ANALYSIS OF FOUR CAPSID PROTEIN GENES OF HSV-1

by

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CONTENTS

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ABSTRACT

The initial goal of this work was the cloning and expression of the genes UL18, UL19 and UL38 of HSV-1. These genes encode the three capsid proteins VP23, VP5 (the major capsid protein) and VP19C respectively, which are all present in cored and in coreless intranuclear capsids. Cloning having been achieved, each gene was expressed in a recombinant vaccinia virus; each recombinant producing a protein profile with a unique band of the correct size, as judged by its comigration with the respective protein in preparations of purified capsids. A further recombinant vaccinia virus was constructed, which expresses the HSV-1 assembly protein VP22a, the product of the gene UL26.5. Expression of VP22a by this recombinant was confirmed using a monoclonal antibody (5010) against VP22a. These viruses were used in a study of HSV-1 capsid structure and assembly.

One aim of this research was to be able to assemble HSV-1 capsids using capsid genes cloned and expressed in a heterologous system. However, electron microscopy demonstrated that the products of the four capsid genes UL18, UL19, UL26.5 and UL38 are insufficient for the assembly of capsids or capsid-like particles in the vaccinia expression system. Possible reasons for this, and implications for the capsid assembly process, are discussed.

VP19C has previously been reported to be a DNA-binding protein. Attempts were made to confirm this using the VP19C expressed from a recombinant vaccinia virus, but VP19C did not exhibit significant DNA-binding activity in this system.

The vaccinia virus expressing VP23 was used to identify VP23 as the target antigen for a previously unassigned

monoclonal antibody (1060). This antibody was then used to study the properties of the UL18 gene product in HSV-1-infected cells. UL18 mRNA has been reported to be regulated with early-late kinetics in lytic infections. In an attempt to confirm this, the kinetics of synthesis of VP23 were examined. VP23 was detectable by immunoprecipitation from 2 to 3 hours post-infection (personal communication, Dr A.M.Cross). Production of VP23 was not affected by the presence of the DNA synthesis inhibitor phosphonoacetic acid. Thus VP23 is produced early in infection and is not dependent on viral DNA synthesis, in agreement with the designation of this gene as an early-late.

To investigate the subcellular distribution of VP5, VP19C, VP23 and VP22a when expressed from the vaccinia vectors, cells infected with the recombinant viruses were separated into cytoplasmic and nuclear fractions. A control experiment using wild-type HSV-1 showed these proteins to separate predominantly with the nuclear fraction. By contrast, in recombinant-vaccinia-infected cells expressing VP5 or VP23, these proteins were almost exclusively cytoplasmic. However, when expressed by a recombinant vaccinia virus, VP19C and VP22a showed intrinsic ability to locate to the nucleus, the site of capsid assembly.

The availability of antibodies against VP23 and VP22a allowed further investigation of the subcellular distributions of these proteins by immunofluorescence experiments on infected cells. These experiments again showed that when expressed by the recombinant vaccinia virus, VP23 remains localised in the cytoplasm in contrast to the situation obtaining during infections with wild-type HSV-1, when VP23 localised to the nucleus.

It was thought unlikely that this failure of the herpes protein to accumulate in the nucleus was a consequence of the vaccinia vector interfering with the normal transport

mechanisms since other herpesvirus proteins expressed from the same vector are known to enter and to accumulate in the nucleus. However, to eliminate this possibility the distribution of VP23 expressed from a plasmid vector was examined. The UL18 coding sequences were placed under the control of the HSV-1 glycoprotein D promoter, and this construct was cotransfected into cells along with plasmids expressing the two HSV-1 immediate early transactivating proteins Vmw110 and Vmw175. When the distribution of VP23 expressed in these cells was examined by fluorescent staining with 1060, it was identical to that in the recombinant vaccinia-infected cells, ie predominantly cytoplasmic.

Use of 5010, directed against VP22a in immunofluorescence experiments showed that when expressed from the vaccinia vector, VP22a localises to the nucleus as in wild-type infections, in direct contrast to the cytoplasmic location of VP23. This observation provides further confirmation that the vaccinia vector does not interfere with the processes of transport of the foreign protein. This result has been confirmed by immunoelectron microscopy.

A significant finding was that immunofluorescence studies suggest that VP22a is able to affect the subcellular location of VP23. Use of 1060 showed that while VP23 adopted a cytoplasmic location when expressed by a recombinant vaccinia virus, in a dual infection with a VP22a-expressing recombinant VP23 localised predominantly in the nucleus. VP22a is thought to function as a scaffolding protein during HSV capsid assembly, and this result suggests that it may also function in intracellular transportation of another capsid protein.

ABBREVIATIONS

A	adenine
Ac	acetate
AcNPV	A.californica Nuclear Polyhedrosis Virus
Amp	ampicillin
APS	ammonium persulphate
ATP	adenosine-5'-triphosphate
BHK	baby hamster kidney cells
BHV	bovine herpesvirus
BSA	bovine serum albumin
BUdR	5-bromo-2'-deoxyuridine
C	cytosine
^{14}C	carbon-14 radioisotope
CAV	cell-associated virus
CCV	channel catfish virus
Ci	Curies
CIP	calf intestinal phosphatase
CMV	cytomegalovirus
c.p.e.	cytopathic effect
c.p.m.	counts per minute
CRV	cell-released virus
CTP	cytidine-5'-triphosphate
Da	Daltons
DATD	diallyltartardiamine
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dNTP	2'-deoxyribonucleoside-5'-triphosphate
DBP	DNA-binding protein
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
E	early (gene)
EBV	Epstein-Barr virus
EDTA	sodium ethylenediamine tetra-acetic acid

EtBr	ethidium bromide
FCS	foetal calf serum
G	guanine
g	grammes
gD	glycoprotein D
GMEM	Glasgow-modified Eagle's medium
GuHCl	Guanidine Hydrochloride
h	hour(s)
HCMV	human cytomegalovirus
HEPES	N-2hydroxyethylpiperazine-N'2ethansulphonic acid
HHV	human herpesvirus
HSV	herpes simplex virus
ICP	infected-cell polypeptide
IE	immediate-early (gene)
IR	internal repeat
kb	kilobase
l	litre
L	late (gene)
LAT	latency-associated transcript
LFP	large (Klenow) fragment of DNA polymerase
LTR	long terminal repeat
M	molar
2ME	2-mercaptoethanol
MEM	Dulbecco-modified Eagle's medium
min	minute(s)
ml	millilitre
mM	millimolar
m.o.i.	multiplicity of infection
mRNA	messenger ribonucleic acid
M_r	molecular weight
N	unspecified nucleotide or amino acid
n	nano
NLS	nuclear localisation signal
NPT	non-permissive temperature
NP40	Nonidet P40
OD	optical density
ORF	open reading frame
ori	origin of DNA replication
^{32}P	phosphorous-32 radioisotope
p.a.	post adsoption

PAA	phosphonoacetic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
p.f.u.	plaque-forming units
p.i.	post infection
PMSF	phenylmethylsulphonyl fluoride
polyA	polyadenylic acid
PRV	pseudorabies virus
PT	permissive temperature
R	purine moiety (A or G)
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
r.p.m.	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
sec	second(s)
SV40	simian virus 40
T	thymidine
TCA	trichloroacetic acid
TEMED	n,n,n',n'-tetramethylethylene diamine
TIF	trans-inducing factor
TK	thymidine kinase
TR	terminal repeat
Tris	tris(hydroxymethyl)aminoethane
ts	temperature sensitive
u	micro
u.v.	ultra-violet
V	volt
Vmw	apparent mol. weight of virus-induced protein
VP	virion protein
v/v	volume/volume
VZV	varicella-zoster virus
wt	wild-type
w/v	weight/volume
Y	pyrimidine moiety (T or C)
X-gal	5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside

INTRODUCTION

1 CLASSIFICATION OF THE HERPESVIRIDAE

Herpesviruses are ubiquitous viruses of vertebrates, with 112 species being listed by Roizman *et al*. (1992) for inclusion in the family *Herpesviridae*. Membership of this family is based on common virion architecture. The typical herpesvirion viewed by electron microscopy is 120-200 nm in diameter, and comprises four principal structural elements: a core, including the nucleic acid complement; an icosahedral capsid approximately 100-125 nm in diameter; an amorphous proteinaceous layer designated tegument, present in variable amounts; the envelope, a bilayered membrane surrounding the tegument. The nucleic acid of herpesviruses is a double-stranded, linear molecule of DNA, of between 32-75 % moles G+C, and a molecular weight of between 80-150 x 10^6 Da, according to species.

