ORFs were purified following agarose gel electrophoresis and ligated to BamHI digested pFJ22ep. The resulting plasmids were named the pUL49ins plasmid series. Apart from the inserted oligonucleotides, these constructs had the same structure as pUL49ep (Fig. 20c).

Each of the pUL49ins plasmids were digested with XbaI and the cassettes containing the epitope-tagged, mutated UL49 ORFs were inserted into the XbaI site present in 1802 virus DNA (Rixon & McLauchlan, 1990). The resultant ligation products were transfected into BHK cells and the virus progeny were screened by Western blot analysis for the synthesis of polypeptides recognised by monoclonal antibody pp65. Individual plaques synthesising proteins of the predicted size were selected and purified to homogeneity for each insertion mutant. Four out of the five UL49 insertion mutants shown in Fig. 37b yielded virus recombinants; these were designated vUL49ins60, vUL49ins159, vUL49ins194 and vUL49ins267 and are collectively referred to as virus series vUL49ins (Fig 37c).

Section 4

Southern Blot Analysis of vUL49ins Viral DNAs

Southern blot analyses were carried out to determine that the correct sequences had been inserted into the HSV-1 genome and that the insertion of a second copy of UL49 did not result in rearrangement of the HSV-1 genome. To ensure that the correct insertions had been incorporated into the recombinant virus genomes XbaI and BglII digests were performed on each of the viral DNAs, and these digests were run alongside the same digests of each of the parental plasmid DNAs. Following electrophoresis, the DNA fragments were transferred to Hybond-N membrane and incubated with a ^{32}P-radiolabelled probe consisting of the 1930bp XbaI fragment from pUL49ep (Fig. 20) which contained the epitope-tagged UL49 cassette. This probe hybridises to all of the sequences within the vUL49ins viruses that are derived from the epitope-tagged UL49 expression cassette, as well as the endogenous UL49 sequences.

Hybridisation with this probe detected a DNA fragment of 9.08Kbp in each of the lanes containing viral DNA (Fig. 39a, lanes 1, 3, 5 & 7) which contained the endogenous copy of UL49. In each of the lanes containing plasmid DNA (Fig. 39a, lanes 2, 4, 6 & 8) a faint band of approximately 5.1Kbp which represented undigested pUL49ep sequences was also detected.

The remaining bands recognised by the probe were derived from the UL49ins gene cassettes. A 0.75Kbp band, corresponding to the BglII fragment containing the HCMV IE promoter sequence was present in each lane. The other bands were unique for each vUL49ins construct; maps for each of these constructs are given in Fig. 39b. Thus UL49ins60 virion and plasmid samples gave rise to 0.23Kbp and 1.08Kbp fragments (Fig. 39a, lanes 3 & 4; Fig. 39b (i)) Bands of 0.78Kbp and 0.52Kbp were detected in the UL49ins159 DNAs (Fig. 39a, lanes 5&6; Fig 39b (ii)). UL49ins194 DNAs were predicted to give fragments of 0.68Kbp and 0.61Kbp (Fig 39b (iii)) which resolved as a single species in the blot (Fig. 39a, lanes 1 & 2). Finally, bands of 0.85Kbp and 0.46Kbp were detected in UL49ins267 DNAs (Fig. 39a, lanes 7&8; Fig 39b (iv)). By comparing the data in Fig. 39a with the predicted maps in Fig. 39b it is evident that the virus recombinants contained the predicted epitope-tagged UL49ins cassettes.

To orientate each of the inserted sequences in the recombinant genomes, BamHI

a)

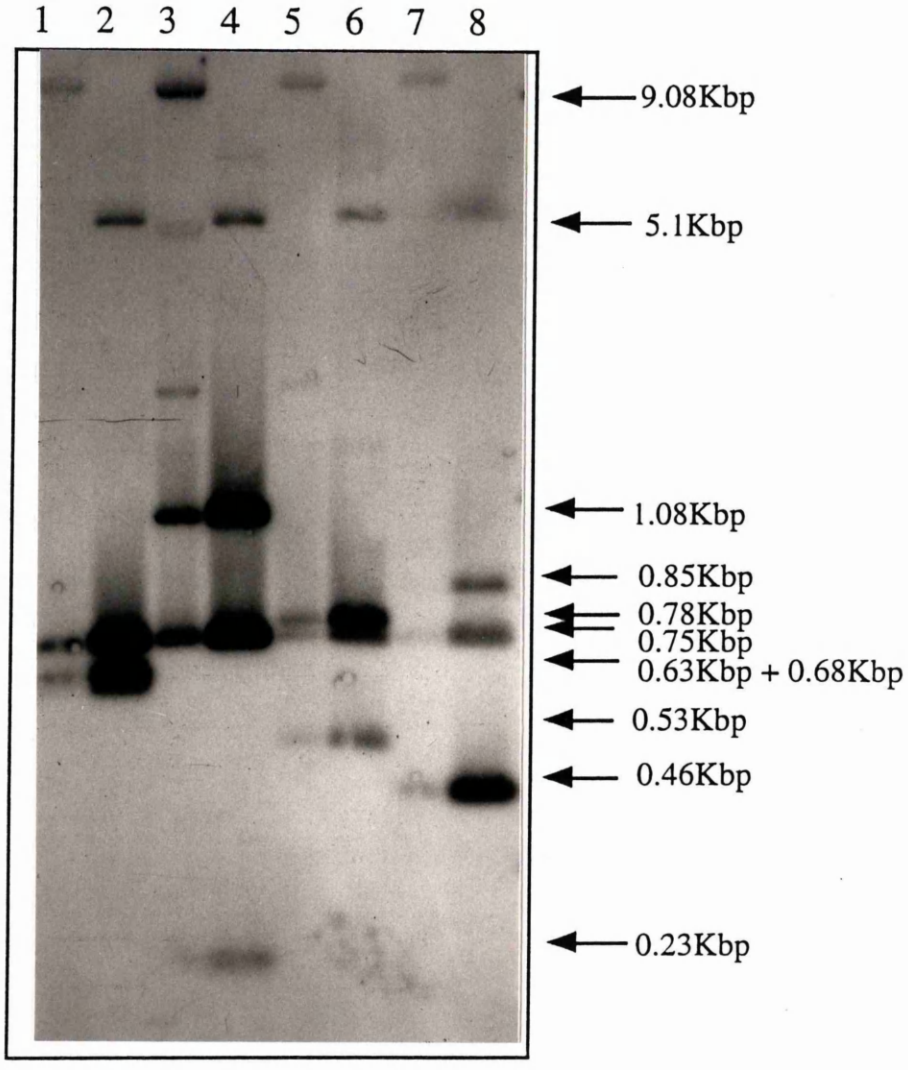
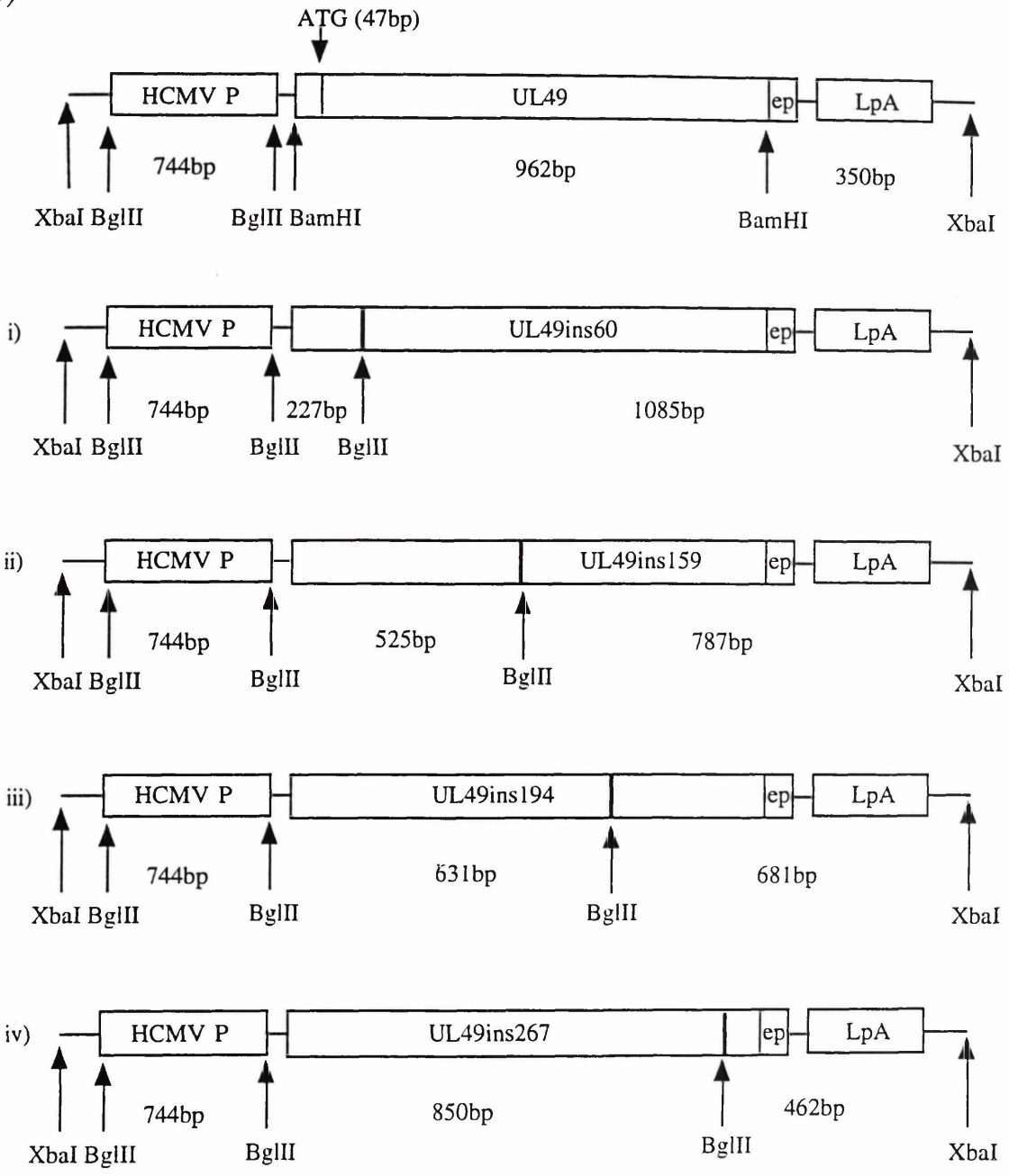


Figure 39. Southern Blot analysis of vUL49ins virus DNAs. a) 0.5µg of DNA was digested with BglII and XbaI and then electrophoresed on a 1.5% agarose gel followed by transfer to Hybond-N membrane. The membrane was then probed with a ^{32}P-labelled probe consisting of the 1.9Kbp XbaI fragment from pUL49ep. The sizes of the major bands detected are indicated. Lane 1, vUL49ins194; lane 2, pUL49ins194; lane 3, vUL49ins60; lane 4, pUL49ins60; lane 5, vUL49ins159; lane 6, pUL49ins159; lane 7, vUL40ins267; lane 8, pUL49ins267. b) Diagramatic representation of the epitop-tagged UL49 expression cassettes that were used to construct the vUL49ins virus series. The sizes of fragments released following BamHI/XbaI digestion of the unmodified UL49 cassette, and of BglII/XbaI digestion of each of the UL49 insertions are indicated.

b)


ATG (47bp)
HCMV P
UL49
ep
LpA
744bp
962bp
350bp
XbaI BglII
BglII BamHI
BamHI
XbaI
i)
HCMV P
UL49ins60
ep
LpA
744bp
227bp
1085bp
XbaI BglII
BglII
BglII
XbaI
ii)
HCMV P
UL49ins159
ep
LpA
744bp
525bp
787bp
XbaI BglII
BglII
BglII
XbaI
iii)
HCMV P
UL49ins194
ep
LpA
744bp
631bp
681bp
XbaI BglII
BglII
BglII
XbaI
iv)
HCMV P
UL49ins267
ep
LpA
744bp
850bp
462bp
XbaI BglII
BglII
BglII
XbaI

a)

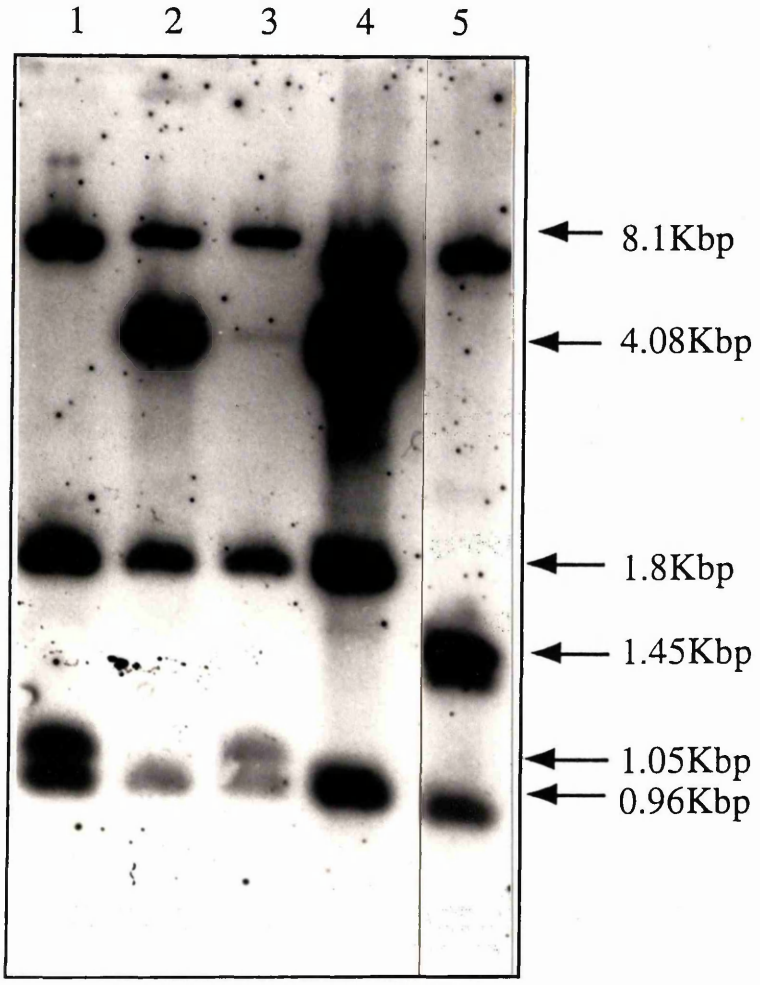
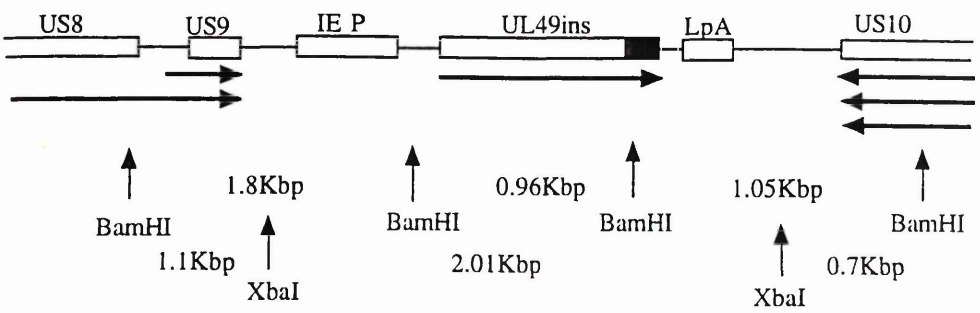


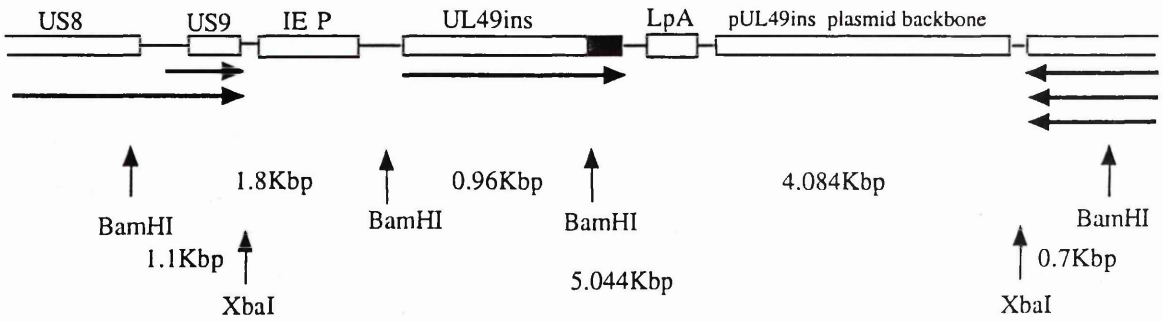
Figure 40. Southern Blot analysis of vUL49ins virus series DNA. a) 0.5µg of DNA purified from of each vUL49ins virus was digested with BamHI and then electrophoresed on a 1% agarose gel followed by transfer to Hybond-N membrane. The membrane was then probed with ^{32}P-radiolabelled pUL49ep. In lane 1, vUL49ins194; lane 2, vUL49ins60; lane 3, pUL49ins159; lane 4, vUL49ins267; lane 5, vUL49ep. The sizes of the major bands detected are indicated. b) Organisation of the genomes of the vUL49ins viruses at the US9/US10 gene locus. The orientation of the UL49ins inserts are shown, as are the sizes of fragments obtained following BamHI and XbaI digestion. i) vUL49ins159 and vUL49ins194 ii) vUL49ins60 and vUL49ins267

b)

i)


US8
US9
IE P
UL49ins
LpA
US10
BamHI
1.8Kbp
1.1Kbp
XbaI
BamHI
0.96Kbp
2.01Kbp
BamHI
1.05Kbp
XbaI
0.7Kbp
BamHI

ii)


US8
US9
IE P
UL49ins
LpA
pUL49ins plasmid backbone
BamHI
1.8Kbp
1.1Kbp
XbaI
BamHI
0.96Kbp
BamHI
5.044Kbp
4.084Kbp
XbaI
0.7Kbp
BamHI

digests were performed on each of the vUL49ins viruses. BamHI digested vUL49ep was used as a size marker. Following electrophoresis and transfer to Hybond-N membrane, the membrane was incubated with ^{32}P-radiolabelled pUL49ep which hybridises to all of the sequences with the viral DNAs derived from the pUL49ins plasmid series as well as the endogenous UL49 sequences. Hybridisation with this probe revealed that the 8.1Kb BamHI f fragment which contained the unmodified copy of UL49 was identical in size for each of the viral DNAs (Fig. 40a). This probe also detected a 0.96Kbp fragment in each of the vUL49ins viral DNAs and vUL49ep viral DNA which correspond to the inserted UL49ins ORFs. Two other bands of approximately 1.8 and 1.05Kb were detected in vUL49ins194 and vUL49ins159 viral DNA samples (Fig. 40a, lanes 1 & 3). The unique XbaI site in 1802 viral DNA lies within the BamHI z fragment and digestion with BamHI and XbaI generates two fragments of 1.1 and 0.7Kbp. Thus the 1.8Kbp fragments are derived from fragments containing the HCMV promoter linked to the 1.1Kbp BamHI z fragment and the 1.05Kbp fragments are derived from the polyadenylation sequences linked to the 0.7Kbp BamHI z fragment.

In lanes 2 and 4 containing vUL49ins60 and vUL49ins267 respectively, bands of 1.8Kbp were detected which correspond to the HCMV promoter sequences linked to the 1.1Kbp BamHI z fragments. In addition, there are bands of 4.08Kbp in each of these lanes which were a result of the 0.35Kbp polyadenylation sequences and the 3.03Kbp pUL49ins plasmid backbone sequences (equivalent to base pairs 4822-2754 in pUL49ep; Fig. 20c) linked to the 0.7Kbp BamHI z fragments. The plasmid backbone sequences were inserted because following XbaI digestion of each of the pUL49ins plasmids, prior to the insertion into XbaI digested viral DNA, the XbaI fragments containing the UL49 expression cassettes were not gel purified. From the sizes of the fragments each of the epitope-tagged UL49ins cassettes are transcribed in the same direction as genes US8 and US9 (as summarised in Fig. 40b).

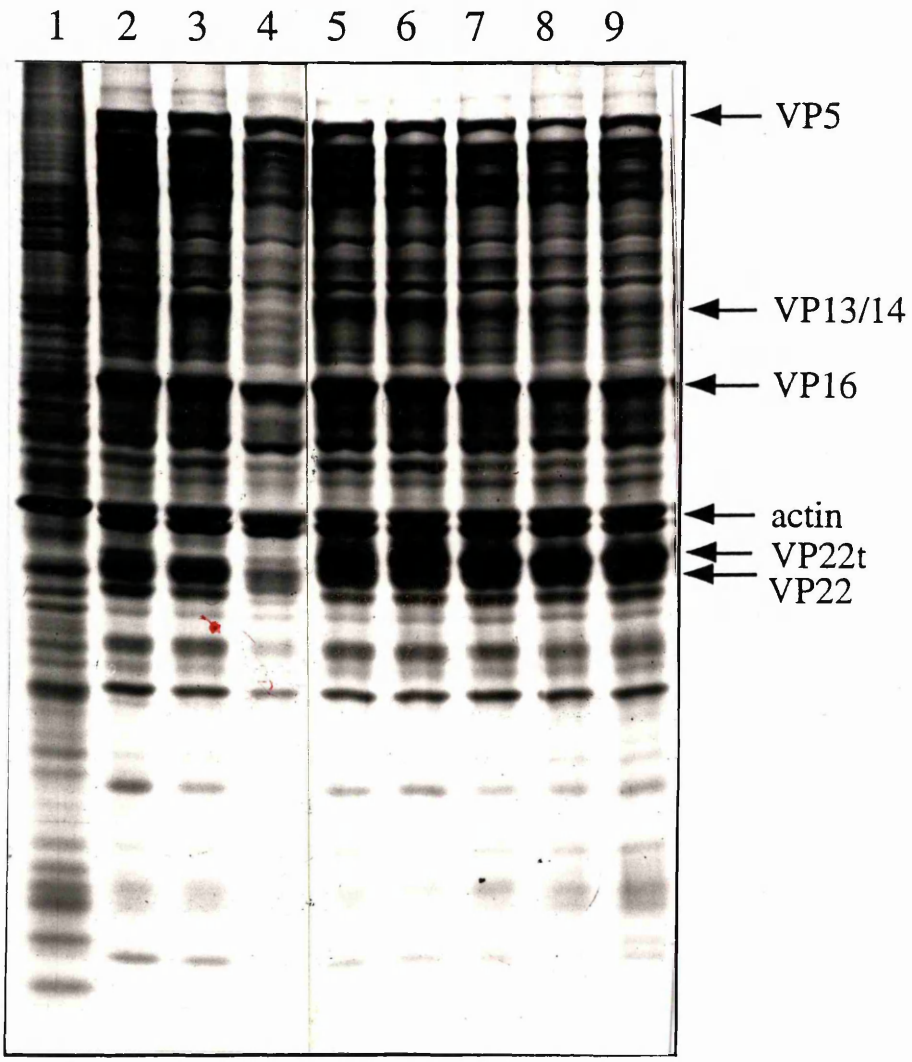


Figure 41. Polypeptides synthesised by vUL49ins viruses. ^{35}S-radiolabelled extracts prepared from 35mm dishes of BHK cells infected with vUL49ins viruses were compared with radiolabelled extracts from HSV-1 strain 17, 1802-, RUL47Δ2-, vUL49ep- and mock-infected cells. Aliquots containing $5x10^5$ cpm were electrophoresed on a 12% polyacrylamide gel. The samples in each lane are as follows; lane 1: mock infected, lane 2: strain 17, lane 3: 1802, lane 4: RUL47Δ2, lane 5: vUL49ep, lane 6: vUL49ins60, lane 7: vUL49ins159, lane 8: vUL49ins194 and lane 9: vUL49ins267. The major polypeptides are indicated (←).