Three subfamilies of herpesviruses are distinguished, on the basis of differences in their biological properties in respect of host range, duration of reproductive cycle, cytopathology and characteristics of latent infection (Roizman *et al*., 1981; Matthews, 1982; Brown, 1989; Francki *et al*., 1991). Members of the subfamily *Alphaherpesvirinae* are primarily distinguished by their relatively permissive *in vitro* host range and ganglionic site of latent infection, although this is not the site of latency in all members. The genus *Simplexvirus* includes the two human herpesviruses HSV-1 and HSV-2. The genus *Varicellovirus* includes the varicella-zoster virus (reviewed by Davison, 1991) of humans. Members of the subfamily *Betaherpesvirinae* are primarily distinguished by their relatively long reproductive cycle and their distinctive cytopathology, especially the phenomenon of cytomegalia. The genus *Cytomegalovirus* contains the human cytomegalovirus (HCMV). Members of the subfamily *Gammaherpesvirinae* are primarly distinguished by a relatively restricted *in vitro* host range and lymphoid

site of latent infection. The genus Lymphocryptovirus includes the human herpesvirus Epstein-Barr virus.

The biological properties used in this scheme do not represent strictly defined criteria, resulting in a classification with many exceptions and inconsistencies. The ever-increasing amounts of sequence data becoming available have largely provided a better basis for the present classification, although some discrepancies have been identified. Two avian herpesviruses, Marek's disease virus (MDV) and the serologically related herpesvirus of turkeys (HVT), previously assigned to the Gammaherpesvirinae because of their tropism for lymphocytes, have significant sequence homology with the Alphaherpesvirinae (Buckmaster et al., 1988; Scott et al., 1989). Similarly, human herpesvirus 6 (HHV-6) (reviewed by Thomson et al., 1991), thought to be a gammaherpesvirus because of its isolation from peripheral blood lymphocytes and its propagation in cultures of lymphoblastoid cells, has been found to have significant sequence similarity to the betaherpesvirus HCMV (Lawrence et al., 1990). The recent sequencing of the entire genome of Channel Catfish Virus (CCV), previously thought to be an alphaherpesvirus, yielded the unexpected finding that there was no detectable significant homology with any known herpesvirus sequence. The lack of homology was so marked that the author was prompted to suggest that this virus should be assigned to a separate subfamily, or even that it be removed from the family Herpesviridae altogether, despite its status as a herpesvirus as judged by the classical criterion of virion morphology (Davison, 1992). Human herpesvirus 7 (HHV-7), recently isolated from peripheral blood lymphocytes, remains unclassified, as HHV-7 DNA did not show any hybridisation with probes derived from other human 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).

2 BIOLOGY OF HSV-1

2.1 Natural host

The natural host of HSV-1 is man, although experimental infections can be achieved in a variety of laboratory animals. A ubiquitous pathogen, HSV-1 is the cause of a variety of human diseases ranging from the common but relatively mild condition of cold sore lesions of the lips and mouth to an occasional life-threatening infection of the central nervous system. The majority of primary infections are asymptomatic (reviewed by Whitley, 1990).

2.2 Latency

An interesting feature of the biology of herpesviruses is the phenomenon of latency. Latency has been defined as a type of persistent infection in which the viral genome is present but infectious virus is not produced except during intermittent episodes of reactivation, and the subject has been reviewed by Stevens (1989) and by Roizman & Sears (1987). HSV-1 establishes latency in peripheral sensory ganglia, although experimental latent infection has been attained in non-neuronal sites (Clements & Subak-Sharpe, 1988). No viral gene product is known to be essential for the establishment of the latent state, but the recent report of Harris & Preston (1991) presents evidence that lack of transactivation of immediate-early transcription by Vmw65 predisposes to latency, which supports the suggestion that failure of Vmw65 to initiate IE transcription is an event leading to latency. Viral DNA in latently infected cells is not integrated into cellular DNA but exists as separate extrachromosomal elements (Mellerick & Fraser, 1987). It is maintained in a non-linear state, probably a circular episome, and associates with nucleosomes in a chromatin-like structure (Rock & Fraser, 1983; Rock & Fraser, 1985; Efstathiou et al., 1986; Deshmane & Fraser, 1989). The only viral RNAs detectable during latency is a related family, the latency-associated transcripts, or LATs.

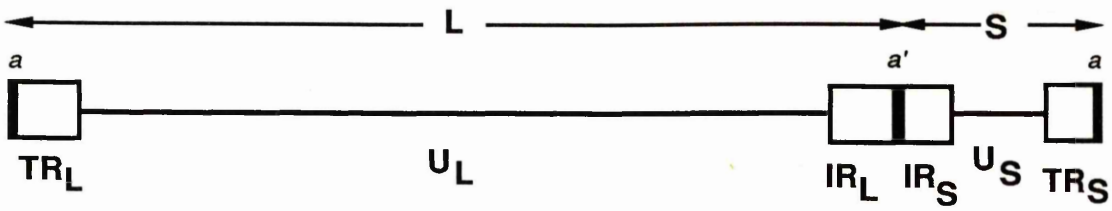


Figure 1. Gross organisation of the HSV-1 genome.

A conventional representation of the HSV-1 genome is shown, with unique sequences (U_L and U_S) as solid lines and major repeat elements (TR_L and IR_L, IR_S and TR_S) as open boxes. The locations of the L and S segments are marked. Terminal *a* sequences and the internal, opposite orientation *a'* sequence are indicated.

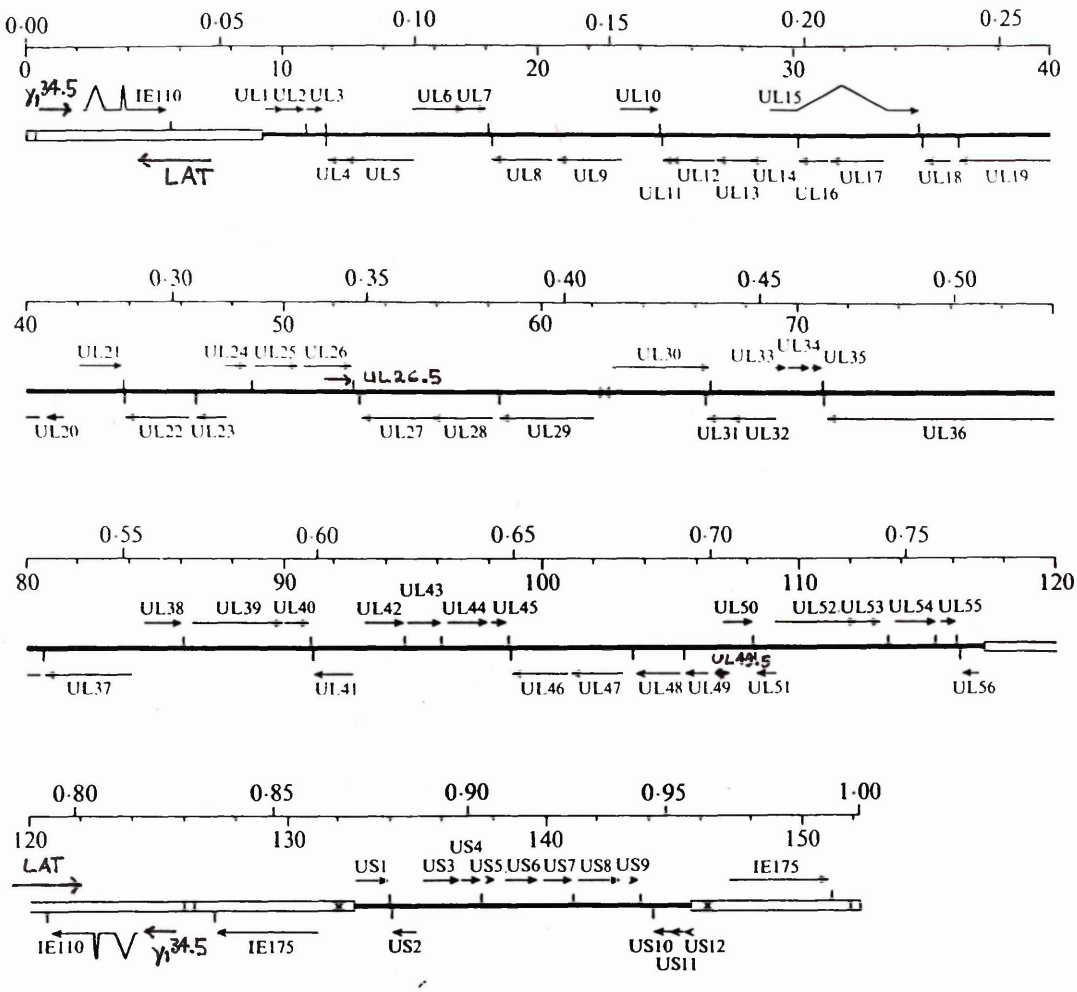
This figure is adapted with permission from McGeoch et al. (1988a).

These transcripts were initially observed by Stevens et al. (1987). The LATs are 2.0, 1.5 and 1.45 kb in length (Spivak & Fraser, 1987), and their 3' ends overlap with and are complementary to the 3' end of the immediate-early gene IE110 in the long internal and terminal repeat regions. This observation led to the suggestion that the LATs may downregulate IE110 by an antisense mechanism (Rock et al., 1987; Stevens et al., 1987). An essential role of the LATs in the establishment of latency was ruled out by the work of Javier et al. (1988) and Steiner et al. (1989). The latter report did present findings suggestive of a role of the LATs in facilitating efficient reactivation from latency in a mouse model; similar findings were made using a rabbit eye model system by Hill et al. (1990). A protein with an apparent molecular weight of 80 kDa has been detected in latently infected neurons, using an antiserum raised against a bacterially expressed fusion protein containing part of a LAT-encoded polypeptide. The function of this protein is not known (Doerig et al., 1991). The molecular basis of the phenomenon of latency remains to be fully resolved.

2.3 Structure of the HSV-1 genome

The DNA of HSV-1 is a double-stranded, linear molecule with molecular weight of 96×10^6 Da. It has a base composition of 68.3% G+C, although this figure is not constant throughout the genome. The entire genome of HSV-1 strain 17 has been sequenced, and was found to consist of 152,260 base pairs (McGeoch et al., 1988a; McGeoch et al., 1986; McGeoch et al., 1985; Perry & McGeoch, 1988). The structure of the HSV-1 genome and those of the other human herpesviruses have been reviewed by McGeoch (1989).

HSV-1 DNA consists of two covalently linked components, termed the long and the short regions. Each component is composed of a unique sequence flanked at each end by an inverted repeat element, as shown in Figure 1. Thus the unique sequence of the long region (U_L) is flanked by its


0·00
0·05
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0
10
20
30
40
γ1 34.5
IE110
UL1 UL2 UL3
UL6 UL7
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LAT
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0·90
0·95
1·00
120
130
140
150
US4
LAT
US1
US3
US5 US6 US7 US8 US9
IE175
IE110
γ1 34.5
IE175
US2
US10 US12
US11

Figure 2. Layout of genes in the genome of HSV-1. The HSV-1 genome is shown on four successive lines, with unique regions represented by solid lines and major repeat elements as open boxes, as in Figure 1. The lower scale represents kilobases, numbered from the left terminus, and the upper scale represents fractional map units. The sizes and orientations of proposed functional ORFs are shown by arrows. Overlaps of adjacent, similarly oriented ORFs are not shown explicitly. Locations of proposed transcription polyadenylation sites are indicated as short vertical bars. Locations of origins of DNA replication are shown as X. In the U_L region, on the first three lines, genes UL1 to UL56 are labelled. In the U_S region, on the bottom line, genes US1 to US12 are labelled. The locations of introns in the coding regions of gene UL15 and the two copies (TR_L and IR_L) of the IE110 gene are indicated. This figure is reproduced with permission from McGeoch et al. (1988a).

repeat elements, termed either the terminal (TR_L) or the internal (IR_L) repeat, depending upon their location in the genome. Thus also there is a short unique region (U_S) with terminal (TR_S) and internal (IR_S) repeat elements. The long and the short regions are covalently linked between IR_L and IR_S. The L and S regions can invert relative to one another. Thus, four sequence-orientation isomers of HSV-1 DNA are possible, and preparations of purified HSV-1 DNA contain these four isomers in equimolar amounts (reviewed by Roizman, 1979). The sequences of R_L and R_S are unrelated, except that they have in common a 400-bp direct repeat present at both genome termini, with one or more copies present in inverted orientations at the junction of IR_L and IR_S. More than one copy can also be present at the long unique terminus. This element is known as the a sequence (Davison & Wilkie, 1981; Roizman, 1979; Mocarski & Roizman, 1982b).

2.4 Genetic content of HSV-1

Seventy-two open reading frames (ORFs) were identified on the HSV-1 genome by McGeoch et al. (1988a). Most of these have been confirmed as functional genes, with gene products being identified. The locations of these ORFs are shown in Figure 2. With 56 genes in U_L, 12 in U_S and one in each copy of R_L and R_S, there would be a total of 72 genes. Since the four genes present in the repeat regions actually represent two copies each of two genes, there were predicted to be only 70 distinct proteins coded by HSV-1. The existence of other functional ORFs is debated, and four instances are of interest. The 3,500-bp region in R_L downstream of the IE110 gene from where the LATs originate contains two partially overlapping 5' co-terminal potential ORFs (Wagner et al., 1988; Wechsler et al., 1989), and a protein product has been detected (Doerig et al., 1991). However, the function of the LATs is uncertain, as discussed above. A gene designated $\gamma_1$34.5 has been proposed in R_L, upstream of the IE110 gene, originating from a proposed promoter in the a

sequence (Chou & Roizman, 1986). This ORF is present in HSV-1 and HSV-2 (Chou & Roizman, 1990; McGeoch et al., 1991; Dolan et al., 1992). A 43.5-kDa protein product, ICP34.5, has been identified (Ackerman et al., 1986; Chou et al., 1990), and evidence suggests it has a role in neurovirulence (Thompson et al., 1989; Taha et al., 1989a; Taha et al., 1989b; Chou et al., 1990; Harland & Brown, 1991; McGeoch & Barnett, 1991; Chou & Roizman, 1992). The work of Liu & Roizman (1991) demonstrates the existence of two transcriptional units within the UL26 gene, and identifies the protein products of each. An ORF, designated UL49.5, has been identified between genes UL49 and UL50 by Barker & Roizman (1992). Promoter activity has been demonstrated, but a protein product has not been identified.

3 THE LYTIC CYCLE

3.1 Entry into target cell

3.1.1 Attachment

The initial stages of infection are attachment of the virion to the cell and fusion of the viral envelope with the plasma membrane (Morgan et al., 1968; Campadelli-Fiume et al., 1988). It is generally assumed that the nine glycoproteins present on the outside of the viral envelope are involved in the infectious process. Whilst only three of these are essential for lytic infection, gB, gD and gH, none is essential for attachment (Cai et al., 1988; Ligas & Johnson, 1988; Johnson & Ligas, 1988; Desai et al., 1988). Infection is initiated by binding to the cell surface heparan sulphate proteoglycans (WuDunn & Spear, 1989), which explains the ability of the related glycosaminoglycan heparin to inhibit infection by HSV in vitro. WuDunn & Spear (1989) thought that binding to heparan sulphate was the first step in a cascade of virion-cell interactions, culminating in fusion of the virion with the plasma membrane. The reasons for this

proposal were that both gB and gD are necessary for penetration of the virus into the cell (Sarmiento et al., 1979; Highlander et al., 1988; Cai et al., 1988; Fuller & Spear, 1987; Highlander et al., 1987; Ligas & Johnson, 1988), and that these molecules are found on morphologically distinct spikes projecting from the virion envelope (Stannard et al., 1987). Glycoprotein H has also recently been shown to be essential for penetration of virus into the cell (Forrester et al., 1992).

A further stage in the attachment process was proposed by Kaner et al. (1990) and Baird et al. (1990). They reported that the cellular basic fibroblast growth factor (FGF) receptor is involved in viral uptake after initial attachment by means of heparan sulphate. This process was said to be mediated by host-cell derived basic FGF present in infecting virions. However, other workers have been unable to confirm these findings (Herold et al., 1991; Mirda et al., 1992; Muggeridge et al., 1992).