Section 5

Polypeptides Synthesised by the vUL49ins Viruses

To analyse the polypeptides synthesised by the vUL49ins series, ^{35}S-methionine labelled infected cell extracts were prepared at 18 hours post infection for each of the vUL49ins viruses and for comparative purposes HSV-1 strain 17, 1802, RUL47Δ2 and vUL49ep. RUL47Δ2 is a HSV-1 deletion mutant lacking the UL47 gene, and was kindly supplied by Dr. J. McKnight (McKnight *et al.*, 1992). Comparison of the profiles of the vUL49ins viruses with those of the control samples revealed an abundant species of approximately 40KDa which was present in each of the vUL49ins and vUL49ep samples but absent in the strain 17, RUL47Δ2 and 1802 samples. These polypeptides corresponded in size to epitope-tagged VP22 (VP22t) synthesised by vUL49ep-infected cells (previously described in *Chapter 8; Results, Part 1, Section 4.*). These species had similar mobilities to VP22t although in samples, vUL49ins159, vUL49ins194 and vUL49ins267 the migration of the additional band was slightly less than that for VP22t in the vUL49ep sample (Fig. 41, compare lanes 7, 8 & 9 with lane 5). These changes in mobility were thought to have been a result of small increases in the molecular weights of the proteins following insertion of the 4 amino acid sequences, and also the nature of the inserted amino acids. No other significant alterations were apparent between the polypeptide profiles of the extracts apart from the intensity of VP13/14 in samples vUL49ins159, vUL49ins194 and vUL49ins267 which was reduced in comparison to vUL49ep and vUL49ins60 (Fig. 41, compare lanes 5 & 6 with lanes 7, 8 & 9) .

To confirm that the abundant 40KDa species in the vUL49ins-infected cell extracts corresponded to epitope-tagged VP22, Western blot analysis was performed on the infected cell extracts shown in Fig. 41. Following separation by electrophoresis and transfer of the proteins onto nitrocellulose, the membrane was then probed with monoclonal antibody pp65. As a positive control and to act as a size marker, the vUL49ep-infected extract was also run on the gel. The antibody recognised a species of approximately 40KDa in each of the samples that corresponded to epitope-tagged VP22 (Fig. 42, compare lanes 2-5 with lane 1). This confirmed that the major species identified in the ^{35}S-methionine polypeptide profiles corresponded to the insertion mutant form of VP22t made by each virus. In this blot, there is also a small but apparent increase in the size of VP22t made by vUL49ins60, vUL49ins159 and vUL49ins194. As described for Fig. 41, these slight changes in the mobility were thought to have been a result of the four amino acid insertions. The antibody

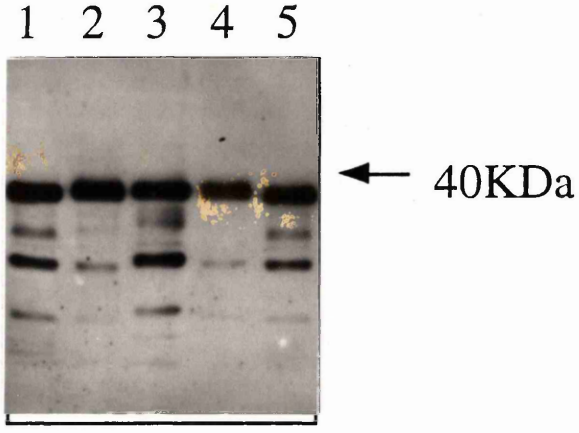


Figure 42. Western blot analysis of ^{35}S-methionine radiolabelled vUL49ins-infected cell extracts. Approximately $6x10^4$ cell equivalents were electrophoresed on a 12% polyacrylamide gel and the polypeptides were transferred to nitrocellolose membrane. The membrane was then probed with monoclonal antibody pp65 (1:2000dilution). Samples were as follows: lane 1: vUL49ep, lane 2: vUL49ins267, lane 3: vUL49ins159, lane 4: vUL49ins194 and lane 5: vUL49ins60.

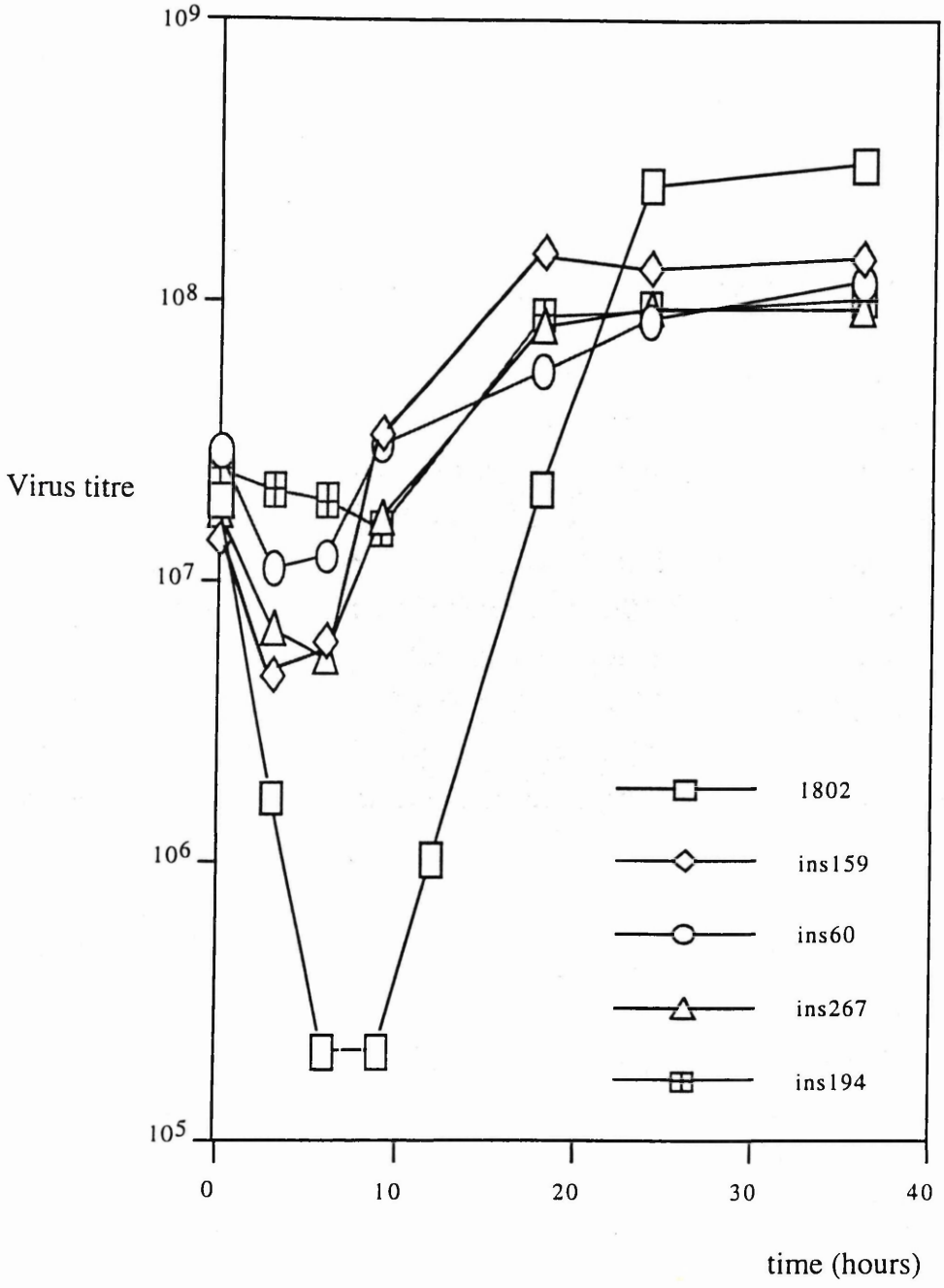


Figure 43. Growth characteristics of vUL49ins virus series compared with the parent virus 1802. 35mm dishes containing approximately $2x10^6$ BHK cells were infected at a moi of 5 pfu per cell and harvested at various time points ranging between 0 and 36 hours after infection. The total amount of virus present at each time point was determined by titration.

also recognises a number of lower molecular weight species that correspond to C-terminal products of VP22 that have been generated either by cleavage of full length protein or internal initiation of translation.

Section 6

Growth Characteristics of vUL49ins Viruses

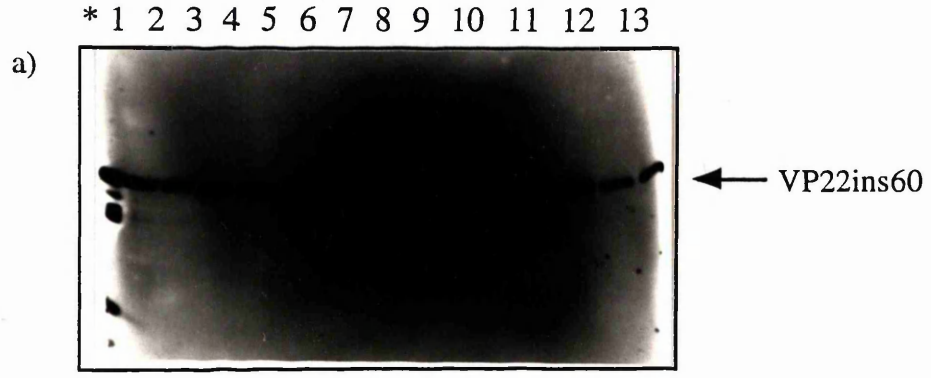
In order to determine whether the growth of each of the vUL49ins virus series was affected by the insertion of a second epitope-tagged partial copy of UL49, the growth characteristics of the vUL49ins viruses were compared to those of the parent, 1802. The gradients of the slopes during the exponential growth phase (between 6 and 24 hours) for each vUL49ins virus mutant suggested that the growth rates were reduced compared to 1802 (Fig. 43). However, for each of the vUL49ins virus mutants the titre during the eclipse phase did not fall as significantly as did that for 1802. The final yields for each vUL49ins mutant were similar and were only slightly less than that for 1802. The data obtained for these viruses were similar to those for vUL49ep (*Chapter 8; Results, Part 1, Section 5*) and suggested that insertion of the mutant epitope-tagged copies of UL49 into 1802 did not have a significant effect on the growth of each virus.

Section 7

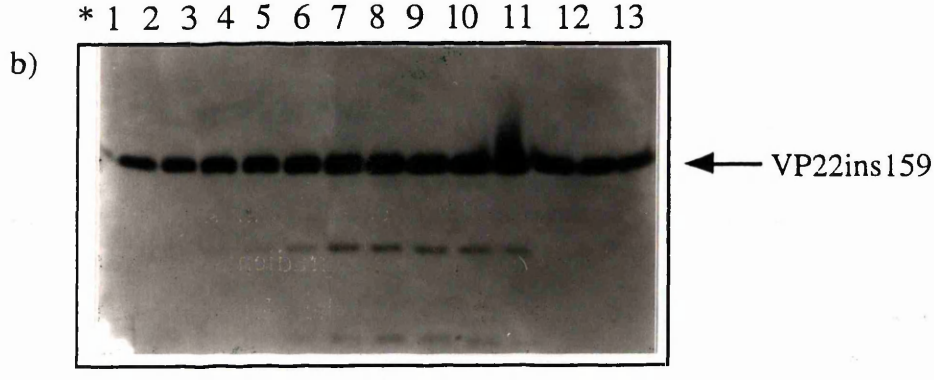
Incorporation of VP22 into vUL49ins Virions and Light Particles

Analysis of the polypeptides produced in cells infected with the vUL49ins viruses had shown that epitope-tagged VP22 was produced to similar levels as that made in vUL49ep-infected cells (Fig. 41). To determine whether these mutated forms of VP22 were incorporated into virions and light particles, Western blot analysis was performed on samples collected from across ficoll gradients containing the material released from infected cells. This material was prepared from four roller bottles of BHK cells that had been infected with each virus at a moi of 1 in 300 and grown at 31°C for 4 days. For comparative purposes, a preparation of vUL49ep was grown in parallel with the vUL49ins viruses. Ficoll gradients of cell-released virus were run and aliquots collected from across each

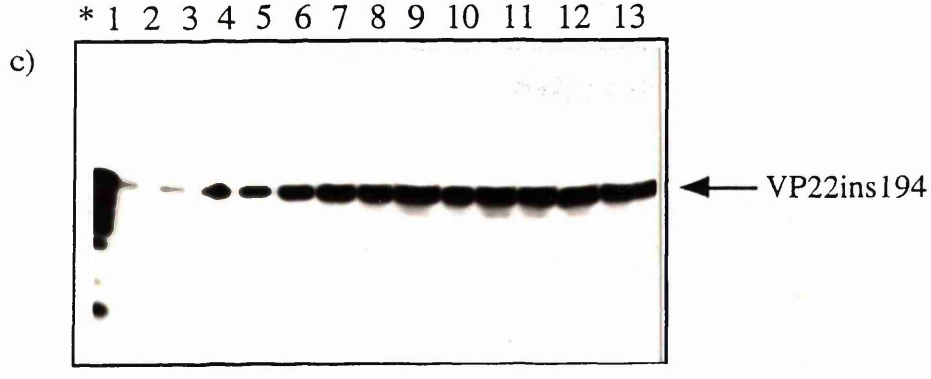
vUL49ins60



vUL49ins159



vUL49ins194



vUL49ins267

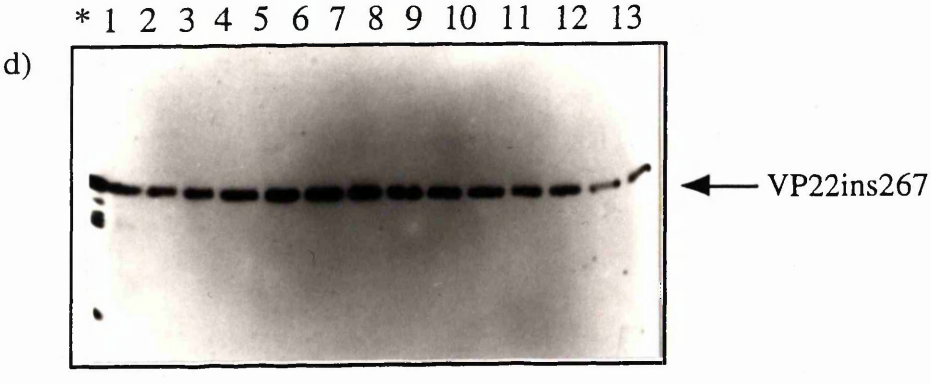


Figure 44. Western blot analysis across gradients containing each of the vUL49ins virus preparations. Material was collected in 13x700µl aliquots from 2cm below the virion band in 13ml, ficoll gradients that were loaded with material from cells infected with each of the vUL49ins virus series. Following electrophoresis of 15µl of each sample on 12% polyacrylamide gels, the polypeptides were transferred to nitrocellulose membranes and each was incubated with pp65 monoclonal antibody. The epitope tagged VP22ins proteins are indicated. In lane * vUL49ep infected cell extracts were included as positive controls and size markers.

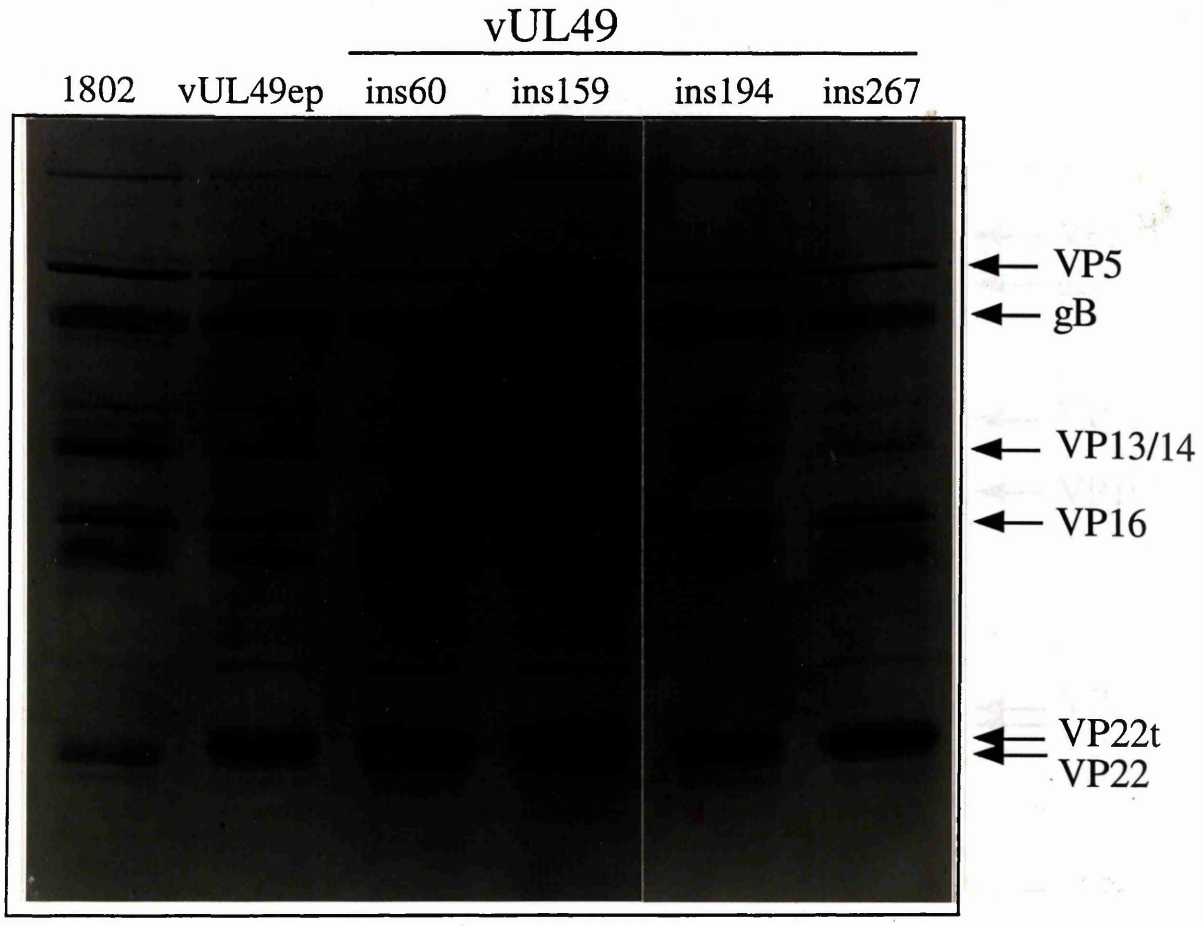


Figure 45. Polypeptide profiles of vUL49ins virions. Approximately $3x10^9$ vUL49ins60, vUL49ins159, vUL49ins194 and vUL49ins267 ficoll purified virions were compared to those of parent virus 1802 and vUL49ep. Following electrophoresis on a 12% polyacrylamide gel, the gel was stained with Coomassie Brilliant blue. The major structural polypeptides are indicated as are the bands corresponding to the epitope-tagged VP22 proteins (VP22t).

Virus	Copy numbers of VP22 + VP22t
1802	3154
vUL49ep	12540
vUL49ins60	13185
vUL49ins159	10939
vUL49ins194	9600
vUL49ins267	12551

Table 9. The copy numbers of VP22 + VP22t were estimated following the excision of the VP22 bands from the Coomassie Brilliant blue stained gel shown in Fig. 45. The relative amount of stain in the gel slice was estimated following elution of the Commassie Brilliant blue stain in each band in 25% piperidine solution followed by spectrophotometric analysis at 605nm. The number of copies of VP22 were calculated relative to the estimated copy number of VP16, calculated by Zhang & McKnight (1993).

gradient as described in *Chapter 7; Results, Part 1, Section 6.* These aliquots were examined for the presence of epitope-tagged VP22 by Western blot analysis. Incubation of each of the membranes with pp65 monoclonal antibody, revealed that VP22t in each of the vUL49ins virus gradients was most abundant throughout the region where virions and light particles migrate (Fig. 44a-d). These data are indistinguishable from those obtained for non-mutated VP22t (*Chapter 8; Results, Part 1, Section 7*) and therefore it was concluded that the epitope-tagged VP22 made by each of the vUL49ins viruses was incorporated into virions and light particles (Fig. 44 a-d).

Section 8

Polypeptide profiles of purified virions and light particles

The polypeptide profiles of the vUL49ins virions purified from ficoll gradients were examined by polyacrylamide gel electrophoresis. This revealed that their polypeptide contents were similar to those for vUL49ep (Fig. 45). Indeed from visual inspection, vUL49ins60 and vUL49ins267 virion polypeptides were apparently indistinguishable from the vUL49ep virion proteins. The most noticeable difference in the polypeptide patterns was a decrease in the abundance of VP22t in vUL49ins194 virions. There was also a sight increase in the amounts of untagged VP22 present in vUL49ins194 virions.

To quantitate the VP22 protein (VP22 + VP22t) in each of the vUL49ins virions, bands containing the tagged and untagged VP22, and VP16 were excised from the gel. The relative amounts of protein in the bands was estimated by spectrophotometric analysis of the stain eluted from each gel slice (Table 9). This revealed that there was approximately a 4-fold rise in the abundance of VP22 in vUL49ep virions as compared to 1802 virions. From examination of the gel the VP22 band in vUL49ep virions consisted mostly of VP22t. These findings are in agreement with those presented in *Chapter 8; Results, Part 1, Section 8.* Analysis of vUL49ins60, vUL49ins159 and vUL49ins267 virions gave essentially identical results to those for vUL49ep. In vUL49ins194 virions, the amount of tagged VP22 incorporated is less, but there remains a substantial rise (~3-fold) in the total amount of VP22 in these particles (Table 9). These data suggest that insertion of the oligonucleotides at positions 60, 159 and 267 have no discernible effect on incorporation of epitope-tagged VP22. The only insertion mutant which does have a noticeable effect on incorporation is ins194 where there is a reduction in tagged VP22. However, even with this mutant, there is relatively more tagged VP22 incorporated as compared to untagged VP22.