Glycoprotein C appears to play a role in attachment, although there seems to be more than one mechanism involved, because gC is not essential for lytic infection (Draper et al., 1984). The demonstration that HSV-2 gC^- strains could have their attachment blocked by polycationic molecules such as neomycin and polylysine whilst gC^+ strains were unaffected, showed that gC can mediate attachment to cells through its own receptor, independent of other routes (Campadelli-Fiume et al., 1990; Langeland et al., 1990). A monoclonal antibody raised against gC reduced attachment of gC^+ strains but did not affect a gC^- mutant. This mutant attached at a reduced rate even in the absence of the antibody (Svennerholm et al., 1991). Thus HSV has alternative mechanisms of attachment, possibly allowing it to increase its tissue tropism.

Kuhn et al. (1990) studied formation of complexes by HSV-1 virion proteins with biotinylated cell membrane components, finding that HEp-2 cells bound gB, gC and gD in a noncooperative manner. Herold et al. (1991) showed that gB and gC showed strong binding to a heparin-Sepharose column. They also found that gC was principally responsible for the binding of wild-type virus to HEp-2 and Vero cells, in that the binding of gB^- mutants was not impaired while binding of gC^- mutants was ten-fold less efficient. Since heparin could also block attachment by gC^- virus, the alternative mechanisms of binding also involved a heparan sulphate receptor. Sears et al. (1991), using a strongly polarised cell line showed that at least two different sets of receptors for HSV-1 were present, asymmetrically distributed on the cell surface. Infection via one of the receptors was dependent upon gC. Thus a gC^- strain was unable to infect when exposed only to the apical surfaces of these cells but could infect via the basal surface. gC^+ virus could infect via either surface.

3.1.2 Penetration

Morgan et al. (1968) observed entry of virus into cells both by endocytosis and by membrane fusion, but were unable to determine by EM which route led to infection. A consensus of opinion had built up that the primary route of infection was by membrane fusion (reviewed by Roizman & Furlong, 1974), but infection by endocytosis could not be ruled out. Results suggesting that infection occurs by membrane fusion only were presented by Addison et al. (1984). The mutant ts1204 with a lesion in gene UL25 had a defect in penetration. At the NPT this virus could attach, and sufficiently high multiplicities could block subsequent infection by wild-type HSV-1. The defect in penetration could be overcome at the NPT by treatment with polyethylene glycol, a membrane fusion agent. Using a cell line which constitutively expressed gD, Campadelli-Fiume et al. (1988) showed that HSV attached to these cells and although EM showed entry of virus was

occurring by endocytosis, no virions were observed in the process of membrane fusion, and no lytic infection resulted. Presumably the cellular gD sequestered all the cellular receptors of gD, showing also that gD is essential for fusion but not attachment. Cai et al. (1988) showed that while gB$^-$ virus retained the ability to attach, infection only resulted following treatment with polyethylene glycol. Thus gB is necessary for penetration.

3.1.3 Release of viral DNA

Following fusion with the cell membrane, the capsids are transported to the vicinity of the nuclear pores. Viral DNA is released from the capsids and translocated into the nucleus. A function essential for this process has been mapped to a region within UL36 (Batterson et al., 1983), which encodes the 273-kDa tegument protein VP1.

3.2 The effect on host cell macromolecular synthesis

3.2.1 Virion host shutoff

Early in infection there is a rapid virus-induced interruption of host cell macromolecular synthesis; DNA, RNA and protein synthesis all being significantly impaired. There are two distinct phases of HSV-induced host shutoff, which have been termed 'early' and 'delayed'; these and other aspects of shutoff have been reviewed by Fenwick (1984).

The first phase is also termed 'virion-associated', after it was shown that one or more components of the infecting virion was responsible for a rapid disaggregation of cellular polyribosomes accompanied by destabilisation and degradation of host mRNA (Fenwick & Walker, 1978; Nishioka & Silverstein, 1978; Schek & Bachenheimer, 1985). A related virion-associated shutoff event is an indiscriminate destabilisation and degradation of viral immediate-early, early and late mRNAs. This interference

with viral mRNA is not easy to explain, but destruction of one regulatory class of RNA may facilitate transition to translation of the next class, since only recently transcribed mRNA would be functional (Kwong & Frenkel, 1987; Oroskar & Read, 1989). These virion-associated functions map to the region of the gene UL41 in HSV-1 (Kwong et al., 1988). The product of UL41 is predicted to have a molecular weight of 54914 (McGeoch et al., 1988a), and a 58-kDa phosphorylated protein has recently been detected in HSV-1-infected cells and in HSV-1 virions using an antiserum raised against a synthetic peptide corresponding to a region of the UL41 predicted amino acid sequence (Smibert et al., 1992). The action of this protein, known as the vhs (virion host shutoff) protein, remains unclear, although it is thought to activate a cellular nuclease (Strom & Frenkel, 1987). Experiments with mutants defective in the shutoff function have demonstrated that this is not an essential function for growth in cell culture (Fenwick & Clark, 1982; Read & Frenkel, 1983). Viruses with the shutoff gene inactivated are also viable in tissue culture, confirming that the vhs function is not essential (Fenwick & Everett, 1990). Since the vhs protein is present in virions it is a point of interest whether it is an essential structural component of the virion. It was conceivable that mutant forms of the vhs protein might still perform an essential structural role despite having lost their shutoff activity. However, since inactivation of the UL41 gene yielded viable, shutoff-defective viruses, it is concluded that the vhs protein does not have an essential structural role (Fenwick & Everett, 1990). The recently developed in vitro RNA degradation assay of Krikorian & Read (1991) has confirmed a marked increase in rates of decay of host and viral cytoplasmic mRNAs induced by a virion component, and it is hoped that this assay system will prove useful in making a more detailed analysis of vhs function.

The second phase of shutoff is also termed 'expression-dependent', as it requires de novo viral protein synthesis. Using mutants deficient in the virion-associated shutoff functions, a further degradation of cellular mRNA has been demonstrated, which is dependent upon synthesis of one or more viral E or L proteins (Read & Frenkel, 1983). Which proteins are involved is not known.

3.2.2 Induction of cellular proteins

Other research has focussed on the induction of specific cellular proteins by HSV. Infection with HSV-1 can activate a range of cellular promoters (Everett, 1985), and a small number of cellular proteins are known to accumulate to high levels (Patel et al., 1986; Kemp et al., 1986). One host cell polypeptide which accumulates in HSV-2-infected cells also accumulates in heat-shocked cells, suggesting that it might function in the shut off of cellular gene expression (LaThangue et al., 1984). The induction of several stress proteins by abnormal forms of the immediate-early protein Vmw175 has led to the suggestion that these proteins may act to eliminate aberrant forms of Vmw175 during infection or to neutralise their damaging effects (Russell et al., 1987). Preston (1990) has demonstrated the induction of a 56-kDa cellular protein which occurs specifically by the binding of HSV-1 to the cell surface. The work of Macnab et al. (1985) has raised the possibility that stress proteins may play a role in transformation by HSV. They demonstrated that an antibody raised against a host cell polypeptide which accumulates in HSV-2-infected cells cross-reacted with several polypeptides expressed by established transformed cell lines. Since these polypeptides are not specific to HSV-transformed cells, but are found in cells immortalised by other transforming agents, it appears that HSV upregulates cell polypeptides which may play a role in immortalisation.

3.3 Regulation of polypeptide synthesis

Herpesvirus DNA is transcribed in the nucleus (Wagner & Roizman, 1969a; Wagner & Roizman, 1969b), and use of a specific inhibitor of cellular RNA polymerase II revealed this enzyme to be the agent of transcription (Costanzo et al., 1977). Herpesvirus mRNAs are capped, methylated and polyadenylated in a similar fashion to cellular mRNAs Silverstein et al., 1976; Moss et al., 1977). Many proteins undergo some form of post-translational processing, such as cleavage, phosphorylation, sulphation, myristilation, glycosylation and poly(ADP) ribosylation (reviewed by Roizman & Sears, 1990).