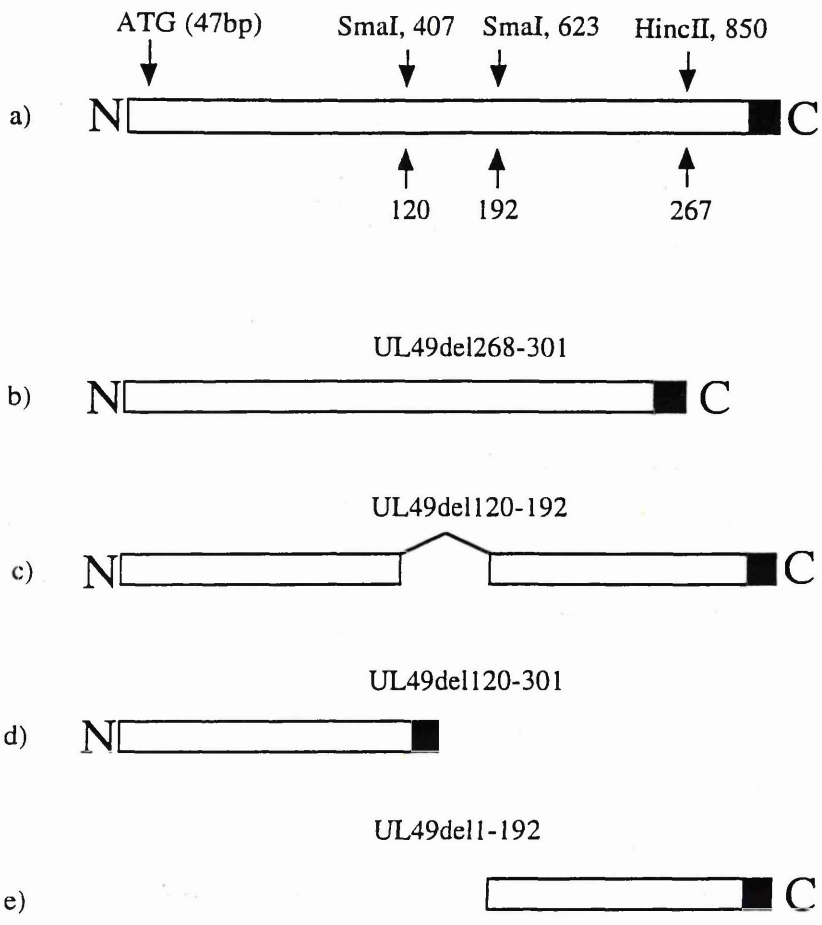


Figure 46. Overview of the UL49 deletion mutants constructed. a) The UL49 ORF showing the restriction enzyme sites utilised in the construction of the deletion mutants, and their base pair positions relative to the 5' terminus of the fragment containing UL49 sequences. Below the ORF the numbers refer to the corresponding amino acid positions at which the restriction enzymes lie in the coding sequence, and are relative to the ATG initiation codon of UL49. b-e) Diagramatic representations of each of the UL49 deletions constructed. The epitope tag sequence at the end of each ORF is shown (■)

Part 2

Deletion Mutagenesis of Epitope-Tagged VP22

Section 1

Plasmid Construction

Coincident with the construction of a panel of viruses containing linker insertions in the epitope-tagged copy of UL49, a parallel series of recombinants was produced in which specific regions of the tagged UL49 coding region were deleted. The construction of these deletion mutants was facilitated by the design of the oligonucleotides used for creating the insertion mutants (*Chapter 9; Results, Part 1, Section 1*). In total, four recombinants were made using epitope-tagged UL49 deletion mutants whose derivation was as follows:

1) pUL49del120-192

As part of the strategy for constructing the plasmid series containing linker insertions, the enzyme SmaI, which cleaves the UL49 coding sequence in three positions, was used to generate partially-digested pUL492 DNA (Fig. 20a). These cleavage sites lie within a region of 287 base pairs and thus, due to the size of pUL492, it was possible that DNA cleaved at more than one site would not separate on agarose gels from molecules cut at a single site. During the course of the screening process for plasmids containing inserted oligonucleotides, one construct was identified in which an oligonucleotide had inserted between the SmaI sites located at base pair positions 407 and 623, thereby removing amino acids 120 to 192 from the UL49 coding region (Fig. 46c). This construct was termed pUL494del120-192. As predicted from the UL49 nucleotide sequence, removal of these sequences and the insertion of the oligonucleotide should result in an in-frame deletion mutant. To verify the location of the oligonucleotide and determine its orientation, the nucleotide sequence of pUL494del120-192 was obtained using a primer that annealed to sequences between base pairs 626 and 648 within the UL49 sequences. The nucleotide sequence data indicated that no frame shift mutation had occurred at the insertion site (Fig. 47). To ensure that the plasmid encoded a polypeptide of the predicted size, the protein was

5' ACC GCC CCC CT**A GAT CTG ATG T**GG GTG GCC 3'

T A P L D L M W V A

Figure 47. Sequence analysis across the oligonucleotide insert within the UL49 ORF in pUL494del120-192. The amino acid sequence across the oligonucleotide insert is indicated, and the BglII site encoded within the oligonucleotide is underlined.

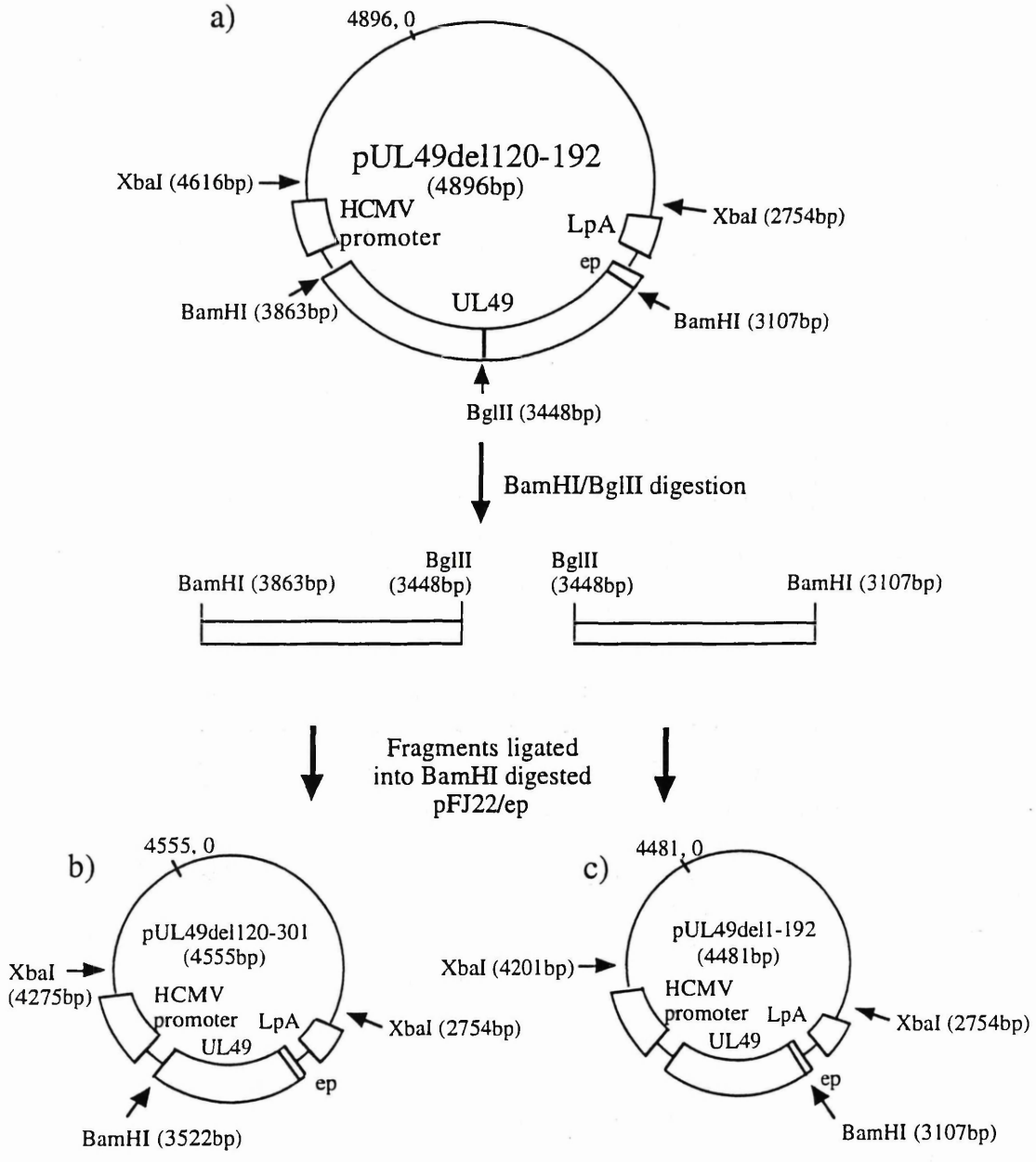


Figure 48. Structures of UL49del plasmids a) pUL49del120-192. b) pUL49del120-301. c) pUL49del1-192. Following the digestion of pUL49del120-192 with BamHI and BglII, the two UL49 fragments of 341bp and 415bp were gel purified and each fragment was then subcloned into BamHI digested pFJ22/ep resulting in plasmids pUL49del1-192 and pUL49del120-301.

translated *in vitro* from pUL494del120-192 using the coupled reticulocyte lysate system. As compared to the VP22 protein synthesised by pUL492, the product from the pUL494del120-192 reaction was smaller with an apparent molecular weight of approximately 32KDa (Fig. 38, lane 8). This size corresponds closely to the predicted size of the VP22 polypeptide that should be encoded by the mutant. It was therefore assumed that the protein detected corresponded to the UL49del120-192 gene product.

Prior to insertion into the virus genome, the BamHI fragment from pUL494del120-192 which contained the UL49 ORF was subcloned into plasmid pFJ22ep that had been cleaved with BamHI (previously described in *Chapter 8; Results, Part 1, Section 1,* Fig. 20b). The resultant plasmid was termed pUL49del120-192 and contained the UL49 deletion mutant under the control of the HCMV IE promoter with the epitope tag sequence linked to the 3' end of the ORF (Fig. 48a).

2) pUL49del120-301

It was evident from the nucleotide sequence data obtained at the insertion site in pUL494del120-192, that ligation between the BglII site in the oligonucleotide linker in this plasmid and the BamHI site in pFJ22ep would directly fuse the N-terminal 119 amino acids of VP22 to the epitope tag, thereby creating a deletion mutant in which residues 120 to 301 had been removed. Thus, a 415bp BamHI/BglII fragment from pUL49del120-192 which contained these 5' terminal sequences was inserted into the BamHI site of pFJ22ep and the resultant construct was termed pUL49del120-301 (Fig. 48b).

3) pUL49del1-192

Due to the orientation of the oligonucleotide inserted into pUL494del120-192, an ATG initiation codon within the linker sequence lies in-frame with base pair residues 623 to 962 of the UL49 ORF (Fig. 47). To construct a plasmid which expressed these sequences, a 341bp BglII/BamHI fragment from pUL49del120-192 which contained these 3' terminal sequences was inserted into the BamHI site of pFJ22ep, thereby generating pUL49del1-192 (Fig. 48c).

4) pUL49del268-301

Introduction of the oligonucleotide at the HincII site in plasmid pUL49ins267 (Fig. 49a) generated a BglII site which could be ligated to the BamHI site in pFJ22ep to give an

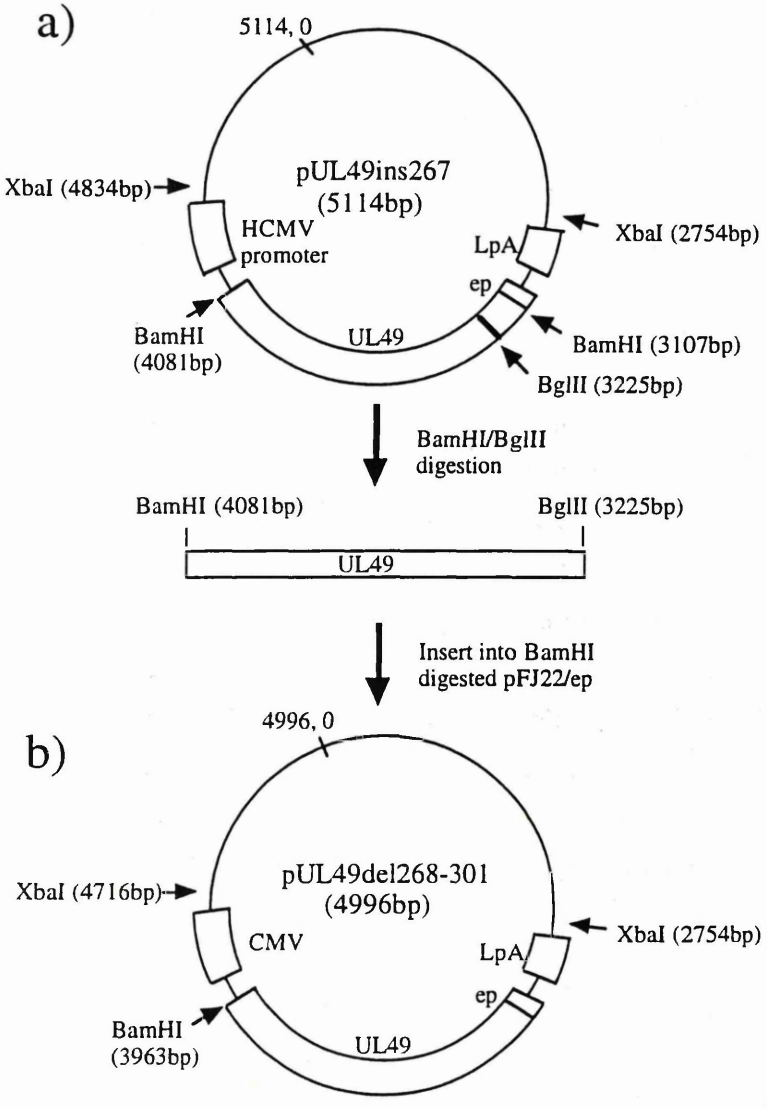


Figure 49. Construction of pUL49del268-301. Plasmid pUL49ins267 was digested with BamHI and BglII which released a fragment of 856bp, containing the UL49 ORF minus the C-terminal 110bp. Following gel purification the 856bp fragment was subcloned into BamHI digested pFJ22ep, resulting in plasmid pUL49del268-301.

in-frame fusion product between base pair residues 1 to 850 and the epitope tag sequence, thereby removing the C-terminal 37 amino acids of the VP22 protein. An 856bp BamHI/BglII fragment from pUL49ins267 was inserted into the BamHI site of pFJ22ep, generating plasmid pUL49del268-301 (Fig. 49b).

Section 2

Construction of Viruses Containing the Epitope-Tagged UL49 Deletions

Viruses expressing the epitope-tagged UL49 deletion mutants were constructed using HSV-1 virus vector 1802 as the parent virus (*Chapter 8; Results, Part 1, Section 2*). Thus, the pUL49del plasmids were digested with XbaI and ligated into the XbaI site within the U_S region of the 1802 viral genome. Following transfection of the ligation products, infected cells were screened by Western blot analysis using monoclonal antibody pp65 for the synthesis of proteins of the predicted sizes. Thereafter, 12 isolated plaques were selected for further screening and purification. Following 3 rounds of plaque purification, single isolates for each deletion mutant were selected and grown. Virus recombinants were designated vUL49del1-192, vUL49del120-192, vUL49del120-301 and vUL49del268-301, collectively termed the vUL49del virus series.

Section 3

Southern Blot Analysis of vUL49del Virus Series DNAs

Southern blot analyses were carried out to determine whether the correct sequences had been inserted into the HSV-1 genome and to ensure that the insertion of a second copy of UL49 did not result in rearrangement of the virus genome. Purified DNA from each of the vUL49del viruses was digested with BamHI and XbaI, and compared with its parent pUL49del plasmid also digested with BamHI and XbaI. As controls vUL49ep and pUL49ep were also digested with BamHI and XbaI. Following electrophoresis, the DNA fragments were transferred to Hybond-N membrane, which was then incubated with ^{32}P-radiolabelled probe consisting of the 1930bp XbaI fragment from pUL49ep (Fig. 20c) containing the epitope-tagged UL49 cassette. This probe hybridises to all of the sequences

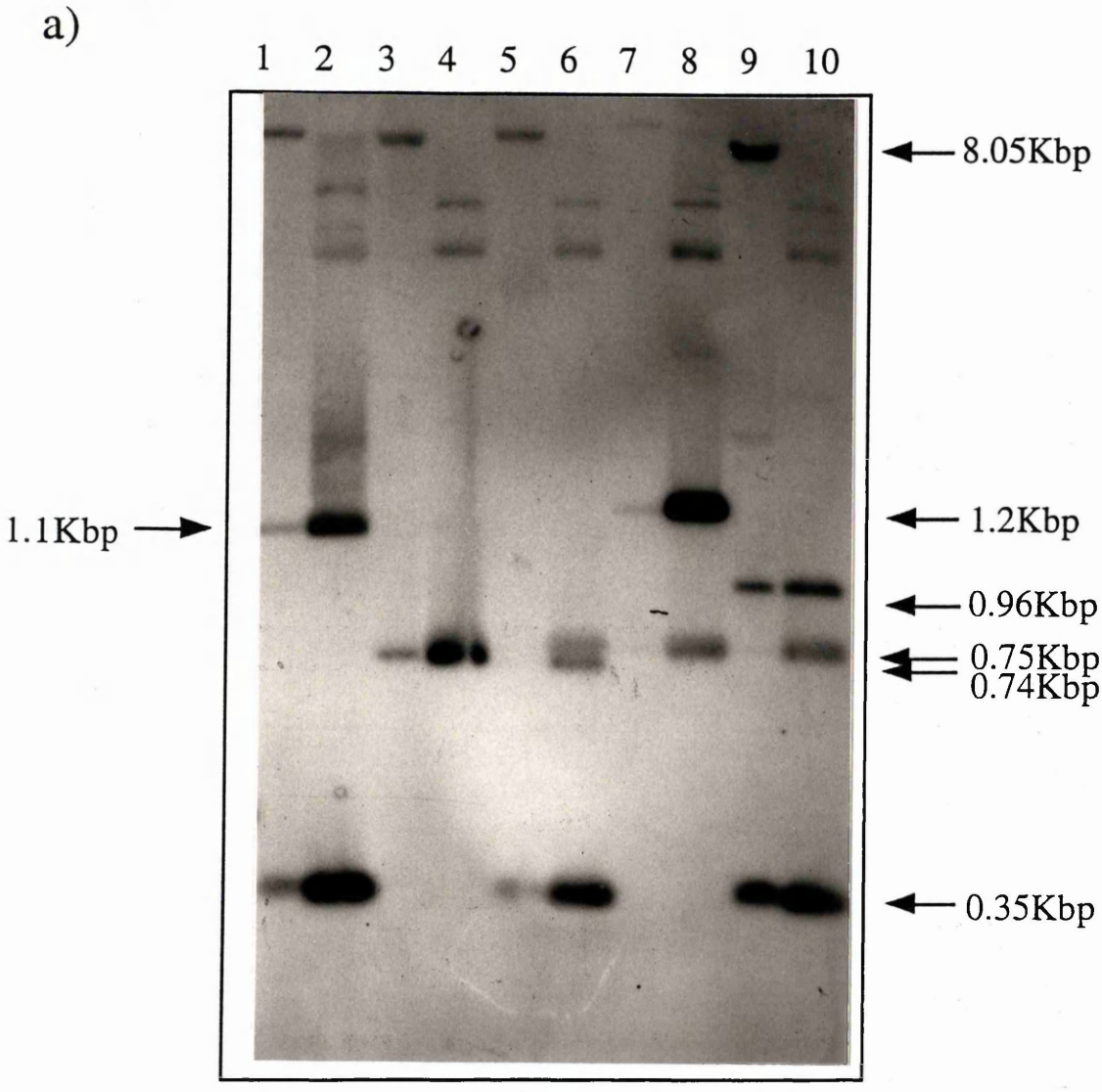
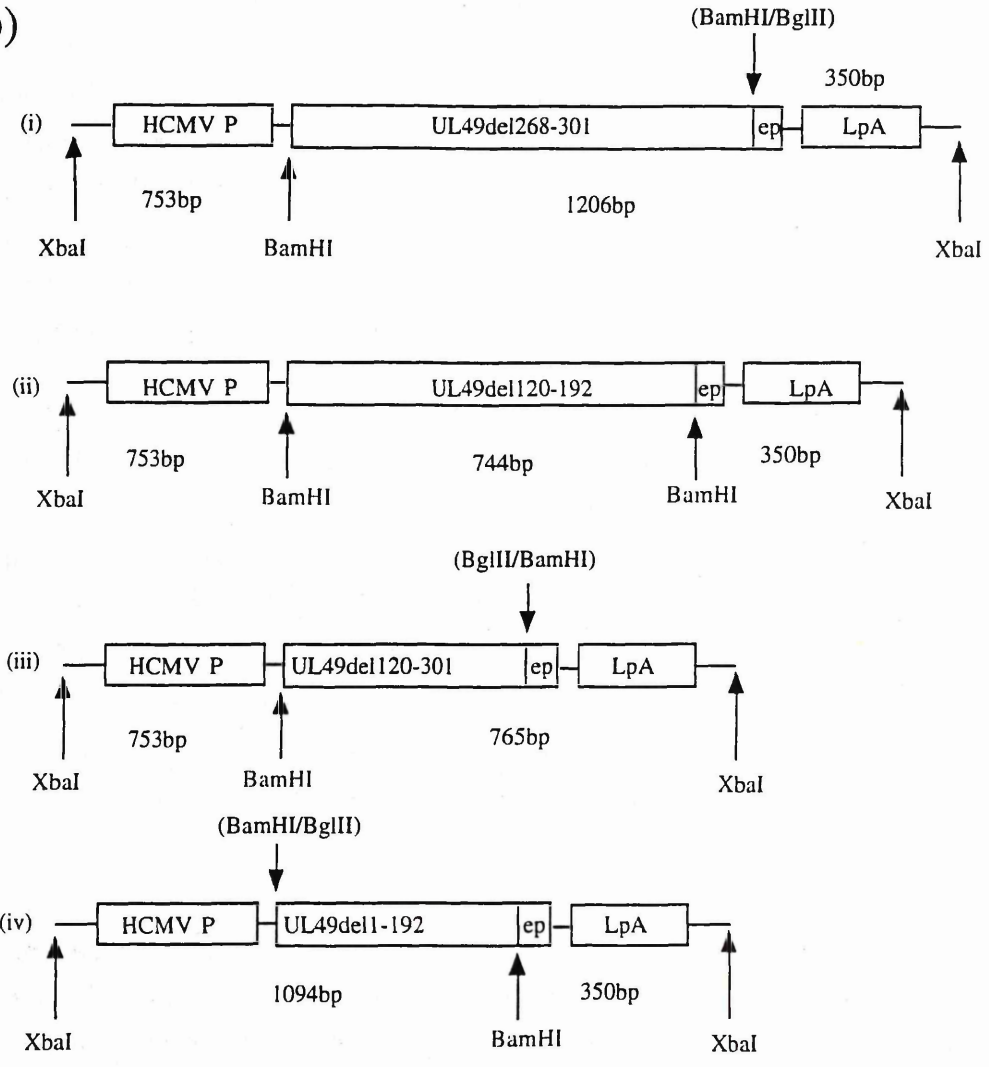


Figure 50. Southern Blot analysis of vUL49deletion virus series DNA. a) 0.5µg of DNA purified from of each vUL49del virus, and 0.2µg of each parental plasmid DNA, was digested with BamHI and XbaI and then electrophoresed on a 1.5% agarose gel followed by transfer to Hybond-N membrane. The membrane was then probed with a radio-labelled probe consisting of the IE HCMV promoter sequence, the entire epitope tagged UL49 ORF and the UL38 late poly A site derived from XbaI digested pUL49ep. In lane 1, vUL49del1-192; lane 2, pUL49del1-192; lane 3, vUL49del120-301; lane 4, pUL49del120-301; lane 5, vUL49del120-192; lane 6, pUL49del120-192; lane 7, vUL49del268-301; lane 8, pUL49del268-301; lane 9, vUL49ep; lane 10, pUL49ep. The sizes of the major bands are indicated. b) Diagramatic representation of the epitope-tagged UL49 expression cassettes that were used to construct the vUL49del virus series. The sizes of fragments released following BamHI/XbaI digestion of each of the UL49deletions are illustrated.

b)


(BamHI/BglII)
350bp
(i)
HCMV P
UL49del268-301
ep
LpA
753bp
1206bp
XbaI
BamHI
XbaI
(ii)
HCMV P
UL49del120-192
ep
LpA
753bp
744bp
350bp
XbaI
BamHI
BamHI
XbaI
(BglII/BamHI)
(iii)
HCMV P
UL49del120-301
ep
LpA
753bp
765bp
XbaI
BamHI
XbaI
(BamHI/BglII)
(iv)
HCMV P
UL49del1-192
ep
LpA
1094bp
350bp
XbaI
BamHI
XbaI

within the vUL49del viruses that are derived from the epitope-tagged UL49 expression cassette, as well as to the endogenous UL49 sequences. Hybridisation with this probe detected a fragment of 8.05Kbp in each of the lanes containing virion DNA (Fig. 50a, lanes 1, 3, 5, 7 & 9) which contained the endogenous copy of UL49.

In all except UL49del1-192 virion and plasmid samples, the HCMV IE promoter sequence is flanked by XbaI and BamHI sites and therefore following digestion with these enzymes a 0.75Kbp fragment should be released (Fig. 50b (i)-(iii)). This 0.75Kbp band was detected in both the virion and plasmid samples of UL49del120-192, UL49del120-301, UL49del268-301 (Fig. 50a, lanes 3 to 8). In lanes 9 and 10 containing pUL49ep and vUL49ep respectively, additional fragments of 0.96Kbp, and 0.35Kbp were detected. These fragments correspond to the full length UL49 ORF (0.96Kbp) and the UL38 late poly A site (0.35Kbp).

In both lanes 7 and 8 containing the UL49del268-301 plasmid and virion samples, a band of 1.2Kbp was detected. This band contained the UL49 ORF (minus the 3' terminal 110bp) linked to the 0.35Kbp poly A site (Fig. 50b (i)). In the UL49del120-192 plasmid and virion samples, two bands of 0.35Kbp and approximately 0.74Kbp were detected (Fig. 50a, lanes 5 & 6). These bands correspond to the UL38 late poly A site and the UL49 ORF minus the internal 216 base pairs (Fig. 50b (ii)). The UL49del120-301 plasmid and virion DNA samples, were predicted to release a doublet of 0.75Kbp and 0.76Kbp corresponding to the IE HCMV promoter sequence and also the UL49 5'terminal 415bp linked to the 0.35Kbp late poly A site (Fig. 50b (iii)). The 0.75Kbp band detected in lanes 3 and 4 containing these plasmid and virion samples agree with this prediction (Fig. 50a). In the UL49del1-192 plasmid and virion DNAs bands of 1.1Kbp and 0.35Kbp were detected (Fig. 50a, lanes 1& 2). The larger band corresponded to the 0.75Kbp HCMV promoter sequence linked to the 3'terminal 341bp of UL49, while the 0.35Kbp fragment corresponded to the UL38 late poly A site (Fig. 50b (iv)). This Southern blot reveals that each of the UL49 deletion viruses contain the same epitope tagged UL49 deletions that are present in their parent plasmids.

In order to orientate each of the inserted sequences within the vUL49del virus series BamHI digests were performed and the restriction enzyme profiles for each of the vUL49del viruses were examined. BamHI digested vUL49ep was included as a size marker. Following electrophoresis and transfer to Hybond-N membrane, the membrane was incubated with ^{32}P-radiolabelled pUL49ep which hybridises to all of the sequences within

a)

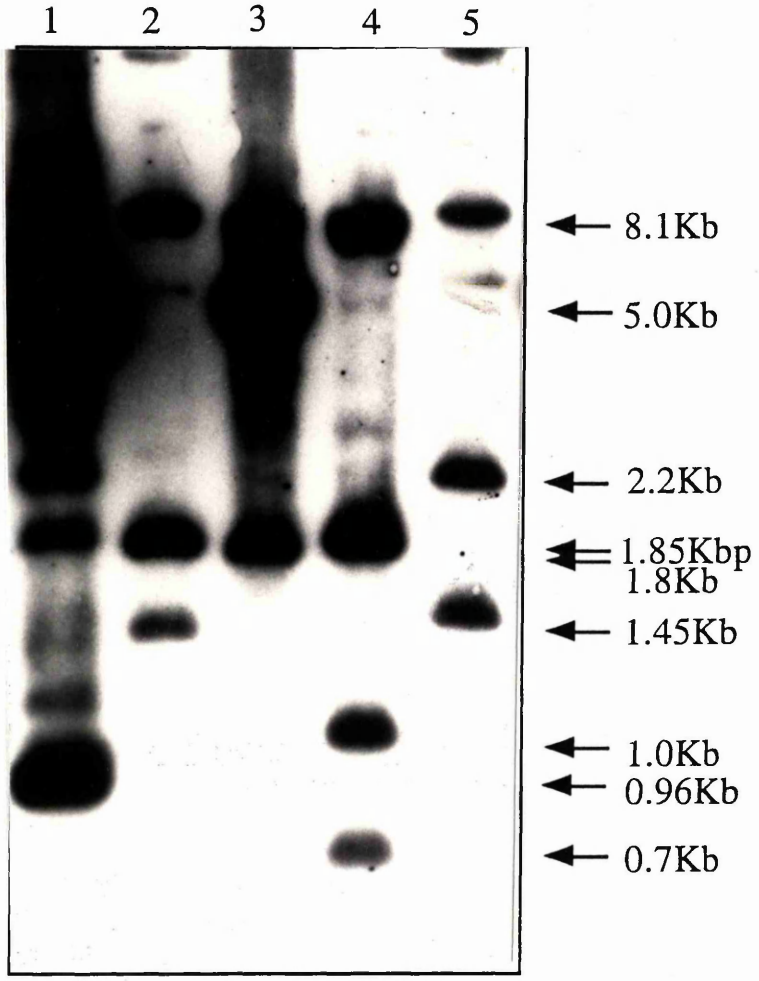
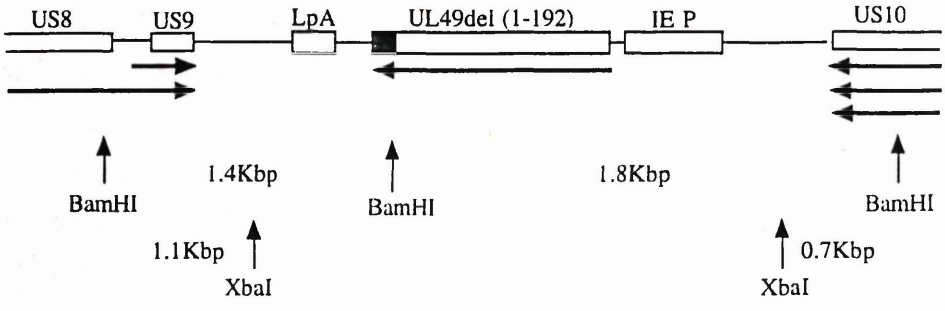


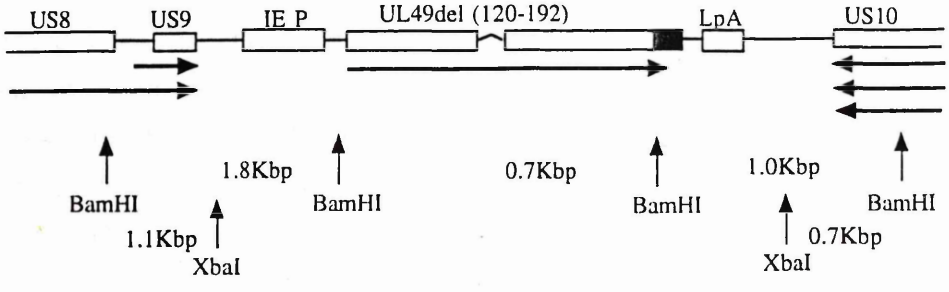
Figure 51. Southern Blot analysis of vUL49deletion virus series DNA. a) 0.5µg of DNA purified from of each vUL49del virus was digested with BamHI and then electrophoresed on a 1% agarose gel followed by transfer to Hybond-N membrane. The membrane was then probed with a radio-labelled pUL49ep. In lane 1, pUL49ep; lane 2, vUL49del1-192; lane 3, vUL49del120-301; lane 4, vUL49del120-192; lane 5, vUL49del268-301. The sizes of the major bands are indicated. b) Diagramatic representations of the genomes of the vUL49 deletion viruses. The positions of the inserted copies of UL49 between genes US9 and US10 are shown, as are the directions in which the UL49del sequences are transcribed. The sizes of fragments obtained following BamHI digestion are also indicated. (i) vUL49del1-192,(ii) vUL49del120-192 and iii) vUL49del268-301.

b)

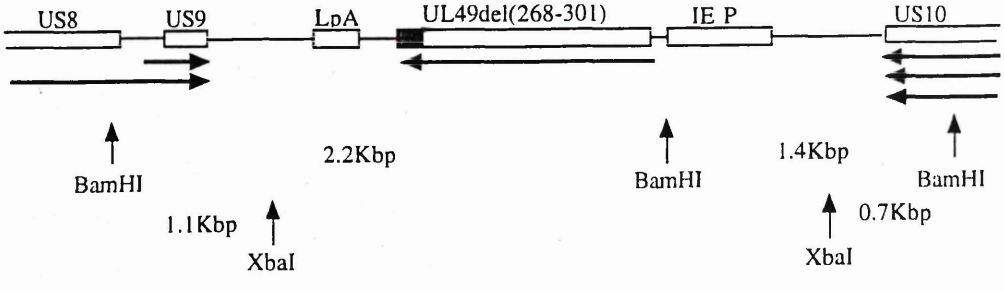
(i)


US8
US9
LpA
UL49del (1-192)
IE P
US10
BamHI
1.4Kbp
BamHI
1.8Kbp
BamHI
1.1Kbp
XbaI
XbaI
0.7Kbp

(ii)


US8
US9
IE P
UL49del (120-192)
LpA
US10
BamHI
1.8Kbp
BamHI
0.7Kbp
BamHI
1.0Kbp
BamHI
1.1Kbp
XbaI
XbaI
0.7Kbp

(iii)


US8
US9
LpA
UL49del(268-301)
IE P
US10
BamHI
2.2Kbp
BamHI
1.4Kbp
BamHI
1.1Kbp
XbaI
XbaI
0.7Kbp

the viral DNAs derived from the pUL49del plasmid series as well as the endogenous UL49 sequences. Hybridisation with this probe revealed that the 8.1Kbp BamHI f fragment which contained the unmodified copy of UL49 was identical in size for each of the viral DNAs (Fig.51a). This probe also detected a 0.96Kbp DNA fragment in the vUL49ep DNA sample which correspond to the UL49 ORF, and acted as a size marker.

The unique XbaI site in 1802 viral DNA lies within the BamHI z fragment and digestion of this fragment with BamHI and XbaI generates two products of 1.1 and 0.7Kb. vUL49del1-192 contains a unique BamHI site at the junction between the epitope-tag and the UL49 sequence (Fig. 51b (iv)), and BamHI digestion resulted in the release of two fragments of 1.45Kbp and 1.8Kbp (Fig. 51a, lane 2). These were composed of the 1.1Kbp BamHI z fragment linked to the 0.35Kbp late poly A site, and the 0.7Kbp BamHI z fragment linked to the 1.1Kbp fragment containing the IE HCMV promoter sequence and the 3' terminal region of UL49 (Fig. 50b(iv)). From the sizes of these fragments the UL49del1-192 ORF is transcribed in the same direction as U_S10 (Fig. 51b (i)).

vUL49del120-301 DNA contains a BamHI site between the HCMV promoter sequence and the UL49 ORF (Fig. 50b (iii)), which following digestion results in the release of a 1.8Kbp fragment (Fig. 51a, lane 3). This band may be composed of the 1.1Kb BamHI z fragment linked either to the 0.75Kbp IE HCMV promoter sequence, or to the 0.76Kbp fragment containing the UL49 sequences and the poly A sequences. It is therefore not possible to orientate the inserted fragment following BamHI digestion of the viral DNA. There is a band of 4.48Kbp which similarly may be composed of either the 0.76Kbp 5' terminal UL49 sequences and the poly A sequences, or the 0.75Kbp HCMV IE promoter sequence linked to the 3.04Kbp pUL49del120-301 plasmid backbone sequences (equivalent to base pairs 4275-2754 in pUL49del120-301; Fig. 48b) linked to the 0.7Kbp BamHI z fragment.

vUL49del120-192 has BamHI sites flanking the UL49 ORF, and following BamHI digestion, three fragments of 1.85Kbp, 1.0Kbp and 0.7Kbp were released (Fig. 51a, lane 4). These were composed of the 1.1Kb BamHI z fragment linked to the 0.75Kbp IE HCMV promoter sequence, the 0.7Kbp BamHI z fragment linked to the 0.35Kbp late poly A site and the 0.7Kbp UL49 ORF with the internal deletion. The sizes of these fragments indicate that the UL49del120-192 ORF is transcribed in the same direction as U_S8 and 9 (Fig. 51b (ii)).

vUL49del268-301 DNA possesses a BamHI site between the HCMV promoter

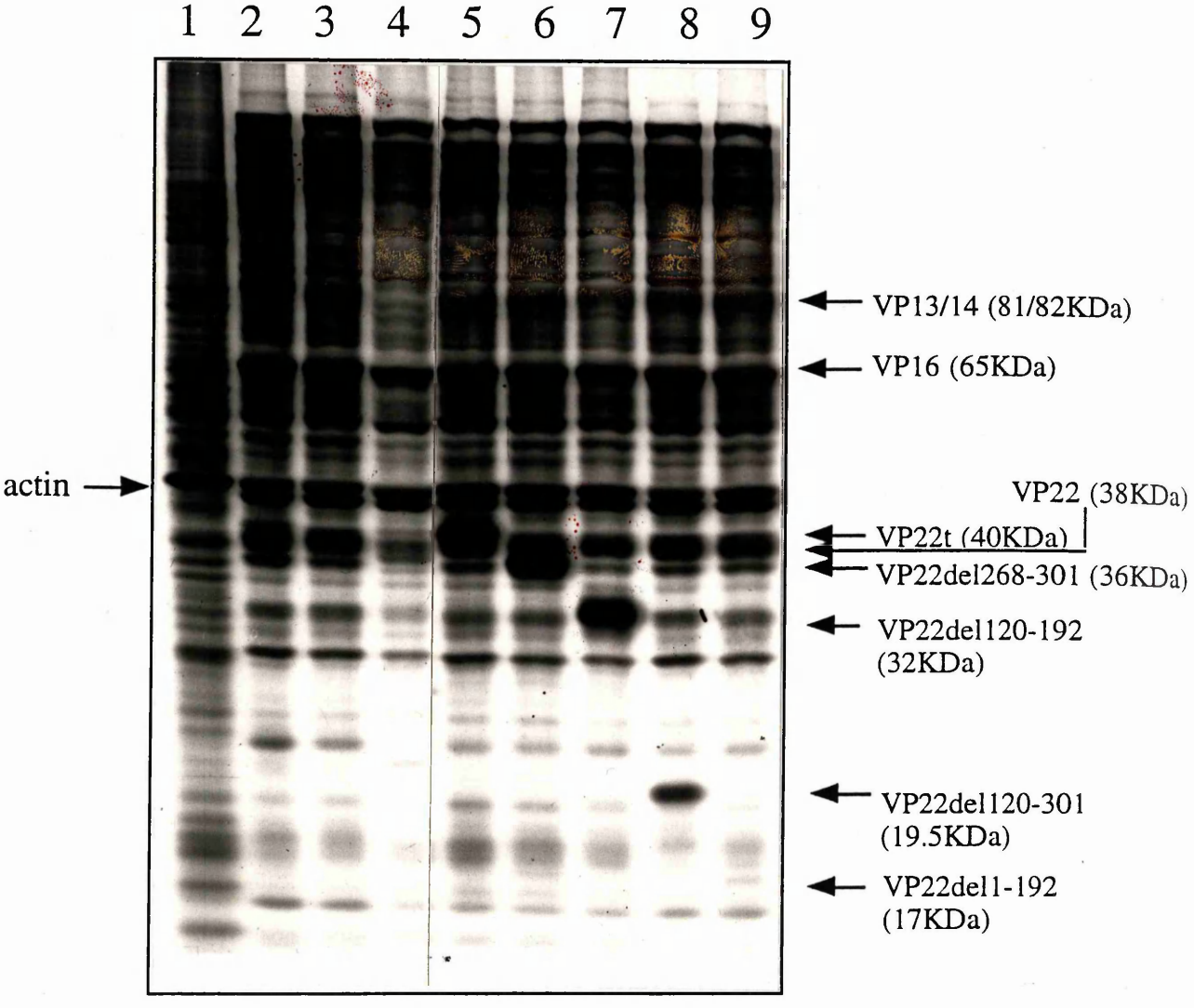


Figure 52. Polypeptides synthesised by vUL49 deletions during an 18 hour infection of BHK cells. Aliquots containing $5x10^5$cpm from ^{35}S-methionine radiolabelled extracts prepared from vUL49del268-301-, vUL49del120-192-, vUL49del120-301- and vUL49del1-192-infected cells were compared with vUL49ep-, 1802-, RUL47Δ2- and mock-infected radiolabelled infected cell extracts. The samples in each lane are as follows: lane 1, mock infected; lane 2, 1802; lane 3, HSV-1 strain 17; lane 4, RUL47Δ2; lane 5, vUL49ep; lane 6, vUL49del268-301; lane 7, vUL49del120-192; lane 8, vUL49del120-301 and lane 9, vUL49del1-192. The UL49-deletion polypeptides are indicated (◄).

plasmid	amino acids encoded	oligos inserted	predicted size of VP22t protein
pUL49ep	1-301	-	33485Da
pUL49del268-301	1-267	(1+2)	29332Da
pUL49del120-192	1-119+193-301	(3+4)	26053Da
pUL49del120-301	1-119	(3+4)	13753Da
pUL49del1-192	193-301	(3+4)	13243Da

Table 10. Details of the plasmid constructs containing the UL49 deletions. The amino acids from the UL49 ORF that are encoded are indicated, as are the oligonucleotides inserted into the UL49 sequence during the construction of these mutants. The predicted sizes of each of the epitope-tagged VP22 deletion mutants are indicated.

sequence and the UL49 ORF which following digestion resulted in the release of two fragments of 1.45Kbp and 2.2Kbp (Fig. 51a, lane 5). These were composed of the 1.1Kbp BamHI z fragment linked to a 1.1Kbp fragment consisting of the UL49 ORF and late poly A sequences, and the 0.7Kbp BamHI z fragment linked to the 0.75Kbp IE HCMV promoter sequence. Therefore from the sizes of these fragments the UL49del268-301 ORF is transcribed in the same direction as U_S10 (Fig. 51b (iii)).

Section 4

Characterisation of the Polypeptides Synthesised by the vUL49del Viruses

To analyse the polypeptides synthesised by the vUL49del virus series, ^{35}S-methionine labelled infected cell extracts were prepared for each of the vUL49del viruses. For comparative purposes 1802, HSV-1 strain 17, vUL49ep, and RUL47Δ2 (Zhang *et al.*, 1991, *Chapter 9; Results, Part 1, Section 5*) infected cell extracts were also prepared. Comparison of the profiles of the vUL49del viruses with those of the control samples revealed the presence of novel species of approximately 36KDa (in the vUL49del268-301 sample; Fig. 52, lane 6), 32KDa (in the vUL49del120-192 sample; Fig. 52, lane 7) and 19.5KDa (in the vUL49del120-301 sample; Fig. 52, lane 8). The apparent molecular weights of these polypeptides are significantly greater than their predicted sizes (see Table 10) which indicates that the proteins made by the vUL49del viruses are post-translationally processed.

In the extract prepared from vUL49del1-192 infected cells, no novel abundant species was found. A minor polypeptide which appears to be absent in other infected cell extracts is indicated in Fig. 52; this species may represent the tagged VP22 protein made by vUL49del1-192. However, it is possible that the tagged protein co-migrates with other low molecular weight polypeptides and is therefore obscured.