3.3.1 Temporal classification of HSV genes

HSV genes (and their products) have been classified into three main groups according to the temporal kinetics of their expression. These groups are expressed in a sequentially ordered cascade (Wagner, 1972; Honess & Roizman, 1974; Honess & Roizman, 1975; Clements et al., 1977). Honess & Roizman (1974; 1975) designated the three groups as α, β and γ. These three groups are also known as immediate-early (IE), early and late respectively, following work which had demonstrated this phenomenon in PRV (Rakusanova et al., 1971; Ben-Porat & Kaplan, 1973). Using inhibitors of mRNA and of protein synthesis, Honess & Roizman (1974; 1975) found that the rates of synthesis of IE polypeptides were highest between 3 and 4 hours post infection and thereafter declined. The rates of synthesis of early polypeptides were maximal from 5 to 7 hours post infection, before a similar decline, and polypeptides of the late class were made at increasing rates until at least 12 hours after infection. They also found that control of switching from synthesis of one group to the next was mediated by one or more polypeptides in each group. IE polypeptides were synthesised without the need for prior viral protein synthesis, but one or more functional IE proteins were required to turn on synthesis of the early and late

groups. In addition, presence of functional polypeptides of the early and late classes was required to shut off synthesis of those of the IE class. It was also observed that synthesis of viral DNA was a requirement for maximal expression of late proteins.

3.3.2 Extensions of the classification

Although this classification of HSV genes and their products has been useful, it is apparent that the distinctions between temporal classes are not as clear as is sometimes implied. The definition of IE genes as those which are expressed in the absence of *de novo* viral protein synthesis is of great significance, but although the peak of expression of these genes ended at about 4 hours p.i., synthesis could still be detected at late times (Honess & Roizman 1974; 1975). More recent studies have shown that significant levels of some IE mRNAs persist late into infection (Harris-Hamilton & Bachenheimer, 1985; Weinheimer & McKnight, 1987). Furthermore, it is now known that in the case of the IE gene IE110, accumulation of the protein product continues late in infection (Everett, 1991; Everett & Orr, 1991), although the functional significance of this is not understood. Early genes require for their expression the presence of functional IE proteins, but their expression is not dependent upon synthesis of viral DNA, indeed, many early proteins are involved in viral nucleic acid metabolism. Pereira *et al*. (1977) divided the early genes into two subclasses, on the basis of sensitivity to the arginine analogue canavanine. Thus β_1 genes were expressed in canavanine-treated cells as well as IE genes and small amounts of the late protein VP5. Genes of the β_2 subclass were not expressed in the presence of canavanine. Expression of β_1 and β_2 genes may be ordered sequentially, with canavanine blocking the transition from β_1 to β_2. Alternatively, there may exist amongst the IE polypeptides different inducers, not all of which are blocked by canavanine, each promoting the synthesis of a different subset of early proteins.

The γ genes have also been divided into two subclasses, although they exhibit much greater variation in the timing of their appearance. The γ_1, $\beta\gamma$, early-late, or leaky-late genes show some expression prior to viral DNA synthesis, whereas the γ_2 or true-late genes are expressed at late times after infection and only appreciably following the onset of DNA synthesis (Holland et al., 1980; Conley et al., 1981; Silver & Roizman, 1985). A commonly cited example of a γ_1 gene is UL19 (Costa et al., 1985a), the gene encoding the major capsid protein VP5. Although readily detected from at least 2 hours p.i. (Honess & Roizman, 1974; Bibor-Hardy et al., 1985a; Harris-Hamilton & Bachenheimer, 1985), VP5 becomes very abundant at late times, following the onset of DNA synthesis, and accumulation of the mRNA transcript is greatly reduced in the presence of inhibitors of DNA synthesis (Holland et al., 1980; Conley et al., 1981). The distinction between an early protein and a γ_1 protein is at times unclear. For example, the 65-kDa DNA-binding protein has been classified as an early protein, despite the fact that accumulation of the protein is greatly reduced in conditions of inhibition of DNA synthesis. The early classification is based on the fact that accumulation of the mRNA transcript peaks at about 6 hours p.i. and that this peak is not appreciably affected by lack of DNA synthesis (Goodrich et al., 1989).

It is also difficult to formulate a definition of the second subclass of late genes, the γ_2 or true-late genes. Following a study of the gene US11, Johnson et al. (1986) reported that even under conditions of the most restricted DNA synthesis attainable, very small amounts of the gene products were still detectable. Thus it is not certain that expression of US11 is absolutely dependent on prior DNA synthesis. These authors offered a definition of a true-late gene as one whose expression is "most severely reduced, compared to all other groups of genes, under conditions of severely inhibited DNA replication." The degree of reduction of expression

implied in the term 'most severely reduced' was stated to be 95%. A satisfactory explanation for this dependence on DNA synthesis is lacking, but it is possible that true-late genes, although transcribed early in infection, have very weak promoters, which require a high copy number achieved through DNA replication for abundant expression.

3.3.3 The α-trans-induction factor, Vmw65

The expression of IE genes prior to de novo viral protein synthesis is induced by a structural component of the infecting virion (Batterson & Roizman, 1983). This protein, known as VP16, αTIF, VF65 or Vmw65, is located in the tegument in 500-1000 copies per particle (Heine et al., 1974). The gene encoding this transactivator was mapped by Campbell et al. (1984) and is now identified as UL48. Vmw65 is an essential structural component of the virion (Moss et al., 1979; Ace et al., 1988). Analysis of a mutant deficient only in transinducing activity suggests that transinduction by Vmw65 is not essential for HSV gene expression at high multiplicities of infection, although it is possible that this mutant did have some residual Vmw65-mediated transinducing activity (Ace et al., 1989). Vmw65 is also essential for virulence after intracranial or intraperitoneal inoculation in mice (Ace et al., 1989).

3.3.4 Mode of action of Vmw65

Vmw65 does not bind DNA directly (Marsden et al., 1987), but participates in the formation of a multiprotein complex involving host proteins (Preston et al., 1988), which initiates transcription of IE genes. Two functional regions of the 490-residue Vmw65 protein have been identified. An acidic domain in the carboxyl-terminal 78 amino acids, including a critical phenylalanine residue at position 442, is responsible for the transactivation function. An amino-terminal domain between residues 49 and 75 confers specificity for IE genes by interaction with host proteins that bind IE cis-regulatory elements (Cress & Triezenberg, 1991; Greaves & O'Hare, 1990).

Details of the Vmw65-IE gene complex are not fully elucidated, but it is known that a cis-acting site present in one to several copies in the 5' transcribed noncoding domains of IE genes binds to the cellular host factor the octamer-binding protein known variously as Oct-1, αH1, NFIII and OTF-1 (Kristie & Roizman, 1988). The IE cis-acting site is of consensus sequence TAATGARAT (where R is a purine), although the flanking sequences affect the magnitude of the response (Mackam & Roizman, 1982; O'Hare & Hayward, 1987; Preston et al., 1988). Many of the TAATGARAT elements have overlapping ATGCTAAT octamer motifs that are similar to the Oct-1 binding site ATGCAAAT, and Oct-1 also binds to these TAATGARAT sites (Aprhys et al., 1989; Spector et al., 1990; Spector et al., 1991). Vmw65 does not bind directly to Oct-1, but at least one other cellular factor is involved, which mediates the complexing of Vmw65 with the Oct-1/IE assembly (Xiao & Capone, 1991; Greaves & O'Hare, 1991).

3.4 The immediate-early genes and their products

3.4.1 IE175

The properties and functions of herpes virus IE polypeptides were reviewed by Everett (1987b). The immediate-early gene IE175, also known as IE-3 and α4, is present in two copies in HSV, being carried in the short repeat region. The polypeptide encoded by this gene is known as Vmw175 or ICP4. Newly translated Vmw175 has an apparent molecular weight of 175 kDa (Preston et al., 1978; Watson et al., 1979), but it is post-translationally modified, and electrophoretic separation yields three bands of apparent molecular weights of 170, 163 and 160 kDa (Pereira et al., 1977; Wilcox et al., 1987). Vmw175 is phosphorylated (presumably with ester phosphate residues) (Pereira et al., 1977; Marsden et al., 1978), poly(ADP) ribosylated (Preston & Notarianni, 1983), and adenylated and guanylated (Blaho & Roizman, 1991). Phosphorylation of Vmw175 is essential for its

interaction with early and late genes, but not for interaction with IE genes (Papavassiliou et al., 1991). Although it carries its own nuclear localisation signal (Paterson & Everett, 1988; DeLuca & Schaffer, 1988) and can readily be demonstrated in the nucleus (Pereira et al., 1977), Vmw175 was recently reported to associate also with the inside of the plasma membrane of infected cells (Yao & Courtney, 1991). Vmw175 was recently reported to be present in the tegument of purified virions in approximately 100-200 copies per particle (Yao & Courtney, 1991). The significance of this is not known but it is possible that these low levels of Vmw175 may result from the presence in virion preparations of L particles, which are known to contain Vmw175 (Szilagyi & Cunningham, 1991; McLauchlan & Rixon, 1991).