To verify that the novel species produced by vUL49del viruses corresponded to epitope tagged protein, Western blot analysis was performed on the ^{35}S-methionine labelled extracts used in Fig. 52. Western blot analysis was also performed on the vUL49del1-192-infected cell extract to determine whether this virus produced an epitope-tagged protein. Following separation by electrophoresis and transfer to nitrocellulose, the membrane was incubated with monoclonal antibody pp65. As a positive control, and to act as a size marker,

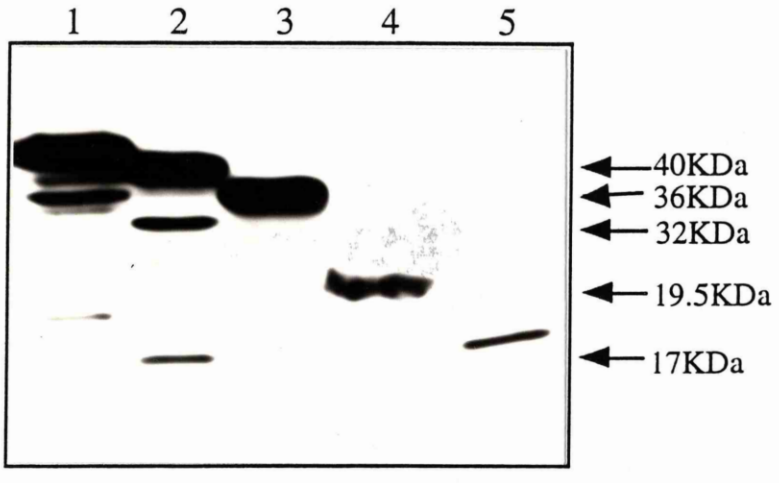


Figure 53. Western blot analysis of vUL49del infected cell extracts. Approximately $2x10^4$ cell equivalents were electrophoresed on a 14% polyacrylamide gel and then transferred to nitrocellulose membrane. The membrane was probed with pp65 monoclonal antibody (1:2000 dilution). In lane 1, vUL49ep; lane 2, vUL49del268-301; lane 3, vUL49del120-192; lane 4,vUL49del120-301 and lane 5, vUL49del1-192. The sizes of the polypeptides detected are indicated.

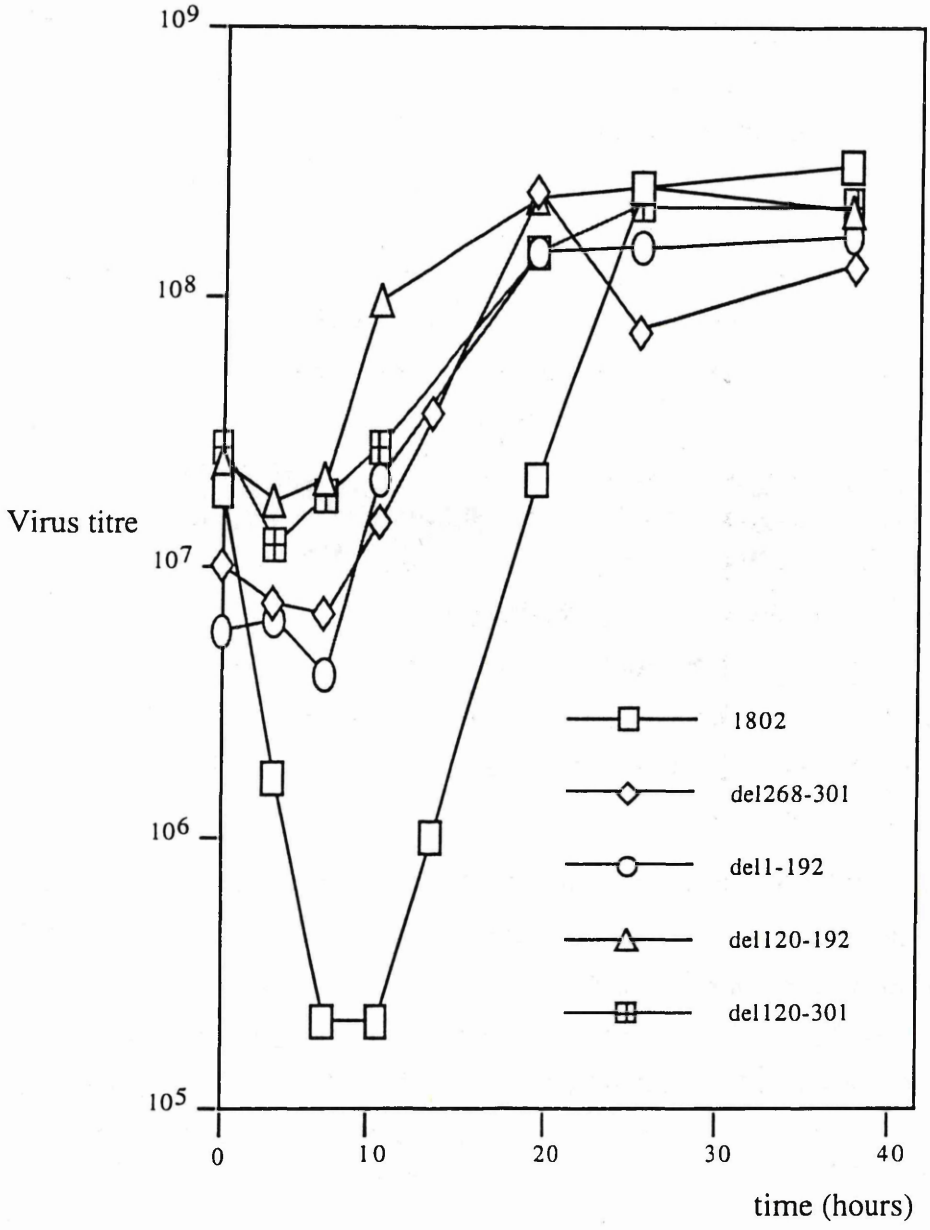


Figure 54. Growth characteristics of vUL49del virus series compared with the parent virus 1802. 35mm dishes containing approximately $2x10^6$ BHK cells were infected at a moi of 5 pfu per cell and harvested at various time points ranging between 0 and 36 hours after infection. The total amount of virus present at each time point was determined by titration.

the vUL49ep-infected cell extract was included on the gel. In the vUL49del268-301 and vUL49del120-192-infected cell extracts, the antibody recognised abundant species of approximately 36KDa (Fig. 53, lane 2) and 32KDa (Fig. 53, lane 3) respectively.

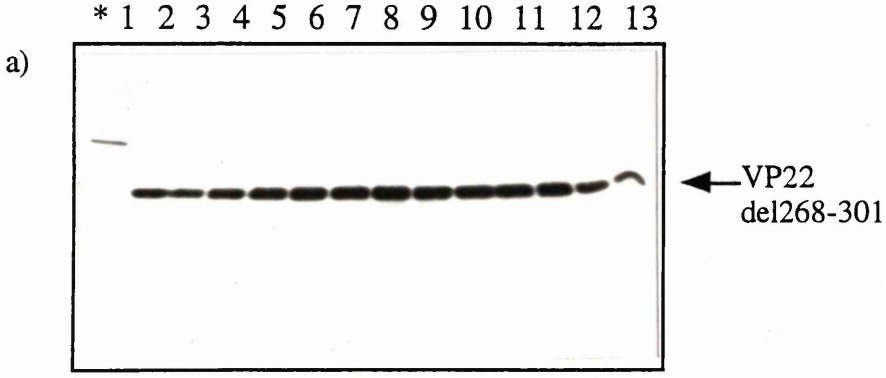
A polypeptide of approximately 19.5KDa was detected in the vUL49del120-301 sample (Fig. 53, lane 4) which was consistently granular in appearance in Western blot analysis. This species was of reduced intensity in comparison to the epitope-tagged forms of protein present in the other virus-infected cell extracts (Fig. 53, compare lane 4 with lanes 1, 2 & 3). The polypeptides detected by the antibody in the vUL49del268-301, vUL49del120-192 and vUL49del120-301 samples correspond to the sizes of the novel proteins identified in the profiles described in Fig.52. In the vUL49del1-192 infected cell extract, a band of approximately 17KDa was detected (Fig. 53, lane 5). This band was of lower intensity than that present in the vUL49del120-301 sample. From these data, it was concluded that the predicted epitope-tagged forms of VP22 were synthesised by the deletion mutant viruses. Both the polypeptide profile analysis and the Western blot data indicated that the quantity of epitope-tagged protein produced by vUL49del268-301 and vUL49del120-192 were similar to the abundance of VP22t produced by vUL49ep. However, vUL49del120-301 and vUL49del1-192 made consistently less epitope-tagged protein compared to vUL49ep. The apparent decrease in the levels of epitope-tagged protein in these infected cell extracts may have been due to a reduction in the stability of these proteins.

Section 5

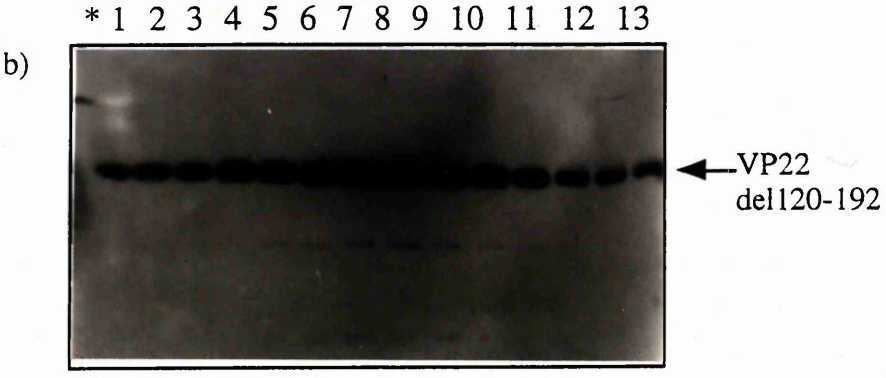
Growth Characteristics of the vUL49del Virus Series

In order to determine whether the growth of each of the vUL49del virus series was affected by the insertion of a second epitope-tagged partial copy of UL49, the growth characteristics of the vUL49del viruses were compared to those of the parent, 1802. The gradients of the slopes during the exponential growth phase (between 6 and 24 hours) for each vUL49del virus mutant suggested that the growth rates were reduced compared to 1802 (Fig. 54). However, for each of the vUL49del virus mutants the titre during the eclipse phase did not fall as significantly as did that for 1802. The final yields for each vUL49del mutant were similar and were slightly less than that for 1802. The data obtained for these viruses were similar to those for vUL49ep (*Chapter 8; Results, Part 1, Section 5*) and suggested that insertion of the mutant epitope-tagged copies of UL49 into virus 1802 did not have a significant effect on the growth of the resulting viruses.

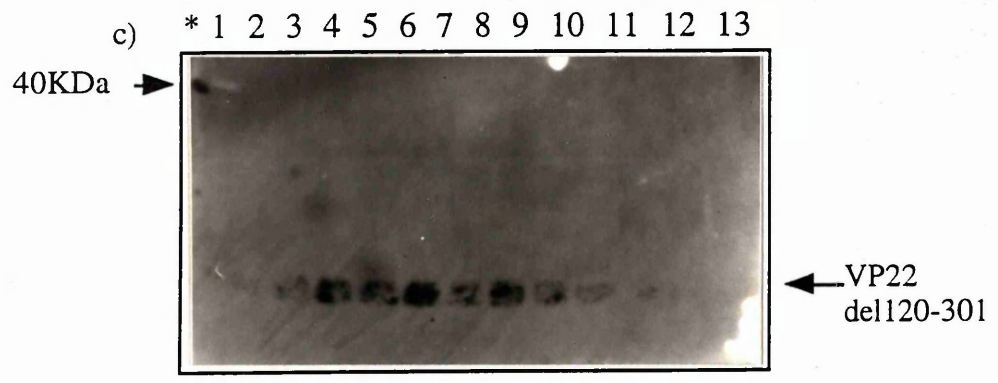
vUL49del268-301



vUL49del120-192



vUL49del120-301



vUL49del1-192

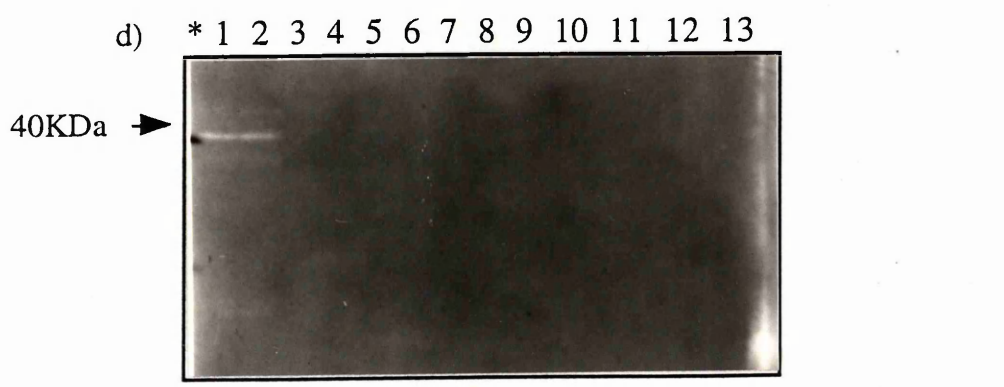


Figure 55. Western blot analysis across gradients containing each of the vUL49del virus preparations. Material was collected in 13x700µl aliquots from 2cm below the virion band to the top of the tube in 13ml, ficoll gradients that were loaded with material from cells infected with each of the vUL49ins virus series. Following electrophoresis of 15µl of each sample on 14% polyacrylamide gels, the polypeptides were transferred to nitroclellulose membranes and each was incubated with pp65 monoclonal antibody. The bands indicated correspond to the epitope-tagged, mutated froms of VP22. In lane * vUL49ep infected cell extracts were included as positive controls and size markers.

Section 6

Incorporation of Epitope-Tagged UL49 Deletions into Virions and Light Particles

Analysis of the polypeptides produced in cells infected with the vUL49del viruses had shown that they synthesised tagged forms of partially deleted VP22. To determine whether these polypeptides were incorporated into virus particles Western blot analysis was performed on fractions collected from across ficoll gradients containing the material released from infected cells. This material was prepared from four roller bottles of BHK cells that had been infected with each virus at a moi of 1 in 300 and grown at 31°C for 4 days. Following separation by electrophoresis, the ficoll purified virus polypeptides were transferred to nitrocellulose membranes. Incubation of each of the membranes with pp65 monoclonal antibody, revealed bands in vUL49del268-301, vUL49del120-192 and vUL49del120-301 virus gradients that were distributed throughout the regions where virions and light particles migrate (Fig. 55a, b & c), as described for tegument proteins such as VP16 (*Chapter 8; Results, Part 2, Section 5*). It was concluded therefore that for these virus mutants each of the VP22t inserts were assumed to have been incorporated into virions and light particles. The intensity of the bands detected across the vUL49del120-301 gradient were reduced in comparison to the gradients containing vUL49del268-301 and vUL49del120-192, and therefore suggested that the level of incorporation of epitope-tagged VP22del120-301 was not as high as for either VP22del268-301 or VP22del120-192. Nothing was detected in the gradient containing vUL49del1-192 cell released material (Fig. 55d) and in the absence of experiments to determine the level of sensitivity no conclusion can be made as to whether epitope-tagged VP22 minus amino acids 1-192 was incorporated into vUL49del1-192 virions and light particles.

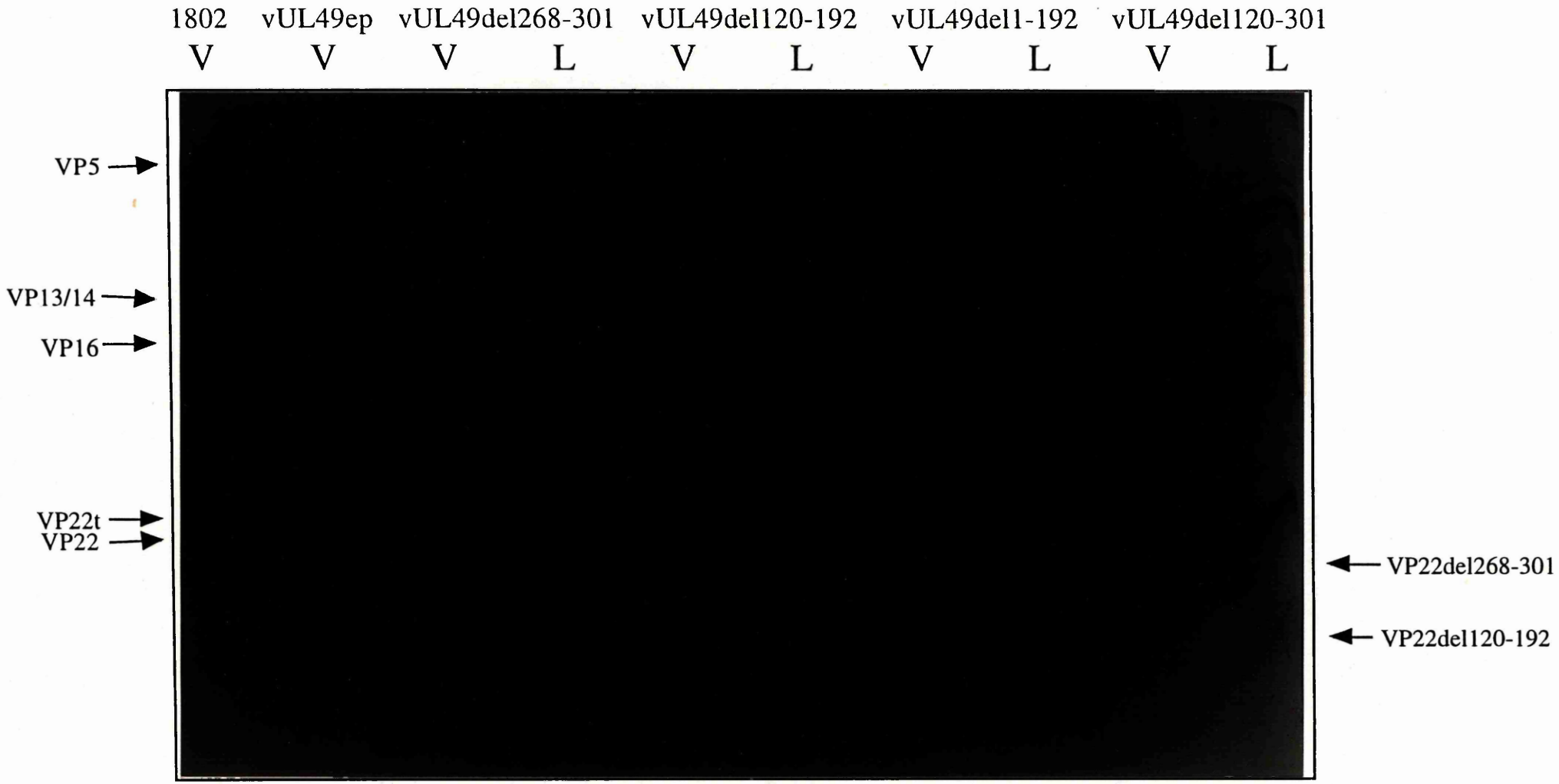

1802
V
vUL49ep
V
vUL49del268-301
V
L
vUL49del120-192
V
L
vUL49del1-192
V
L
vUL49del120-301
V
L
VP5
VP13/14
VP16
VP22t
VP22
VP22del268-301
VP22del120-192

Figure 56. Polypeptide profiles of vUL49del virions and light particles. Approximately $3x10^9$ vUL49del268-301, vUL49del120-192, vUL49del120-301 and vUL49del1-192 ficoll purified virions and light particles were compared to vUL49ep and 1802 virions. Following electrophoresis on a 12% polyacrylamide gel, the gel was stained with Coomassie Brilliant Blue. The samples in each lane are as shown. The major structural polypeptides are indicated as are the bands corresponding to VP22del proteins.

		Polypeptides analysed					
		VP5[c]	gB, gC, gH[c]	VP13/14[c]	VP16[b]	VP22[c]	VP22t
predicted copy numbers (average)[b]		960	1750	1846	1647	?	
1802[a]	V	895	1307	1691	1647	2872	
	L		1828	1730	1647	4349	
vUL49ep[a]	V	477	1177	992	1647	7745	
	L		1462	1076	1647	8668	
vUL49del 268-301	V	596	1698	673	1647	1312	6113
	L		2104	843	1647	2487	6289
vUL49del 120-192	V	775	1795	1538	1647	2515	3787
	L		1439	1705	1647	3670	4537
vUL49del 1-192	V	944	2342	1531	1647	2987	
	L		1983	2195	1647	4167	
vUL49del 120-301	V	1180	1534	1414	1647	3755	
	L		1305	1808	1647	2748	

Table 11. The copy numbers of VP5, gB(gC, gH), VP13/14, VP22 and where possible VP22t were estimated following the excision of these bands from the Coomassie Brilliant blue stained gel shown in Fig. 56. The protein concentration was estimated following the elution of the Coomassie Brilliant blue stain in each band in 25% piperidine solution followed by spectrophotometric analysis at 605nm. The copy number for each polypeptide was calculated assuming that the copy number of VP16 remains constant in each virus. .

a vUL49ep and 1802 virus particles corresponding to preparation 1 in *Chapter 9; Results,* Table 6.

b average number of copies of major structural species was taken from Zhang & McKnight (1993)

c number of major structural species was calculated relative to the estimated copy number of VP16

Section 7

Polypeptide Profiles of vUL49del Virus Series Virions and Light Particles

To further characterise the virus particles made by the vUL49del virus mutants, the polypeptide profiles of their virions and light particles were compared to those present in vUL49ep and 1802 virions (Fig. 56). Novel species of 36KDa and 32KDa were found in the vUL49del268-301 and vUL49del120-192 samples; these correspond in size to the tagged VP22 proteins made by these viruses. The 36KDa protein in vUL49del268-301 virus particles was the most abundant species incorporated and appeared to be present in similar amounts to the VP22t protein in vUL49ep particles. By contrast, there was a marked reduction in abundance of the tagged protein in vUL49del120-192 particles, however the protein did remain clearly visible on stained gels. The polypeptide profiles of vUL49del1-192 and vUL49del120-301 particles were indistinguishable from those of 1802.

The relative abundances of the major proteins in the samples in Fig. 56 were examined by excising bands from the gel and measuring the amount of stain that could be eluted from each band (Table 11). This revealed that the levels of untagged full-length VP22 were reduced by approximately 50% in vUL49del268-301 virus particles as compared to those of 1802. The amount of tagged protein as compared to full-length VP22 in these particles was 4.5-fold greater in virions and 2.5-fold greater in light particles. Interestingly, combining the estimated copy numbers of tagged and untagged proteins indicates that the total amount of VP22 incorporated into vUL49del268-301 particles is very similar to that for vUL49ep particles (Table 11). In vUL49del120-192 particles, the amount of tagged protein found was reduced by about 40% as compared to the tagged protein in vUL49del268-301 virus particles. This was mirrored by an approximately equivalent rise in the abundance of untagged VP22 such that vUL49del120-192 particles contained similar levels of VP22 to 1802 virus particles. Combining the copy numbers of tagged and untagged VP22 for vUL49del120-192 suggested that the amount of protein incorporated in particles made by this mutant were slightly reduced as compared to vUL49ep and vUL49del268-301 but markedly greater than in 1802. For both vUL49del1-192 and vUL49del120-301 virus particles the abundance of untagged VP22 protein was similar to that found in 1802 virions and light particles. In addition to the increase in VP22 abundance, analysis of vUL49ep virus particles had shown a marked decrease in the abundance of VP13/14 (*Chapter 8;*

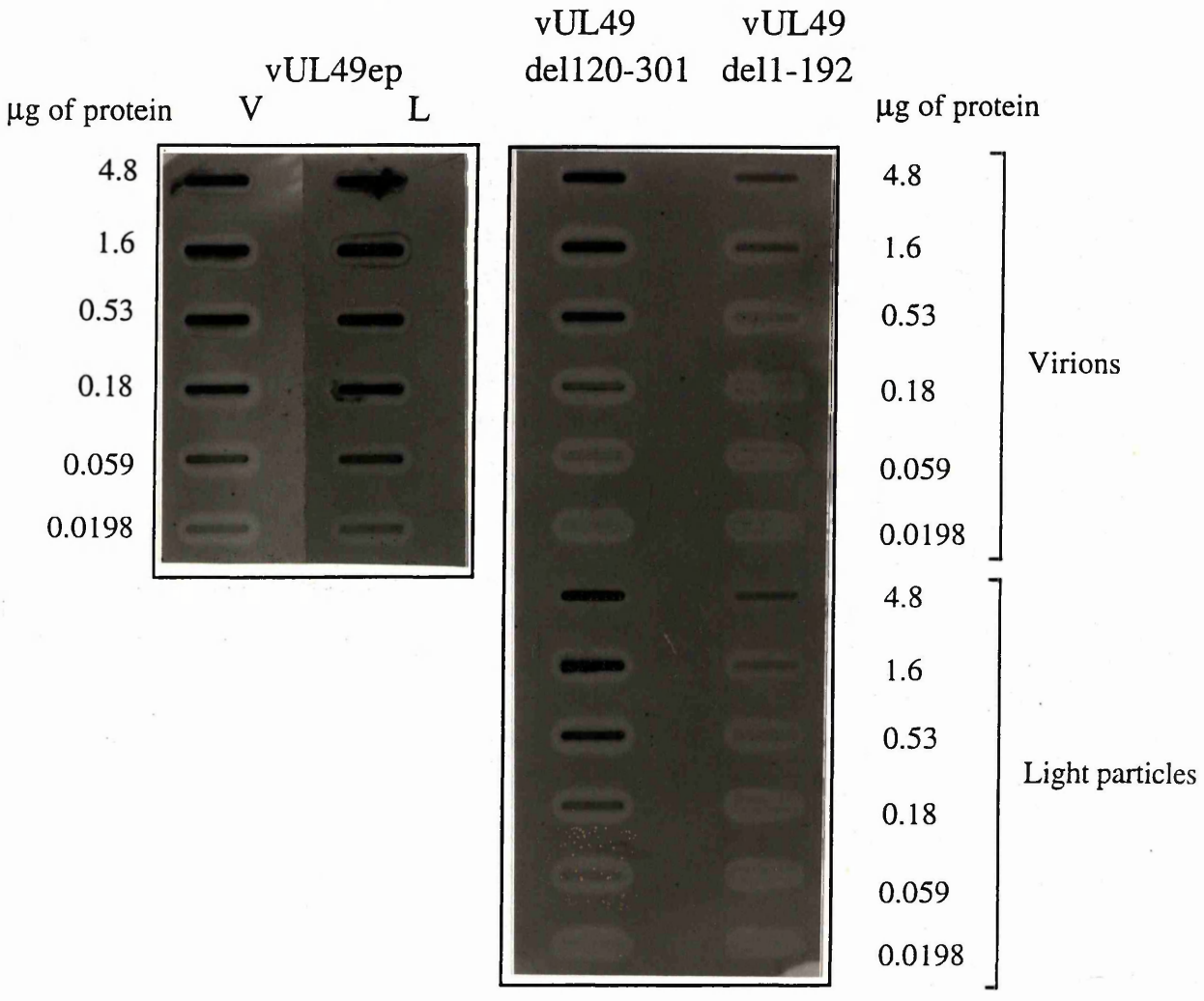


Figure 57. The relative efficiency of epitope-tagged VP22 to enter vUL49del120-301 and vUL49del1-192 virus particles was estimated by Western blot analysis. Following Bradford assay of the purified stock preparations of vUL49ep, vUL49del120-301 and vUL49del1-192 virions and light particles, approximately 12µg (+/-4.3µg) of viral protein was diluted in a total volume of 400ml of TBS + 0.1% NP40, and 3-fold dilutions of these samples were loaded onto nitrocellulose membrane using a slot-blot apparatus. The amount of protein in each slot is shown. The membrane was probed with pp65 monoclonal antibody.

Results, Part 1, Section 8) Comparison of the relative amounts of VP13/14 in vUL49del268-301 had reduced quantities of these proteins which were similar to the estimates for vUL49ep, while particles made by the other 3 mutants contained quantities similar to those for 1802.

For vUL49del120-301, Western blot analysis had suggested that the tegument protein was incorporated into virus particles (Fig. 55c) but was not detected in the Coomassie Brilliant blue stained gels. In addition, there was no evidence that the tagged protein made by vUL49del1-192 did incorporate (Fig. 55d). To try to provide some estimate of the relative efficiency of the tagged proteins produced by these viruses to enter virus particles, 3-fold dilutions of virus particles were blotted onto nitrocellulose membranes and probed with pp65 monoclonal antibody. This showed that VP22t could be detected in 0.02μg of vUL49ep virions and light particles while epitope-tagged VP22 in vUL49del120-301 virions and light particles was detected in 0.18μg of viral protein (Fig. 57), suggesting a reduction of 9-fold in the level of incorporation of these proteins in comparison to vUL49ep. The faint detection of epitope-tagged protein in 4.8μg of vUL49del1-192 virions and light particles (Fig. 57) may have been a result of the detection of contaminating cellular material migrating throught the gradient, or alternatively it could be concluded that vUL49del1-192 virus particles contained minimal amounts of VP22del1-192.

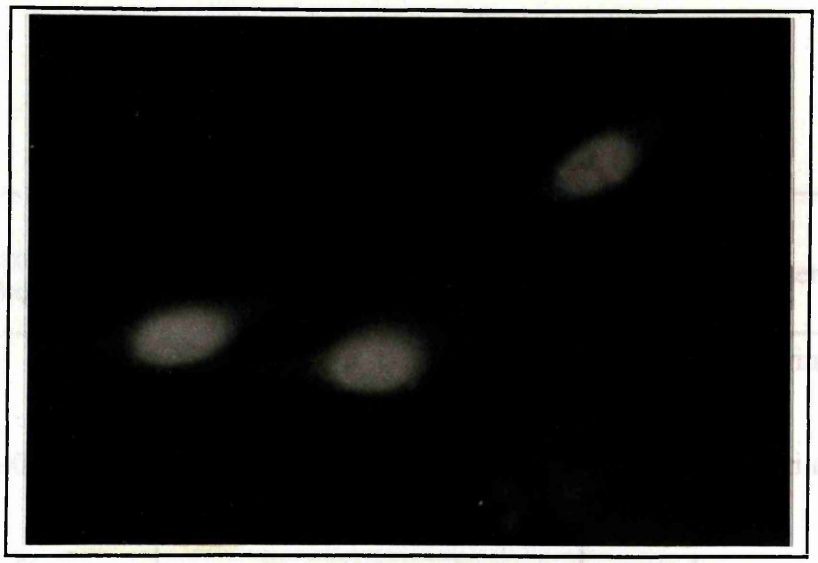
plasmid transfected	location of fluorescence		staining pattern
	nucleus	cytoplasm	
pUL49ep	++	+	nuclear, perinuclear & network
pUL49ins60	++	+	nuclear, perinuclear & network
pUL49ins159	++	+	nuclear, perinuclear & network
pUL49ins194	-	+++	network & diffuse
pUL49ins267	+	++	nuclear, perinuclear & network
pUL49del268-301	-	++++	network
pUL49del120-192	-	+++	aggregates
pUL49del120-301	-	+++	diffuse
pUL49del1-192	-	+++	diffuse

Table 12. Summary of results obtained following the tranfection of plasmid DNA into BHK cells and staining with pp65 monoclonal antibody.

- \- 0% of cells exhibited this patten of fluorescence
- \+ 15% of cells exhibited this patten of fluorescence
- ++ 35% of cells exhibited this patten of fluorescence
- +++ 55% of cells exhibited this patten of fluorescence
- ++++ 70% of cells exhibited this patten of fluorescence

Examples of staining patterns are illustrated i) edge of nucleus: Fig. 58a)
ii) network: Fig. 58b)
iii) aggregates: Fig. 60
iv) diffuse: Fig. 60 c) & d)

a)



b)

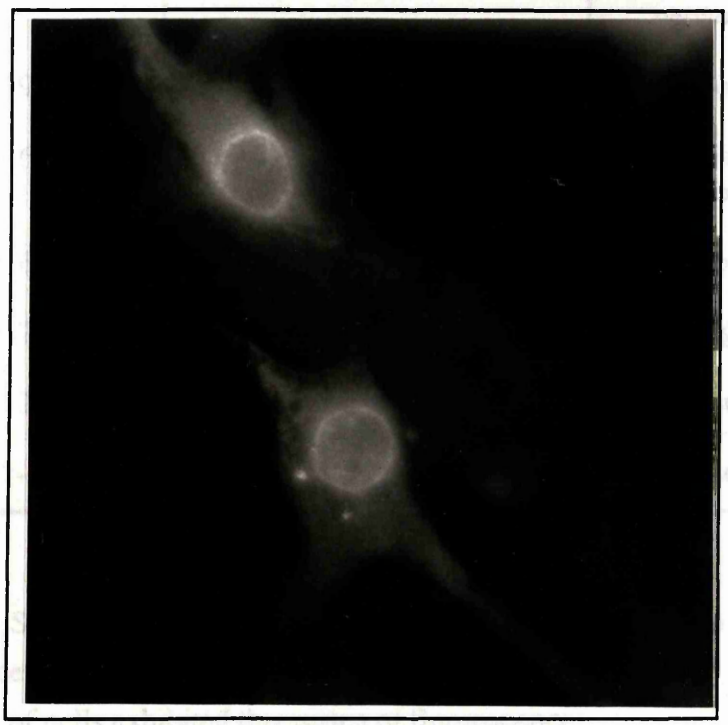
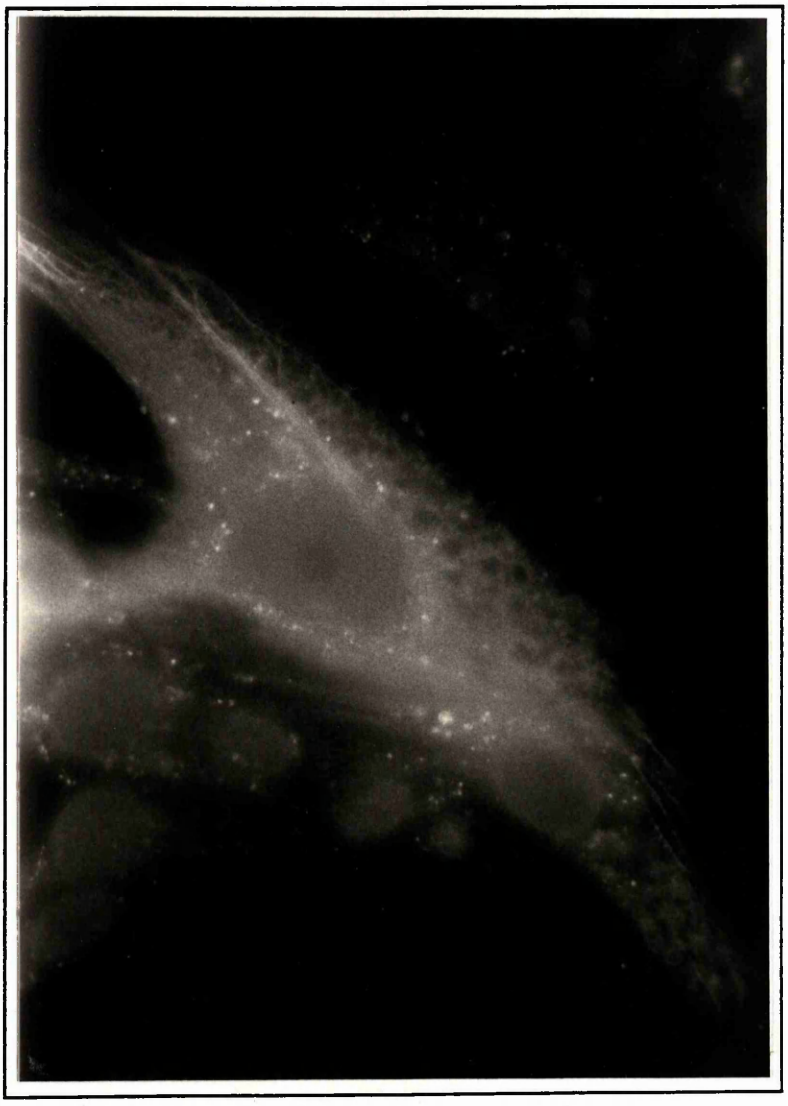


Figure 58. Immunofluorescent staining of pUL49ep transfected BHK cells using pp65 monoclonal antibody. BHK cells were transfected with 0.5μg of plasmid DNA and were incubated at 37°C for 18 hours. Cells were stained with a 1:300 dilution of pp65 monoclonal antibody followed by a 1:100 dilution of FITC conjugated goat anti-mouse antibody to detect the epitope tag sequence. a) Nuclear fluorescence , b) perinuclear fluorescence , c) cytoplasmic network, and the presence of VP22 in adjacent cells.

c)



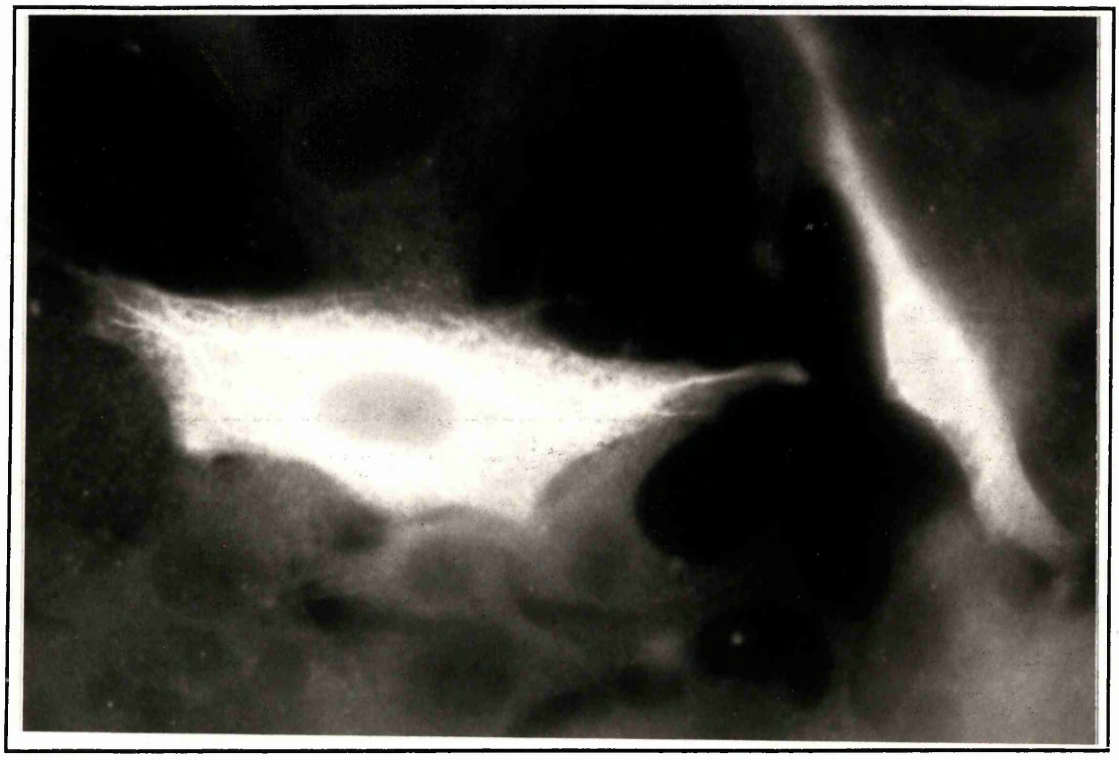


Figure 59. Immunofluorescent staining of pUL49ins194 transfected BHK cells using pp65 monoclonal antibody. BHK cells were transfected with 0.5μg of plasmid DNA and were incubated at 37°C for 18 hours. Cells were stained with a 1:300 dilution of pp65 monoclonal antibody followed by a 1:100 dilution of FITC conjugated goat anti-mouse antibody to detect the epitope tag sequence. The filamentous cytoplasmic network is evident in the large cell on the right hand side of the photograph and a diffuse cytpolasmic flourescence is shown in the cell on the left.

Part 3

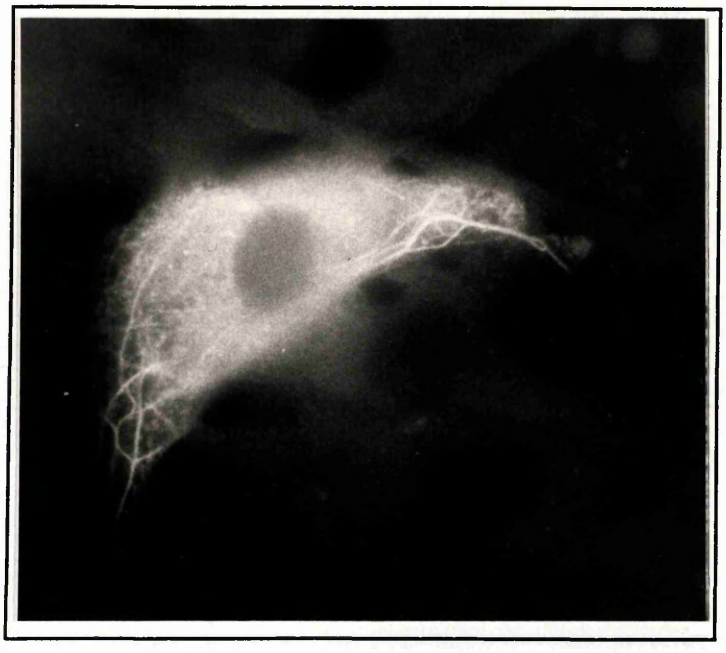
Intracellular Location of the Epitope-Tagged VP22 Mutants

To further characterise the epitope-tagged UL49 insertion and deletion mutants, the intracellular distribution of each of the epitope-tagged VP22 proteins was investigated by indirect immunofluorescence. Coverslips with sub-confluent BHK cells were infected at a moi of 0.1 pfu per cell with each of the vUL49ins and vUL49del virus mutants, and following incubation at 31°C for 3, 6, 9, 12 and 15 hours the coverslips were harvested and processed as described in *Chapter 6; Methods, Section 4.6*. Repeated attempts failed to produce intracellular patterns of fluorescence following virus infection. It was therefore decided to investigate the intracellular location of the epitope-tagged VP22 proteins following plasmid transfection. BHK cells grown on 13mm coverslips were transfected with plasmid DNA (*Chapter 6; Methods, Section 1.9*) and after overnight incubation (18 hours) the coverslips were harvested and processed. The plasmids transfected into BHK cells were pUL49ep, the pUL49ins plasmid series and the pUL49del plasmid series. The results are summarised in Table 12.

Full length epitope-tagged VP22 synthesised in cells transfected with pUL49ep typically exhibited a nuclear localisation (Fig. 58a), however two distinct patterns of cytoplasmic fluorescence were also seen. These were a perinuclear pattern of fluorescence (Fig. 58b) and a distinct network pattern that had the appearance of a cytoskeletal architecture (Fig. 58c). In addition, a speckled pattern of fluorescence that was also detected in adjacent cells was also evident. This pattern of fluorescence suggested that VP22 may move between cells, however this property of VP22 was not further studied. The intracellular patterns of fluorescence for the UL49 insertion mutants synthesised in cells transfected with pUL49ins60, pUL49ins159 and pUL49ins268 were the same as those for full length VP22 (data not shown).