Vmw175 is involved in the regulation of expression of its own gene. This autoregulation is mediated by a cis-acting site, including the motif ATCGTC, at the cap site of IE175, which is specifically bound by Vmw175 (Roberts et al, 1988; Paterson & Everett, 1988; Everett et al., 1991a). The gene IE110, although possessing an identical site, was not downregulated by Vmw175 in these experiments. However, viruses with mutations in IE175 do overproduce other IE proteins (Preston, 1979a; Preston, 1979b; Dixon & Schaffer, 1980; DeLuca et al., 1985), and recent studies have shown that Vmw175 does act to repress the IE110 promoter in transfection assays, although it does not appear to do so during infection in tissue culture (Resnick et al., 1989; Everett & Orr, 1991). Mutants in IE175 also fail to produce early and late proteins, and this function of Vmw175, as the major transactivator of HSV genes (Preston, 1979a; Preston, 1979b), is of great importance. Vmw175 is continuously required for both early and late transcription (Watson & Clements, 1980), and has been shown to participate in complexes with promoter-regulatory regions of many early and late genes (Tedder et al., 1989), but the mechanisms responsible for repression and activation are not known.

However, the sites involved differ substantially from the site involved in autoregulation of IE175, and it would seem that additional proteins are involved in these complexes (Papavassiliou & Silverstein, 1990).

3.4.2 IE110

The immediate-early gene IE110, also known as IE-1 and α0, is present on the long repeat regions of the genome and is therefore, like IE175, diploid. The gene is spliced, being composed of three exons encoding a 775 amino acid protein (Perry et al., 1986). The product of IE110, known as ICP0 or Vmw110, has an apparent molecular weight of 110 kDa (Preston et al., 1978; Watson et al., 1979). It is phosphorylated, and locates to the nucleus of infected cells (Pereira et al., 1977). Unlike Vmw175, Vmw110 is not essential for viral growth in tissue culture, although viruses lacking this protein grow very poorly at low multiplicities of infection, and it is required for fully efficient infection (Stow & Stow, 1985).

Vmw110 is an efficient transactivator of early genes (Gelman & Silverstein, 1985; O'Hare & Hayward, 1985) and of late genes (Mavromara-Nazos et al., 1986b). In concert with Vmw175 there is a strong synergistic effect: in a transient expression assay using the glycoprotein D gene promoter, cells transfected with both IE genes accumulated 12-60 times more reporter RNA than cells transfected with either one alone (Everett, 1984). Other workers have produced comparable results (Gelman & Silverstein, 1986; Quinlan & Knipe, 1985). Efficiency of promoter activation by Vmw110 alone and its degree of synergy with Vmw175 vary with cell type, promoter DNA sequence and method of transfection (Everett, 1988b).

There are several reports of detailed mutational analyses of IE110. Several functional domains have been identified, including those involved in its intrinsic and synergistic (in association with Vmw175) activation

effects (Everett, 1987a; Everett, 1988a). This work was performed using a plasmid transfection system, and subsequent work using mutant viruses derived from the same plasmids has showed similar results (Everett, 1989; Cai & Schaffer, 1989). Purified Vmw110 expressed by a recombinant baculovirus forms multimers, including dimers, in solution. Although it is able to bind DNA in a non-specific manner, it is apparently unable to bind to specific sequences or to form a stable interaction with DNA in solution (Everett *et al.*, 1991b).

3.4.3 IE-2

The immediate-early gene IE-2, also known as α27, encodes a protein of an apparent molecular weight of 63 kDa (Honess & Roizman, 1973; Preston *et al.*, 1978; Watson *et al.*, 1979), which is designated ICP27 and Vmw63. The protein is phosphorylated, and locates to the nucleus of infected cells (Fenwick *et al.*, 1978). Vmw63 is encoded by gene UL54 (Watson *et al.*, 1979; Whitton *et al.*, 1983).

Vmw63 appears to have an essential role in the modulation of early and late gene expression at the level of transcription. Temperature-sensitive and deletion mutants in IE-2 fail to complete the lytic infectious cycle, overproducing early proteins but not synthesising many late proteins, especially those of the γ_2 class (Sacks *et al.*, 1985; McCarthy *et al.*, 1989). Work using plasmid transfection assays has identified the region responsible for repression of early genes to the carboxy-terminal 78 amino acids, and the region determining activation of late genes to the carboxy-terminal half of Vmw63 (Hardwicke *et al.*, 1989). A further series of mutant viruses has been constructed from a series of plasmids containing UL54 truncated to various extents at the carboxy terminus. This has enabled identification of genetically separable transactivation functions: one activity stimulates, but is not required for, the expression of γ_1 genes, the other is a requirement for expression of γ_2 genes. These mutants also showed a down-

regulation of early genes late in infection, supporting the view that Vmw63 has a role in switching to late gene expression (Rice & Knipe, 1990).

3.4.4 IE-4 and IE-5

The immediate-early genes IE-4 (α22) and IE-5 (α47) are both spliced, each having one intron, and display the interesting feature of having identical promoters. The genes lie at opposite ends of the short unique region of the genome, and their promoters and splice sites are located in the repeat sequences bounding this region (Rixon & Clements, 1982).

The product of IE-4, Vmw68, or ICP22, has an apparent molecular weight of 68 kDa (Preston et al., 1978), and was mapped to the region of US1 by Watson et al. (1979). Little is known about the role of this protein in natural infections. Vmw68 is not essential for growth in some cell lines (Post & Roizman, 1981), but in other lines Vmw68$^-$ virus grows poorly; viral DNA is made but some late genes are not efficiently expressed. It would seem that some cell lines express a function similar to that of Vmw68, and are able to complement it (Sears et al., 1985).

Vmw12, or ICP47, is the product of the gene US12, or IE-5, and has an apparent molecular weight of 12 kDa (Watson et al., 1979). It is the only one among the IE proteins which is not phosphorylated; nor does it localise to the nucleus (Marsden et al., 1982). It is not essential for growth in some cell lines (Longnecker & Roizman, 1986). However, in a similar fashion to Vmw68, some cell lines do not support efficient growth of Vmw12$^-$ virus. It may be that Vmw68 and Vmw12 act to complement functions missing in some natural host cells (Mavromara-Nazos et al., 1986a).

3.5 Synthesis of viral DNA

Synthesis of herpesvirus DNA occurs in the nucleus (Munk & Sauer, 1964; Rixon et al., 1983), and is detectable from about 3 hours p.i., continuing until at least 12-15 hours (Roizman et al., 1963; Roizman et al., 1964) . This process was reviewed by Challberg & Kelly (1989). Restriction endonuclease profiles of viral DNA synthesised from about 4 hours p.i. reveal that the DNA lacks free ends and consists either of circles or head-to-tail concatemers (Jacob et al., 1979; Jongeneel & Bachenheimer, 1981). The high sedimentation rate of this DNA in comparison to that of genome-length DNA indicates that it exists in a concatemeric state (Ben-Porat & Tokazewski, 1977; Jacob & Roizman, 1977; Jacob et al., 1979; Ben-Porat & Rixon, 1979).

HSV DNA synthesis conforms well to the accepted model of prokaryotic and eukaryotic DNA replication (Bramhill & Kornberg, 1988), whereby sequence-specific initiation begins with the recognition of and binding to an origin of replication by an initiator protein. There are three origins of DNA replication on the HSV-1 genome. Two copies of ori_S are located in the short repeat regions, ori_S1 between the promoters of IE genes IE175 and US1, ori_S2 between the promoters of IE genes IE175 and US12 (Spaete & Frenkel, 1982; Mocarski & Roizman, 1982a; Stow & McMonagle, 1983). The third, ori_L, maps in the long unique segment between the promoters of the two early genes UL29 and UL30 (Weller et al., 1985). Ori_L is not essential for viral growth (Polvino-Bodnar et al., 1987), and at least one copy of ori_S is dispensible (Longnecker & Roizman, 1986; Smith et al., 1989). To date, no HSV variant lacking both copies has been reported.