In contrast to pUL49ep, pUL49ins194 produced exclusively cytoplasmic patterns of fluorescence. 70% of the fluorescing cells examined exhibited a network pattern similar to that seen following transfection of pUL49ep, while the remainder of the cells had a diffuse cytoplasmic pattern of fluorescence. Fig. 59 shows one cell exhibiting filamentous network

a)



b)

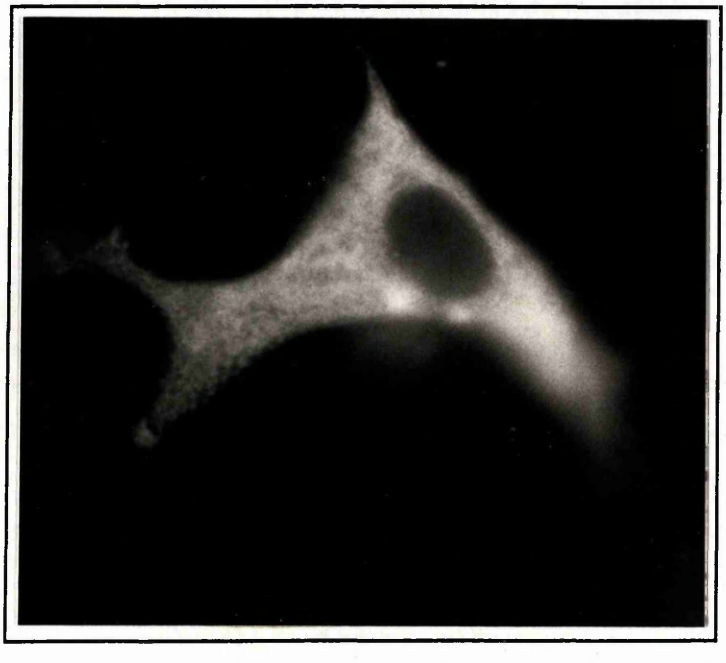
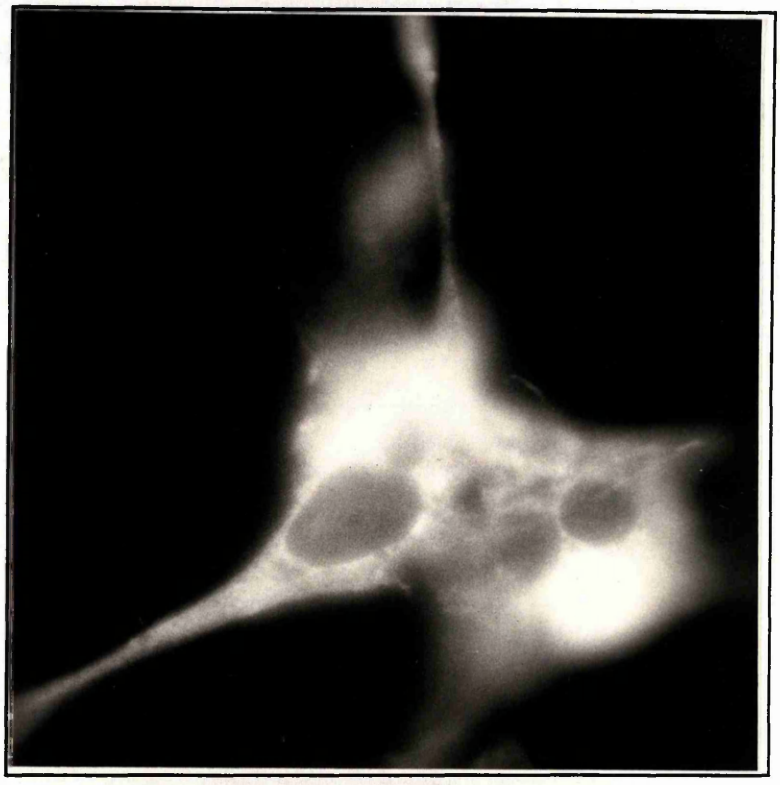
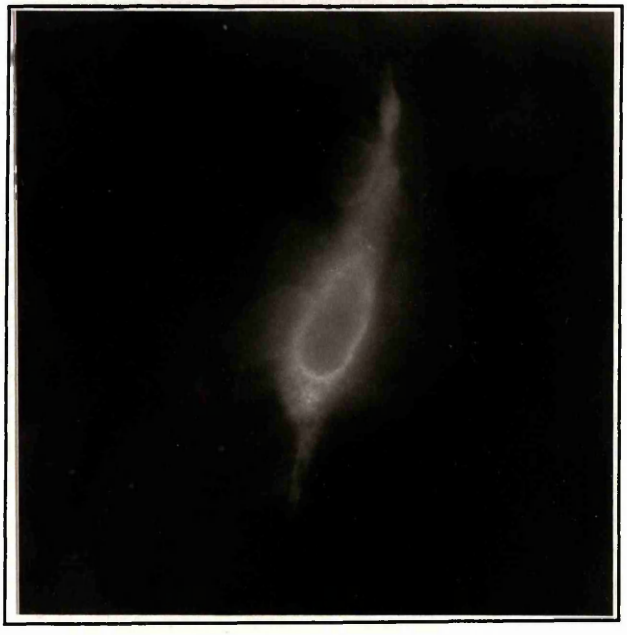


Figure 60. Immunofluorescent staining of a) pUL49del268-301, b) pUL49del120-192, c) pUL49del1-192, d) pUL49del120-301 transfected BHK cells using pp65 monoclonal antibody. BHK cells were transfected with 0.5μg of plasmid DNA and were incubated at 37°C for 18 hours. To detect the epitope tag sequence cells were incubated with a 1:300 dilution of pp65 monoclonal antibody followed by a 1:100 dilution of FITC conjugated goat anti-mouse antibody.

c)

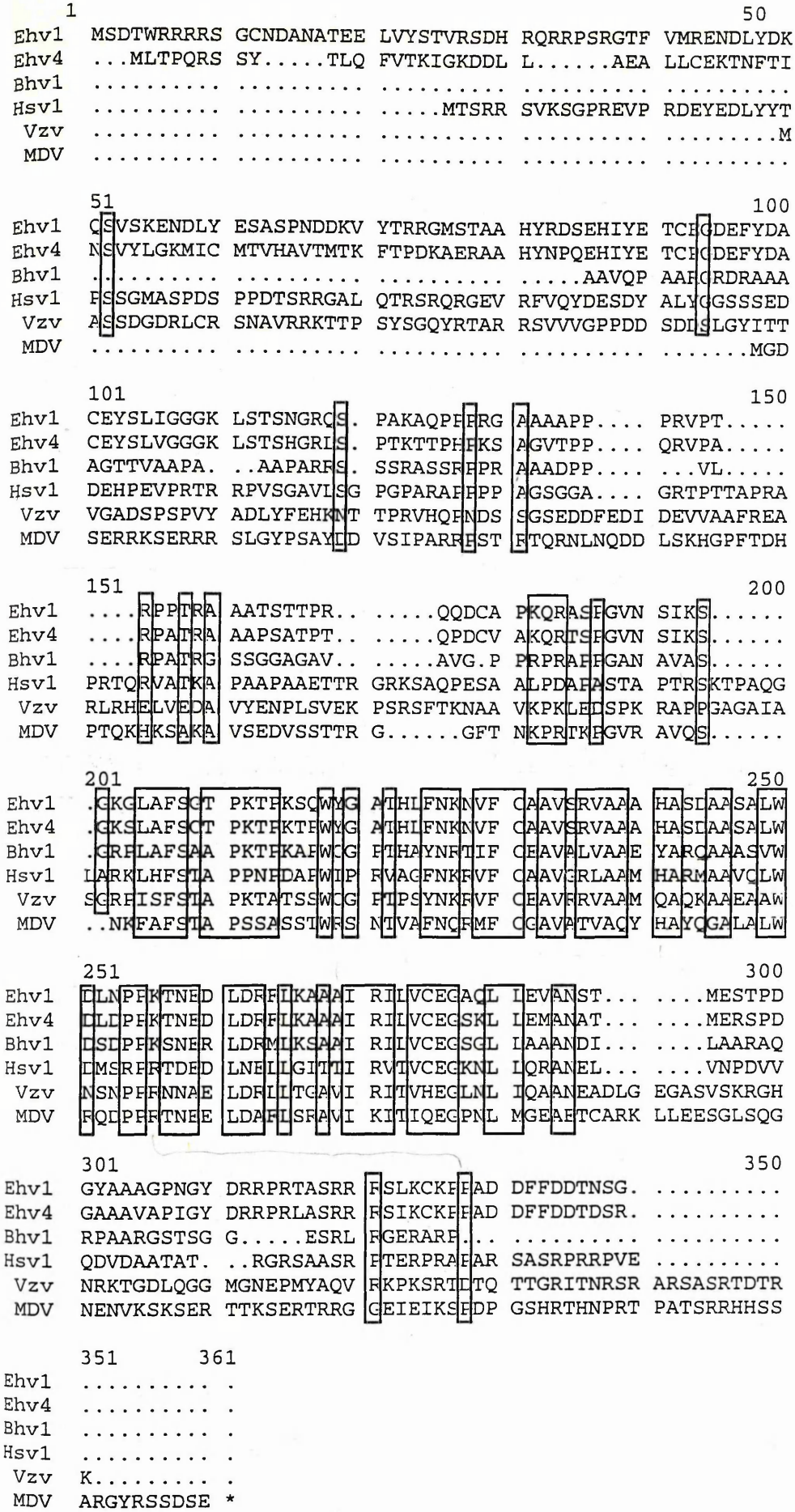


d)



structure and another with a diffuse cytoplasmic fluorescence. The epitope-tagged versions of VP22 produced in cells transfected with each of the pUL49del plasmids all had exclusively cytoplasmic patterns of fluorescence. Interestingly, cells transfected with pUL49del268-301 only formed cytoplasmic filamentous network structures (Fig. 60a). pUL49del120-192 only formed cytoplasmic aggregates that were distinct from the network structures (Fig. 60b). The plasmids containing the large N- and C-terminal UL49 deletions pUL49del1-192 and pUL49del120-301, gave only diffuse cytoplasmic patterns of fluorescence (Fig. 60c & 60d).

Figure 61. Polypeptides were selected from EHV-1, EHV-2, BHV, VZV and MDV that had significant levels of homology with HSV-1 VP22. From the sequence alignment, conservation of four out of the six residues was considered to be a significantly high level of homology, and the region was classified as being highly conserved. The highly conserved regions are boxed.



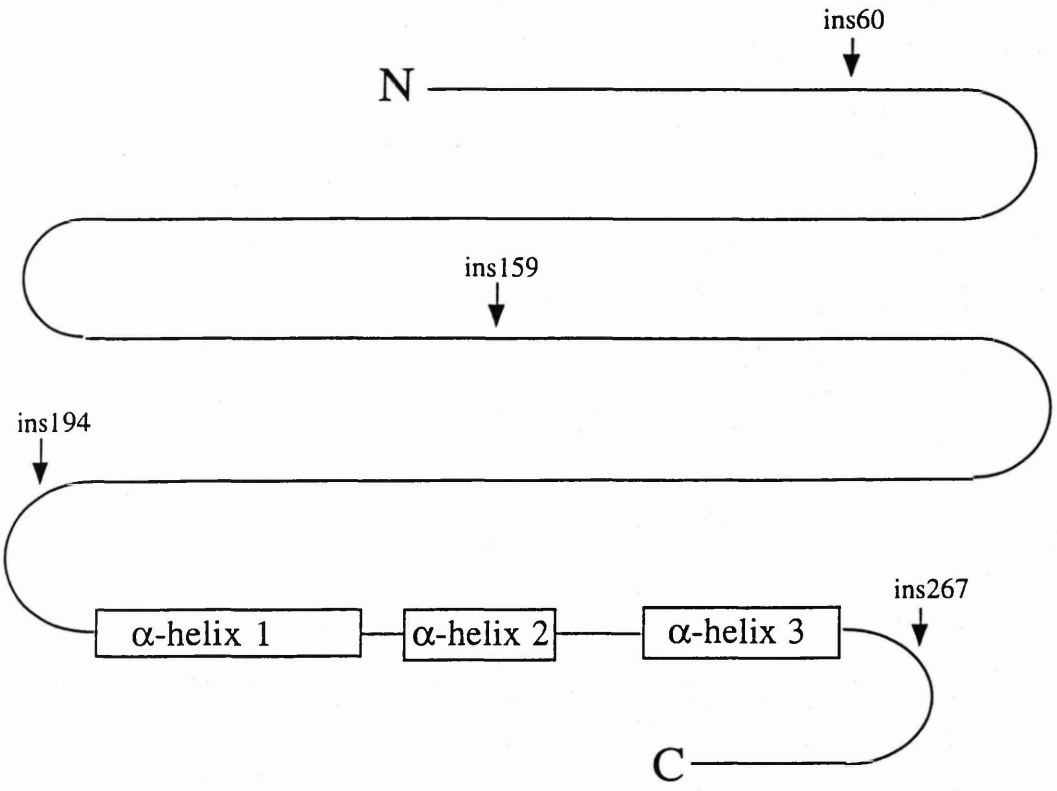


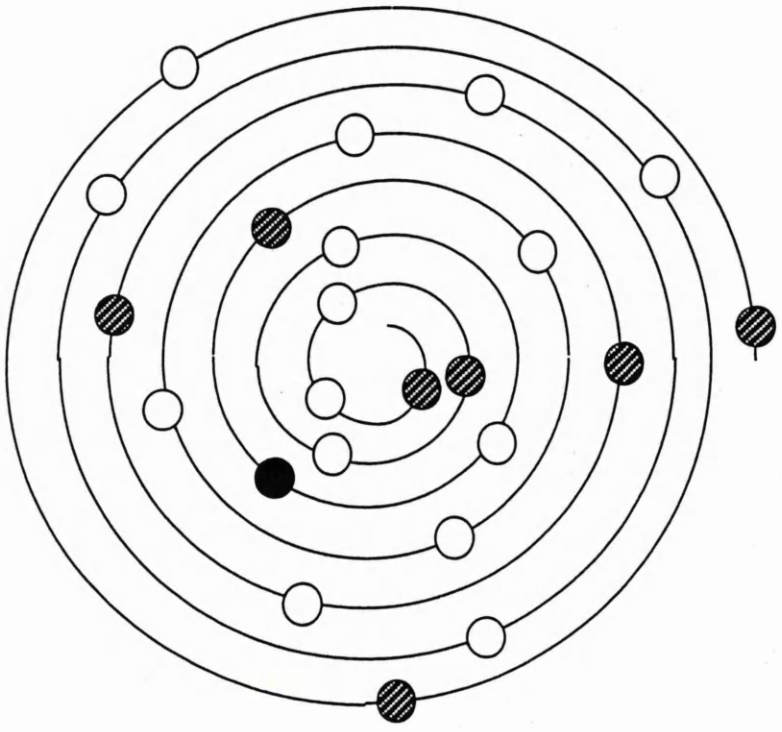
Figure 62. Diagramatic representation of the predicted secondary structure of VP22. The three predicted α-helices are indicated, as are the positions of the oligonucleotide insertions.

Discussion

This chapter describes the construction and characterisation of a series of HSV-1 recombinant viruses that contained mutated versions of epitope-tagged VP22. It was hoped that these inserted oligonucleotides would affect the ability of VP22 to incorporate into the tegument which would enable the identification of regions within the protein coding sequence important for the incorporation of the protein into the tegument. In the following discussion, the effects of these alterations to the VP22 protein will be discussed in the context of the predicted secondary structure of VP22. The structure of VP22 was predicted using Homology Derived Secondary Structure of Proteins (Version 1.0 1991) which derives the secondary structure of proteins by a system of neural networks with an accuracy of (up to) 70%. The input is a multiple sequence alignment, also completed by the program (Rost & Sander, 1993a, 1993b; Rost & Sander, 1994). The polypeptides selected by the program that had significant levels of homology with HSV-1 VP22 were sequences from BHV-1, EHV-1, EHV-4, VZV and MDV. Examination of the predicted secondary structure of VP22 (Fig. 62) and the sequence alignment data (Fig. 61), show that from residues 1-200 VP22 is predicted to consist of a random coil structure, with little conservation between other polypeptides selected for the sequence alignment. From residues 198 to 258, there are three predicted α-helical structures (Fig. 62), and this region is also highly conserved (Fig. 61). The largest of these α-helices, from residues 201-220, is almost entirely composed of hydrophobic residues, which might suggest that it may be inserted into a membrane, or that it forms an interface in a protein/protein interaction (Fig. 63a). Following an eight residue sequence with no structure, a second α-helix composed of eight residues is predicted. This helix is amphipathic, with polar residues located on one side of the helix and hydrophobic residues on the other (Fig. 63b). A short region bridges the gap between this second α-helix and a third. The third helix is eleven residues in length and is polar at the N-terminal end, and hydrophobic at the C-terminal end (Fig. 63c). Finally, the C-terminal 40 residues have lower homology and no predicted secondary structure.

The characteristics of mutant viruses, vUL49ins60, vUL49ins159 and vUL49ins267 were essentially indistinguishable from vUL49ep. They each synthesised approximately 5-fold higher levels of VP22 than wild type virus in infected cells, and the elevated levels of expression of VP22 resulted in an approximately 3-fold increase in the amount of VP22 present in virions and light particles. These insertions were all located in regions of no predicted secondary structure, as shown in Fig. 62, and the results suggested that disruption of these sites did not affect the ability of the epitope-tagged proteins to incorporate into virus

a)



α-helix 1

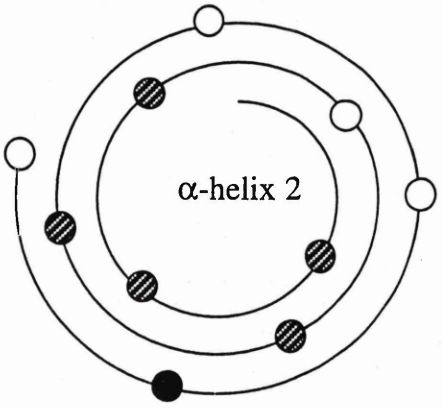
R - V - F - C - A - A - V - G - R - L - A - A - M - H - A - R - M -
A - A - V - L - Q - L - W

Figure 63. Helical wheels of each predicted α-helix in VP22. Amino acid residues are plotted every 100° consecutively around the wheel.

The following code is used:

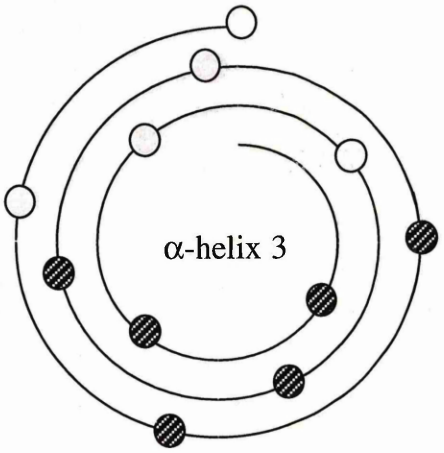
- amino acids with a hydrophobic side chain
- amino acids with polar or charged side chains
- Glycine residue

b)



D - E - D - L - N - E - L - L - G - I

c)



K - N - L - L - Q - R - A - N - E - L - V

particles. In addition, the polypeptide profile of vUL49del268-301 showed that deletion of the C-terminal 34 amino acids of VP22 also had no discernible effect on the ability of the epitope-tagged protein to be incorporated into virus particles. Similar to the three vUL49ins viruses described above, the region of VP22 deleted during the construction of vUL49del268-301 contained no predicted secondary structure and low levels of homology.

The epitope-tagged UL49 deletion present in vUL49del120-192 appears to be synthesised in infected cells to equivalent levels as the epitope-tagged VP22 protein in vUL49ep however, the virion and light particle polypeptide profiles of vUL49del120-192, and the quantitation results, suggest that there is a reduction of approximately 4-fold in the level of incorporation of this protein in virus particles. The region deleted contains no predicted secondary structure (Fig. 62), but is in close proximity to the highly conserved large hydrophobic α-helix. The majority of residues within the region of no predicted secondary structure between positions 192-200 are capable of being components of α-helices, as they are each present within the α-helix between residues 200-220; therefore the start of the large hydrophobic α-helix may be closer to residue 192 than predicted by the program and this deletion may result in the removal of part of the α-helix. In addition, the deletion also brings the proline residue from position 119 into close proximity of the predicted start of this α-helix, which may provide some steric hindrance to the α-helical conformation. Proline is known to fit well into the first turn of an α-helix but anywhere further on it usually produces a significant bend in the helix thereby destabilising the structure. Residues 177-189, of no predicted secondary structure, are also highly conserved which may imply that there is a functional requirement for this length of the random coil structure before the start of the α-helix. Each of these explanations for the significant reduction in the ability of VP22del120-192 to be incorporated into the tegument of virus particles suggest that the highly conserved region between residues 119-192 is important for the efficient incorporation of VP22 into virus particles.

The total amount of VP22 incorporated into vUL49del120-192 virus particles was reduced in comparison to each of the vUL49ins viruses but was still elevated in comparison to virus 1802. Therefore the deletion only partially removed or partially disrupted the sequences required for incorporation of VP22 into the tegument. In an attempt to determine whether the residual VP22 incorporation sequences resided in the N- or C-terminal portions of VP22del120-192, vUL49del1-192 and vUL49del120-301 were constructed. These mutant viruses were derived from the plasmid that was used to construct vUL49del120-192, and contained only the N-terminal and C-terminal regions of VP22 that flanked residues 120-192. Characterisation of these viruses showed that only VP22del120-301 was detected

in virions and light particles, and that the level of incorporation was reduced by approximately 9-fold in comparison to the amount of epitope-tagged VP22 present in vUL49ep virus particles. These results suggested that the region of VP22 with little homology and no predicted secondary structure contains sequences that enable limited incorporation of VP22 into the tegument, and that the highly conserved α-helical region of VP22 by itself is apparently not capable of directing VP22 to the tegument. These results may have been a result of the reduced levels of VP22del120-301 and VP22del1-192 synthesised in infected cells in comparison to each of the other vUL49ins and vUL49del viruses. The small amounts of these proteins in infected cells may not have permitted the efficient recognition of sequences that enabled their incorporation into the tegument, therefore it cannot be concluded that the inability to detect VP22del1-192 within virus particles was a result of the inability of this protein to be incorporated. The reduced levels of synthesis of VP22del1-192 may have been due to the introduction of a foreign ATG initiation codon, the context of which may not have resulted in the efficient expression of the gene within the infected cell. However, UL49del120-301 contains the original VP22 initiation codon and the reduced levels of expression of VP22del120-301 are likely to be a result of reduced protein stability.

It is interesting that the only insertion (ins194) to have altered the characteristics of epitope-tagged VP22 lies four amino acids upstream from the predicted α-helical region. Insertion at position 194 appeared to have a noticeable effect on the ability of VP22ins194 to be incorporated into virus particles, however the amount of epitope-tagged VP22 in vUL49ins194 virions remained greater than the amount of untagged VP22. In addition, immunofluorescent staining revealed that this mutant exhibited an exclusively cytoplasmic pattern of fluorescence, the distribution of which appeared similar to the subset of pUL49ep transfected cells exhibiting cytoplasmic fluorescence. Investigation of the residues introduced by the oligonucleotide insertion at position 194 show that a proline and a glycine residue are introduced in close proximity to the predicted first turn of the α-helix. Different side chains have been found to have weak but definite preferences either for or against being in α-helices, and both proline and glycine are very poor α-helix formers. Thus insertion of the oligonucleotide at this position may have disrupted the conformation of the α-helix. These results further support the data suggesting that the highly conserved region between residues 120-192 is important for the incorporation of VP22 into the tegument.

There is some evidence to show that tegument proteins are able to interact with each other (Smibert *et al.*, 1994; Elliot *et al.*, 1995, Lam *et al.*, submitted) and these interactions may influence the incorporation of proteins into the tegument. Several pieces of evidence

suggest that VP22 is able to interact with itself. These include data from Western blot analysis, experiments with recombinant baculovirus and biochemical data. Firstly, Western blot analysis of vUL49ep virions and light particles using the pp65 antibody specific for the epitope-tag sequence, recognised species that were estimated to be double and three times the molecular weight of VP22. These were thought to be dimers and trimers of VP22, and because they were detected following electrophoresis on denaturing polyacrylamide gels, the interaction was resistant to fairly harsh denaturation (1% SDS and boiling). Far Western blot analysis of VP22 has since confirmed these initial results (B. Verbeek, personal communication). Secondly, insect cells infected with a baculovirus recombinant which expresses high levels of VP22 were shown to contain large irregular aggregates of the protein (J. McLauchlan, personal communication). Thirdly, gel filtration analysis of *in vitro* transcribed/translated VP22 showed that VP22 migrated only as a dimer and a trimer (P O'Hare, personal communication). In addition, increasing the salt concentration, in order to minimise non-specific aggregation of VP22, resulted in the migration of the protein as a tetramer (P O'Hare, personal communication). Fourthly, a bacterially expressed portion of VP22 (amino acids 159-267) appears to exist in dimer and higher molecular weight forms; gel filtration and preliminary NMR analysis has failed to detect monomeric protein (G. Hope, J. McLauchlan & P. Barlow, personal communication). The ability of VP22 to interact with itself is likely to influence the incorporation of the mutated epitope-tagged forms of VP22 into the tegument.

There is compelling evidence that VP22 also interacts with the major transcriptional activator, VP16 (Elliott *et al.*, 1995). A strong interaction between VP16 and VP22 was first demonstrated by immunoprecipitation of both proteins from infected cells using a VP16-specific antibody. Transfection of VP22 and VP16 expressing plasmids into Vero cells resulted in the relocalisation of both proteins into novel 'doughnut' structures termed tegument bodies situated in the cytoplasm (Elliot *et al.*, 1995). The functional significance for this interaction is not yet known. A paper by Hensel *et al.* (1995) described similar large aggregated structures early in infection in the nucleus and latterly in the cytoplasm of cells infected with HCMV. These structures were identified as being composed of the pp150 tegument protein and it was suggested that the acquisition of the tegument protein pp150 seems to take place in these special nuclear subcompartments where nucleocapsids assemble. These structures are distinct from dense bodies, the formation of which is a characteristic feature of CMV replication (Severi *et al.*, 1992). Dense bodies contain an abundance of the major tegument protein pp65, accounting for approximately 95% of their protein mass (Irmiere & Gibson, 1983). The remaining 5% consists of several host cell

Mutant	Location	Pattern	+VP16
pUL49ep	most nuclear some cyto.	edge of nucleus network	O
pUL49del268-301	cytoplasmic	network & diffuse	O
pUL49del120-192	cytoplasmic	aggregates	VP16 also aggregates
pUL49del120-301	cytoplasmic	diffuse	-
pUL49del1-192	cytoplasmic	diffuse	-
pUL49ins60	as pUL49ep	as pUL49ep	O
pUL49ins159	as pUL49ep	as pUL49ep	O
pUL49ins194	cytoplasmic	network & diffuse	together form aggregates
pUL49ins267	as pUL49ep	as pUL49ep	O

Table 13. Results obtained by G. Elliot following the cotransfection of a VP16 expressing plasmid with pUL49ep, and each of the pUL49ins and pUL49del mutant plasmids.
Location; intracellular location of the fluorescence
Pattern; intracellular pattern of fluorescence
+VP16; cotransfection of the mutant plasmid with a VP16 expressing plasmid.
(O; indicates the formation of tegument bodies).

proteins as well as DNA and RNA. The functional significance of dense bodies during the replicative cycle of CMV is not clear. They may be directly involved in virus morphogenesis , however, due to their composition, it has been suggested that they represent the site where surplus cellular, viral or virus induced molecules are stored before being eliminated from the cell (Severi *et al.*, 1992).

Collaborative studies with G. Elliot were performed to further investigate the interaction between VP22 and VP16. Cotransfection of cells using a VP16 expressing plasmid and each of the pUL49ins and pUL49del mutant plasmids yielded the results summarised in Table 13. The VP22ins mutants encoded by pUL49ins60, pUL49ins159 and pUL49ins267 when expressed in cells with VP16 were all able to redistribute VP16 into large aggregates at the edge of the nucleus which were indistinguishable from the tegument bodies formed by interaction of VP16 with full length VP22 (Table. 13). Therefore, none of these three insertions altered the phenotype from that exhibited by wild type VP22 synthesised in cells transfected with pUL49ep. Both VP22ins194 and VP22del120-192 were unable to redistribute VP16 into large aggregates, but these proteins were able to co-localise with VP16, indicating that an interaction between VP16 and each of these proteins still occured. These results imply that the ability of VP22 and VP16 to interact is not sufficient for tegument body formation and that other interactions play a part. Assuming that no cellular proteins are involved in tegument formation, and from the results that suggest that VP22 can oligomerise, it is reasonable to conclude that the inablity of these mutants to form tegument bodies is caused by a disruption in the ability of VP22 to interact with itself. Such a disruption may account for the reduced levels of incorporation of VP22del121-192. In the case of VP22ins194, the reduced levels of incorporation are less marked but there is a noticeable increase in the amount of untagged endogenous VP22 in virus particles; which would suggest that insertion at this position had an effect on its ability to compete with the endogenous form of VP22 for incorporation.

VP22del120-301 was incorporated into virus particles, albeit to a limited extent, but cotransfection of the plasmid expressing this protein with a VP16 expressing plasmid showed that these proteins were unable to interact. These results suggest that the incorporation of VP22 into the tegument in this experimental system is not dependant on an interaction between VP16 and VP22. This epitope-tagged VP22 deletion mutant does not contain the sequences 159-267 which allow dimerisation, however it is possible that other sequences exist within residues 1-119 that enable VP22 to form dimers which would enable VP22del120-301 to enter the tegument following an interaction with the endogenous VP22 protein, which is in turn able to interact with VP16. Otherwise VP22del120-301 may be

entering the tegument following an interaction with an as yet unspecified tegument protein which may or may not require an association with VP16 for entry into the tegument. Alternatively, within VP22del120-301 there may be a tegumentation signal sequence which enables the incorporation of this protein without the requirement for any protein/protein interactions. It is interesting that VP22del1-192, which contains most of the residues (159-267) in the bacterially expressed VP22 protein that does dimerise is not capable of being incorporated into the tegument. This would suggest that residues 159-192 contribute to the dimerisation of VP22, however further studies are necessary to clarify the precise location of these sequences.

The distinctive network patterns of immunofluorescence obtained following the transfection of plasmids pUL49ep, each of the pUL49ins plasmid series and pUL49del268-301 into BHK cells suggest that epitope-tagged VP22 may be interacting with a protein that forms, or interacts with a cellular filamentous structure. BHK cells contain microfilaments composed of actin and tubulin, and intermediate filaments; the majority of which are composed of vimentin (Franke *et al.*, 1979), however the major intermediate protein of muscle cells, desmin is also present (Frank *et al.*, 1982). Microfilaments are involved in the formation of the cellular cytoskeleton that imparts structure and elasticity to eukaryotic cells, while intermediate filaments appear to be responsible for anchoring the nucleus. Intermediate filaments may have other fuctions however these are not known. The possibility that VP22 is interacting with either tubulin or vimentin are thought to be unlikely because the patterns of flourescence exhibited by these proteins are different from the networks observed following transfection of the pUL49ep plasmids (G. Elliot & J. McLauchlan personal communications). Alternatively epitope-tagged VP22 may be interacting with an antigenically related group of polypeptides, IFAP's (intermediate filament-associated proteins) ranging from 70 to 280KDa, which are closely associated with intermediate filaments (Yang *et al.*, 1992), and appear to regulate the arrangements of intermediate fillaments within cells (Yang *et al.*, 1990). The *in situ* localisation of some of these different molecular weight forms of IFAP may more closely reflect the network patters of fluorescence observed following transfection of the pUL49ep expressing plasmids into cells. The functional significance of the interaction between VP22 and these cellular proteins, and whether these structures are seen during normal infection of cells is not known, but knowledge of the nature of the cellular protein involved in this interaction may help to elucidate a function for VP22.

VP22 is extensively post-translationally processed. It is highly phosphorylated, ADP-ribosylated and has also been proposed to be nucleotidylylated. The functions of these

modifications within VP22 are not known, but are frequently seen in proteins that regulate biochemical processes. Within VP22, residues 115 to 126 are proposed to be a nucleotidylylation site (Blaho *et al.*, 1994). These residues are partially removed in VP22del120-192, present in vUL49del120-301 virus particles. The nucleotidylylation of proteins is rare and has not been extensively studied, however the functions fall into three categories: i) proteins where nucleotidylylation represents an intermediatory covalent attachment of a nucleotide to a protein during the course of an enzymatic reaction, eg. guanyltransferases involved in the capping of mRNA (Venkatesan & Moss, 1982), and DNA ligases (Weiss & Richardson, 1967). ii) Nucleotides covalently attached to viral genomes via stable nucleotidyl-protein linkages that function in viral nucleic acid synthesis, eg. adenovirus terminal protein (Desiderio & Kelley, 1981), and iii) covalent attachment of a nucleotide to the protein as a regulatory mechanism, eg. glutamine synthetase of E.coli (Shapiro *et al.*, 1967), where the activity of the enzyme is determined by the adenylated state (Stadtman, 1990). The function of VP22 may be regulatory, indeed due to its association with VP16, it may act to regulate the transactivating functions of VP16. It has also been shown to bind to DNA, and although it is not essential for DNA replication, it may be involved in packaging of DNA or nucleic acid metabolism. Further characterisation of VP22del120-192 in which the nucleotidylylation signal has been disrupted may help to elucidate the function of nucleotidylylation within VP22.

In conclusion, these results demonstrate that the incorporation of VP22 into the tegument is likely to involve a series of interactions between itself and/or VP16. It is unlikely that these proteins form tegusome like structures as seen with HHV-6 (Roffman *et al.*, 1990), as such invaginations of the nuclear membrane are not seen during HSV-1 infection. However, the production of light particles during HSV-1 infection, and the fact that the integrity of the tegument within light particles is not dependent on the presence of the envelope, implies that the tegument is capable of self assembly (Rixon *et al.*, 1992). This evidence for a self-assembling tegument together with the formation of large aggregates in cells coexpressing VP16 and VP22, provides several possible mechanisms for tegument assembly within the cell. VP16 may function as a nucleation signal for the rest of the tegument proteins to build themselves around, such that VP16 would be the only essential protein for the process, with VP22 and the rest of the components able to compensate for each other in their relative amounts. For example, it has been demonstrated that neither VP11/12 (encoded by UL46) or VP13/14 (encoded by UL47) is essential for virus assembly and that virions lacking either of these proteins appear to have increased levels of the other (Zhang & McKnight, 1992). Alternatively, the VP16-VP22 interaction together

with one or more additional minor tegument proteins may form the scaffold of the tegument, in which case VP22 would also be essential for assembly. The argument against other major tegument proteins being included in a scaffold formation is provided by the characterisation of a HSV-1 UL46/UL47 double deletion mutant made by Zhang & McKnight, (1992). The polypeptide profile of this mutant virus, showed that VP16 and VP22 were the only major tegument species of this virus mutant, and therefore only minor components of the tegument can be proposed to be candidates essential for scaffold formation. Recent chemical cross-linking experiments on HSV-1 virions have shown that the tegument proteins VP22, VP16 and VP13/14 can also associate with the envelope glycoprotein gB (Zhu & Courtney, 1994), which may suggest a possible mechanism for a signal in the association of the tegument with the envelope during virus assembly. The large aggregates of VP16 and VP22 seen during transfection may represent an extension of the interactions which occur during normal tegument assembly, whereby the presence of the many other virus proteins within the cell and the interactions therein would affect or limit the size of and/or organisation of the complexes formed.

The mutational analysis of VP22, presented in this section, failed to identify a minimum region required for the incorporation of VP22 into the HSV-1 tegument, however the results presented have enabled the the preliminary identification of regions that are important as well as the elimination of regions of VP22 that are not involved in the incorporation of the protein into the tegument. With regards to the mechanism by which VP22 is incorporated into the tegument the results presented do not exclude the possibility that VP22 is incorporated into the tegument via an interaction with other proteins, and it is likely that by multimerising with the endogenous form of VP22 some of the mutants constructed were escorted into the tegument. The construction of a mutant virus that does not contain the endogenous copy of UL49 is therefore essential for the further analysis of the incorporation of VP22 into the tegument.

Conclusions

The initial aim of this project was to devise a manipulatable experimental system that would enable the identification of sequences which direct proteins to the tegument of the HSV-1 particle. A minor tegument protein, vhs, was fused to a bacterial reporter gene, CAT, and the resulting fusion protein was shown to be incorporated into the recombinant vVHS-CAT virus particles. This was a significant result, demonstrating for the first time that foreign proteins could be incorporated into the tegument of HSV-1 virions and light particles. In the second experimental system (*Chapter 8*) the tegument protein chosen, VP22, was a major component of the tegument, and thus easily detected on Coomassie Brilliant blue stained gels. In this case, the tag fused to VP22 consisted of a ten amino acid epitope and the expression of the fusion protein was under the control of the the strong IE HCMV promoter sequence. Similar to vVHS-CAT, the vUL49ep recombinant virus contained the epitope-tagged copy of VP22 in both virions and light particles but there was also a dramatic increase in the levels of VP22 in these virus particles. The mutagenesis of epitope-tagged UL49, described in *Chapter 9*, was attempted in order to identify sequences which direct VP22 into the tegument of virus particles. This did not identify a minimum region required for the incorporation of VP22 into the HSV-1 tegument, however the results enabled the elimination of regions of VP22 that are not involved in the incorporation of the protein into the tegument. Thus, this investigation has shown that tegument proteins are amenable to alteration without loss of ability to incorporate into virus particles. The tagging systems developed therefore provide the basis for further work on the sequences which direct proteins to the tegument and on their modes of entry.

With regard to the mechanism by which tegument proteins are incorporated into virus particles, the results obtained from this study and those of others suggest that proteins may be directed into the tegument by interaction with other structural components. Both vhs and VP22 have been shown to interact with VP16 (Smibert *et al.*, 1992; Elliot *et al.*, 1995; Schmelter *et al.*, 1996), the only tegument protein to date which is essential for virus assembly (Ace *et al.*, 1988; Weinheimer *et al.*, 1992). This implies that, at least for HSV-1, VP16 plays a pivotal role in tegument formation. It is unlikely however that a mechanism of assembly based on interactions with VP16 and its homologues is universal among herpesviruses. Firstly, VP16 homologues are not readily identified by sequence comparisons in β- and γ-herpesviruses. Secondly, the VZV VP16 homologue encoded by ORF10, is a tegument protein but is not essential for growth or assembly of the virus

particle (Cohen & Seidel, 1994). While interactions with VP16 may play a role during assembly, this does not preclude the possibility that other as yet unidentified interactions may also be occurring. For example, studies using chemical cross-linking reagents were performed by Zhu & Courtney, (1994) in order to determine the relationship between gB on the viral envelope and other virion proteins. Four virion structural proteins, likely to be tegument proteins were seen to be associated with gB as well as gD and gH, but not with gC. One of these four proteins was positively identified as being VP16 and the others were predicted to be VP11/12, VP13/14 and VP22. These interactions may represent an extension of the interactions which occur during normal tegument assembly.

Results have shown that VP22 interacts with itself, and since VP22 is the most abundant tegument protein this may provide a framework for providing structural integrity to the tegument. Since the endogenous copy of UL49 was not deleted in any of the vUL49 mutant viruses constructed, it is possible that the epitope-tagged copies of VP22 were able to incorporate into the tegument following interactions with the unmodified forms of VP22. In order to prevent such an interaction it would be necessary to remove the endogenous copy of UL49 in each of the vUL49 mutant viruses, and this could further enable the identification of regions within VP22 that are critical for its incorporation into the tegument. Thus far, one such mutant has been constructed which expresses only the VP22del268-301 polypeptide. (Y. San & J. McLauchlan, personal communication). This protein is readily detected in virus particles further implying that the C-terminal domain, which is not highly conserved, is not necessary for incorporation. However, the virus mutant does grow significantly less well than the parent construct, suggesting that the C-terminal region influences the function of VP22.

In conjunction with the literature available, the results presented in this thesis suggest that it is likely that the incorporation of tegument proteins may be mediated through interactions between components of virus particles rather than there being a 'tegumentation signal sequence' common to all tegument proteins. It is also possible however, that sequences which direct proteins to a particular cellular compartment also play an important role, and specific cellular compartments may serve as sites for the tegumentation of capsids.

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Appendix.

The HSV-1 UL49 sequence.

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    ATGACCTCTCGCCGCTCCGTCAAGTCGTGTCCGCGGGAAGCGCCGCGCGGGACCCACGAG
1   ---------+---------+---------+---------+---------+---------+ 60
    TACTGGAGAGCGGCGAGGCAGTTCAGCACAGGCGCCCTTCGCGGCGCGCCCTGGGTGCTC

    M  T  S  R  R  S  V  K  S  C  P  R  E  A  P  R  G  T  H  E  -

    GAGCTGTACTATGGCCCGGTCTCCCCGGCGGACCCAGAGAGTCCGCGCGACGACTTCCGC
61  ---------+---------+---------+---------+---------+---------+ 120
    CTCGACATGATACCGGGCCAGAGGGGCCGCCTGGGTCTCTCAGGCGCGCTGCTGAAGGCG

    E  L  Y  Y  G  P  V  S  P  A  D  P  E  S  P  R  D  D  F  R  -

    CGCGGCGCTGGCCCGATGCGCGCGCGCCCGAGGGGCGAGGTTCGCTTTCTCCATTATGAC
121 ---------+---------+---------+---------+---------+---------+ 180
    GCGCCGCGACCGGGCTACGCGCGCGCGGGCTCCCCGCTCCAAGCGAAAGAGGTAATACTG

    R  G  A  G  P  M  R  A  R  P  R  G  E  V  R  F  L  H  Y  D  -

    GAGGCTGGGTATGCCCTCTACCGGGACTCGTCTTCGGACGACGACGAGTCCCGGGATACC
181 ---------+---------+---------+---------+---------+---------+ 240
    CTCCGACCCATACGGGAGATGGCCCTGAGCAGAAGCCTGCTGCTGCTCAGGGCCCTATGG

    E  A  G  Y  A  L  Y  R  D  S  S  S  D  D  D  E  S  R  D  T  -

    GCGCGACCGCGTCGTTCGGCGTCCGTCGCGGGCTCTCACGGCCCCGGCCCCGCGCGCGCT
241 ---------+---------+---------+---------+---------+---------+ 300
    CGCGCTGGCGCAGCAAGCCGCAGGCAGCGCCCGAGAGTGCCGGGGCCGGGGCGCGCGCGA

    A  R  P  R  R  S  A  S  V  A  G  S  H  G  P  G  P  A  R  A  -

    CCTCCACCCCCCGGGGGCCCCGTGGGCGCCGGCGGGCGCTCGCACGCCCCTCCCGCGCGG
301 ---------+---------+---------+---------+---------+---------+ 360
    GGAGGTGGGGGGCCCCCGGGGCACCCGCGGCCGCCCGCGAGCGTGCGGGGAGGGCGCGCC

    P  P  P  P  G  G  P  V  G  A  G  G  R  S  H  A  P  P  A  R  -

    ACCCCCAAAATGACGCGCGGGGCGCCTAAGGCCTCCGCGACCCCGGCGACCGACCCCGCC
361 ---------+---------+---------+---------+---------+---------+ 420
    TGGGGGTTTTACTGCGCGCCCCGCGGATTCCGGAGGCGCTGGGGCCGCTGGCTGGGGCGG

    T  P  K  M  T  R  G  A  P  K  A  S  A  T  P  A  T  D  P  A  -

    CGCGGCAGGCGACCCGCCCAGGCCGACTCCGCCGTGCTCCTAGACGCCCCCGCTCCCACG
421 ---------+---------+---------+---------+---------+---------+ 480
    GCGCCGTCCGCTGGGCGGGTCCGGCTGAGGCGGCACGAGGATCTGCGGGGGCGAGGGTGC

    R  G  R  R  P  A  Q  A  D  S  A  V  L  L  D  A  P  A  P  T  -

    GCCTCGGGAAGAACCAAGACACCCGCCCAGGGACTGGCCAAGAAGCTGCACTTCAGCACC
481 ---------+---------+---------+---------+---------+---------+ 540
    CGGAGCCCTTCTTGGTTCTGTGGGCGGGTCCCTGACCGGTTCTTCGACGTGAAGTCGTGG

    A  S  G  R  T  K  T  P  A  Q  G  L  A  K  K  L  H  F  S  T  -
```

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    GCCCCACCGAGCCCCACGGCGCCGTGGACCCCCCGGGTGGCCGGGTTCAACAAGCGCGTC
541 ---------+---------+---------+---------+---------+---------+ 600
    CGGGGTGGCTCGGGGTGCCGCGGCACCTGGGGGGCCCACCGGCCCAAGTTGTTCGCGCAG

    A  P  P  S  P  T  A  P  W  T  P  R  V  A  G  F  N  K  R  V  -

    TTCTGCGCCGCGGTCGGGCGCCTGGCGGCCACGCACGCCCGGCTGGCGGCGGTACAGCTG
601 ---------+---------+---------+---------+---------+---------+ 660
    AAGACGCGGCGCCAGCCCGCGGACCGCCGGTGCGTGCGGGCCGACCGCCGCCATGTCGAC

    F  C  A  A  V  G  R  L  A  A  T  H  A  R  L  A  A  V  Q  L  -

    TGGGACATGTCGCGGCCGCACACCGACGAAGACCTCAACGAGCTCCTCGACCTCACCACC
661 ---------+---------+---------+---------+---------+---------+ 720
    ACCCTGTACAGCGCCGGCGTGTGGCTGCTTCTGGAGTTGCTCGAGGAGCTGGAGTGGTGG

    W  D  M  S  R  P  H  T  D  E  D  L  N  E  L  L  D  L  T  T  -

    ATTCGCGTGACGGTCTGCGAGGGCAAGAACCTCCTGCAGCGCGCGAACGAGTTGGTGAAT
721 ---------+---------+---------+---------+---------+---------+ 780
    TAAGCGCACTGCCAGACGCTCCCGTTCTTGGAGGACGTCGCGCGCTTGCTCAACCACTTA

    I  R  V  T  V  C  E  G  K  N  L  L  Q  R  A  N  E  L  V  N  -

    CCCGACGCGGCGCAGGACGTCGACGCGACCGCGGCCGCCCGGGGCCGCCCCGCGGGGCGT
781 ---------+---------+---------+---------+---------+---------+ 840
    GGGCTGCGCCGCGTCCTGCAGCTGCGCTGGCGCCGGCGGGCCCCGGCGGGGCGCCCCGCA

    P  D  A  A  Q  D  V  D  A  T  A  A  A  R  G  R  P  A  G  R  -

    GCCGCCGCGACCGCACGGGCCCCCGCCCGCTCGGCTTCCCGTCCCCGCCGCCCCCTCGAG
841 ---------+---------+---------+---------+---------+---------+ 900
    CGGCGGCGCTGGCGTGCCCGGGGGCGGGCGAGCCGAAGGGCAGGGGCGGCGGGGGAGCTC

    A  A  A  T  A  R  A  P  A  R  S  A  S  R  P  R  R  P  L  E  -
```