3.5.1 The UL9 origin-binding protein

The initiator protein is the HSV-specified origin-binding protein (OBP), the 94-kDa product of UL9. DNase footprinting and gel retardation experiments have

demonstrated that the OBP binds to two sites within ori_S (Elias & Lehman, 1988; Olivio et al., 1988; Weir et al., 1989). These sites lie within a 45-base-pair imperfect palindrome and are of identical sequence YGYTCGCACT (Koff & Tegtmeyer, 1988; Deb & Deb, 1989). More recently it has been shown that the presence of both sites is necessary for efficient origin activity, and that a third cis-acting site, also within ori_S, does not bind the OBP but is essential for efficient DNA replication (Weir & Stow, 1990; Martin et al., 1991). Dabrowski & Schaffer (1991) have found that one or more cellular factors, as yet unidentified, bind to one of the origin-binding sites and also enhance complex formation of the OBP with DNA at this site.

3.5.2 Other trans-acting viral gene products essential for replication of viral DNA

Plasmid DNAs containing either ori_S or ori_L are replicated when introduced into HSV-infected cells (Stow, 1982). Analysis in a complementation assay of cloned restriction fragments of HSV-1 DNA identified seven genes - all in the long unique region - without which there was no detectable origin-dependent amplification of DNA (Wu et al., 1988; McGeoch et al., 1988b). The seven protein products of these genes, when expressed by recombinant baculoviruses, have recently been shown to be competent for replication of a plasmid containing the viral ori_S (Stow, 1992). These genes are reviewed by Weller (1991). One of these genes, UL9, has already been discussed. The other six genes are listed, and their products are discussed below: UL5, UL8, UL29, UL30, UL42 and UL52.

UL30: DNA polymerase

The 140-kDa polypeptide product of UL30 (Powell & Purifoy, 1977; Knopf, 1979; Quinn & McGeoch, 1985) exhibits novel DNA polymerase activity readily distinguishable from host enzymes. It is, for instance, inhibited by the pyrophosphate analogue phosphonoacetic acid (PAA) (Hay & Subak-Sharpe, 1976), thus affording the

basis for a convenient experimental method of preventing viral DNA replication. The enzyme has intrinsic 3'-5' exonuclease activity serving a proofreading function (Knopf, 1979; O'Donnell et al., 1987b). It also has intrinsic ribonuclease activity that specifically degrades RNA-DNA heteroduplexes as well as DNA duplexes in the 5'-3' direction (Crute & Lehman, 1989), thus enabling removal of the RNA primers that initiate synthesis of Okazaki fragments during lagging strand synthesis. When expressed in yeast or by in vitro transcription and translation the polymerase is enzymatically active (Haffey et al., 1988; Dorsky & Crumpacker, 1988). However, other virus-encoded proteins may act to increase the efficiency of the polymerase or to modify its activity. In one report, the HSV-2 DNA polymerase was co-purified with a 55-kDa protein later identified as the product of the gene UL42, also essential in DNA synthesis (Vaughan et al., 1984), and this finding has been confirmed by Gallo et al. (1988) in respect of the type 1 proteins (see section 5.2.3). DNA polymerase also associates with ICP8, the product of UL29, and its activity is greatly stimulated by this protein (O'Donnell et al., 1987a). Recently it has been shown that although DNA polymerase exhibits intrinsic nuclear localising capability, correct localisation of the polymerase to prereplicative sites within the nucleus is effected by ICP8 (Bush et al., 1991).

UL29: Single-strand-specific DNA-binding protein

The 128-kDa product of UL29 is known as the major DNA-binding protein and infected-cell protein 8 (ICP8). It binds strongly to single-stranded DNA with a stoichiometry of one ICP8 molecule per 12 nucleotides; it also binds to double-stranded DNA but not so tightly (O'Donnell et al., 1987a).

ICP8 has intrinsic ability to localise to the nucleus of infected cells. Before onset of viral DNA synthesis, it locates to nuclear framework-associated structures called prereplicative sites. During viral DNA synthesis, ICP8

localises to randomly distributed replication compartments, where it is bound to replicating viral DNA. Migration of ICP8 from prereplicative site to replication compartment is initiated with the onset of DNA synthesis, and prereplication sites are thought to serve as nuclear reservoirs of ICP8 (Quinlan et al., 1984). The work of de Bruyn Kops & Knipe (1988) has shown that ICP8 sequesters components of the cellular DNA replication apparatus to the replication compartments, and these authors suggest that the organising of structure and composition of these compartments may be a function of ICP8. In this connection the work of Bush et al. (1991) is interesting, in that they demonstrate the role of ICP8 during infection in directing HSV DNA polymerase to the replication compartments.

Evidence has been presented which implicates ICP8 in the down-regulation of expression of the IE protein Vmw175 (Godowski & Knipe, 1986), and recently results were presented suggesting that, notwithstanding the indirect requirement for ICP8 in true-late gene expression in respect of the need for prior DNA synthesis, ICP8 has a distinct role in the direct stimulation of late gene expression (Gao & Knipe, 1991). In these experiments, cells expressing a mutant ICP8 protein with a trans-dominant phenotype synthesised reduced amounts of viral DNA which should still have been sufficient to support some true-late gene expression, but virtually no true-late expression was detected. By contrast, infection in wild-type cells when viral DNA replication was reduced using PAA to a similar level to that in the mutant-ICP8 cells, significant amounts of true-late protein were expressed.

UL42: Double-stranded DNA-binding protein

The product of UL42 is a 65-kDa protein (Parris et al., 1988) known as the 65K DNA-binding protein ($65K_{DBP}$). It binds strongly to double-stranded DNA (Marsden et al., 1987), and localises to the nucleus during infection

(Olivo et al., 1989). Gallo et al. (1988) observed a physical association of the $65K_{DBP}$ with the DNA polymerase, and noted that highly purified $65K_{DBP}$ was free from polymerase activity and did indeed have intrinsic DNA-binding capabilities. The $65K_{DBP}$-polymerase complex is composed of the two proteins in a 1:1 ratio (Crute & Lehman, 1989). Purified polymerase, which clearly does possess intrinsic polymerase activity, is stimulated up to 10-fold by the addition of purified $65K_{DBP}$; similar stimulation is exhibited by plasmid-expressed polymerase in the presence of plasmid-expressed $65K_{DBP}$ (Gallo et al., 1989). The recent isolation of a deletion mutant in UL42 and a stably-transformed UL42-expressing cell line has enabled confirmation that the $65K_{DBP}$ is essential for virus replication, and suggests potential for further work in elucidation of the function of the $65K_{DBP}$ and its relationships with other DNA replication proteins by the construction of defined UL42 mutants (Johnson et al., 1991). The UL42 protein acts to increase the processivity of the DNA polymerase (Gottleib et al., 1990).

UL52, UL5, UL8: Helicase-primase complex

The three remaining genes essential for HSV-1 DNA replication, UL52, UL5 and UL8, encode proteins having molecular weights of 120, 97 and 70 kDa respectively. These three proteins copurify from infected cells and constitute the three subunits of a helicase-primase enzyme which exhibits DNA-dependent ATPase, DNA-dependent GTPase, DNA helicase and DNA primase activities (Dodson et al., 1989; Crute et al., 1988; Crute et al., 1989). All the enzymic activities of the complex are associated with the UL5 and UL52 proteins (Calder & Stow, 1990; Dodson & Lehman, 1991). A ts and a null mutant in UL8 have been used to confirm the requirement for the 70-kDa protein in viral DNA synthesis (Carmichael & Weller, 1989), and it seems that one role of this protein within the complex is to effect efficient nuclear localisation of the complex (Calder et al., 1992).

3.5.3 Other virus-specified proteins involved in DNA synthesis

HSV specifies several other proteins which play a significant role in viral DNA synthesis, although most are not essential for growth in tissue culture. An exception is the alkaline deoxyribonuclease, the product of UL12. Thus, although the seven genes discussed above are necessary and sufficient for DNA replication in transient expression assays, the DNase has been shown by marker rescue of a type 2 ts mutant in DNase activity to be essential for virus replication in tissue culture (Moss, 1986). However, experiments with a UL12 insertion mutant have shown that the DNase is not essential for viral DNA synthesis but may play a role in the processing or packaging of viral DNA into infectious virions (Weller et al., 1990).

The gene UL23 encodes the viral thymidine kinase (TK), which is not essential for growth in dividing cells but is required in serum-starved cells (Jamieson et al., 1974). However, TK is essential for normal virus multiplication in vivo (Tenser & Dunstan, 1979). The successful anti-herpetic drug acyclovir is an acyclic nucleoside analogue which is phosphorylated by the viral TK but not to any significant degree by equivalent host cell enzymes. Following conversion from the monophosphate to triphosphate form by cellular enzymes, it exhibits potent inhibition of the viral DNA polymerase (Elion, 1983). A single amino acid change in the TK protein is sufficient to eliminate sensitivity to acyclovir; such resistant strains retain full neuropathogenicity (Chatis & Crumpacker, 1991).

The gene UL2 specifies a uracil DNA-glycosylase. In a virus such as HSV with an extremely high G+C content, this enzyme plays a significant role, in that its function is to remove uracil residues from DNA during proofreading. However, this enzyme has been shown to be dispensible for growth in tissue culture (Mullaney et al., 1989).

The product of UL50 is a deoxyuridine triphosphatase (dUTPase), hydrolysing dUTP to dUMP, thus preventing dUTP

being incorporated into DNA (Wohlrab & Francke, 1980; Preston & Fisher, 1984). This enzyme is not essential for growth in tissue culture (Fisher & Preston, 1986).

HSV types 1 and 2 encode their own ribonucleotide reductases (Dutia, 1983; Preston et al., 1984), which catalyse conversion of the four ribonucleoside diphosphates to their corresponding deoxyribonucleoside diphosphates, creating a pool of substrates for DNA synthesis (Thelander & Reichard, 1979). The active HSV-1 enzyme consists of two subunits, R1 the large subunit, and R2 the small subunit. Each subunit is itself a dimer: R1 of the 140-kDa UL39 gene product, R2 of the 40-kDa UL40 gene product (Ingemarson & Lankinen, 1987). A nonapeptide of sequence YAGAVVNDL, representing the C-terminal nine amino acids of R2, has been shown to inhibit the activities of both the type 1 and type 2 enzymes (Dutia et al., 1986; Cohen et al., 1986). This inhibition results from competition between the nonapeptide and R2 for interactive sites with R1 (Paradis et al., 1988). Although the viral enzyme is largely dispensible for virus growth in actively dividing cells maintained at 37°C, it is required for efficient growth in resting cells or cells maintained at 39.5°C (Preston et al., 1988; Goldstein & Weller, 1988; Jacobson et al., 1989). It is also required for the virulence of HSV in mice, and for reactivation from latent infection (Cameron et al., 1988; Jacobson et al., 1989). Thus the inhibitory nonapeptide is considered to have potential as an antiviral drug, especially in view of the fact that it also inhibits the ribonucleotide reductases of PRV (Cohen et al., 1987) and EHV (Telford et al., 1990). The investigation of this potential requires structural studies of the enzyme, and to this end both the large and small subunits have been expressed in a bacterial system and purified (Furlong et al., 1991; Lankinen et al., 1991).

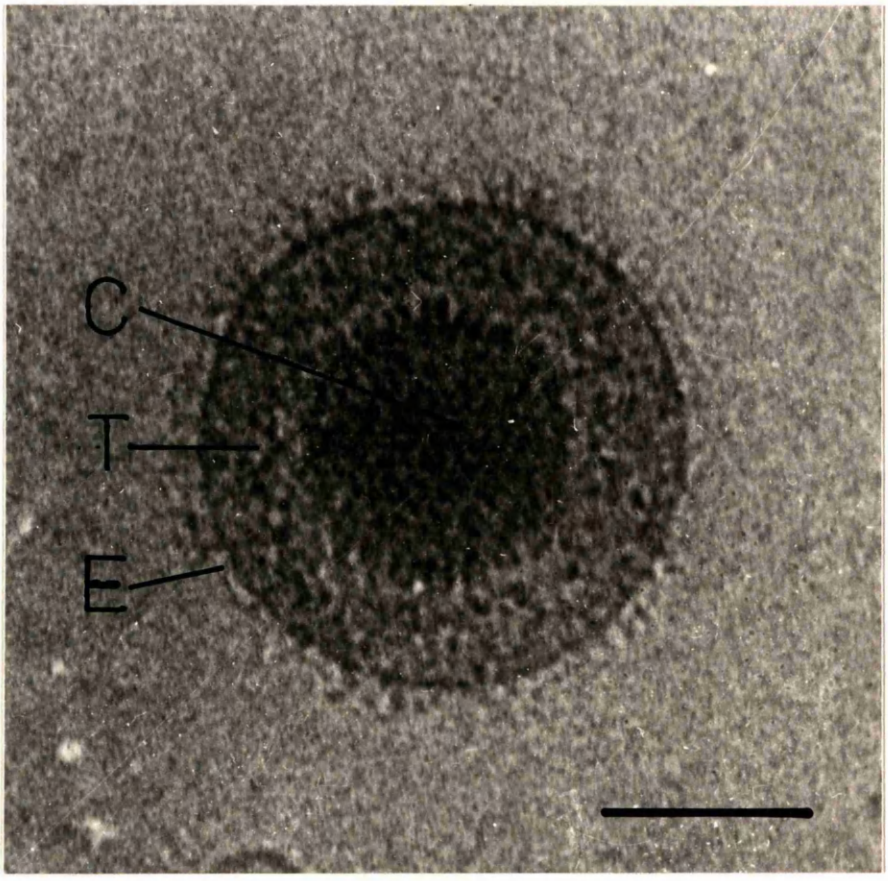

C
T
E

Figure 3. Cryoelectron micrograph of a virion of HSV-1. The major structural features, capsid (C), tegument (T) and envelope with associated glycoprotein spikes (E), are indicated. Scale bar is 100 nm. This figure is reproduced with permission from Schrag et al. (1989).

4 STRUCTURE AND ASSEMBLY

This section will contain a discussion of the main structural components of the herpes virion, and in particular of the capsid and its assembly. There will be a concentration on features of HSV-1, but because much structural research has been carried out on other herpesviruses, relevant details will be noted, and these other viruses specified. Two major reviews of structural aspects of herpesviruses are those of Dargan (1986) and O'Callaghan & Randall (1976). A recent review covering some aspects of capsid assembly and virion maturation is that of Roizman & Sears (1990).

The four structural components of herpesvirions, DNA-containing core, capsid, tegument and envelope have already been mentioned. The characteristic 'fried egg' morphology in many electron micrographs of negatively stained virus is the result of fixation and dehydration procedures; Schrag et al. (1989) present micrographs of undamaged, ice-embedded virions manifesting a spherical shape which probably represents the natural state (Figure 3). The herpes virion is a highly complex structure, purified HSV-1 virions containing approximately 33 polypeptide species (Spear & Roizman, 1972; Heine et al., 1974; Cassai et al., 1975; Powell & Watson, 1975; Marsden et al., 1976).

4.1 Core

The DNA of mature virions is contained within the capsid in a structure termed the core. Until recently the core was thought to consist of a cylindrical protein plug around which the DNA was spooled. Details of this model are reviewed by Dargan (1986). The plug was first described by Furlong et al. (1972), and was conjectured to be composed of the capsid protein VP21 (Furlong et al., 1972; Gibson & Roizman, 1972).

Although the cylindrical plug continued to be reported by many workers in a variety of herpesviruses, an inconsistency was noted in this model in that the estimated volume of the DNA molecule was almost as large as the observed volume of the core, thus leaving little space for any appreciable amount of core protein (Gibson & Roizman, 1973). Core protein was felt to be necessary in order to effect condensation of the viral DNA into a sufficiently tight configuration to allow packaging inside the capsid. The demonstration by Gibson & Roizman (1971; 1973) that the highly basic polyamine spermine is present in the capsid in amounts sufficient to neutralise 40% of the electronegativity of the DNA offers a partial solution. The small size of the spermine molecule would allow it to fit between the strands of the DNA spool. More recently doubt was cast over the status of the core by Puvion-Dutilleul *et al*. (1987). They showed, in several herpesviruses including HSV-1, that the configuration of encapsidated DNA was critically determined by the dehydrating agent used in the fixation procedures for electron microscopy. Thus by using methanol as the dehydrating agent the plug with spooled DNA previously observed was replaced by a swollen mass with an intricate internal structure of DNA fibres. This structure completely filled the internal cavity of the capsid. They were unable, however, to determine which structure most closely represents the state of the DNA *in vivo*.

The most recent model of the herpesvirus core is presented by Booy *et al*. (1991). They also had noted that the volume of the internal cavity of the capsid is probably not sufficient to accomodate a protein plug. Using the data of Lepault *et al*. (1987) they calculated the packing density of DNA inside the capsids of phages T4 and λ. The same calculation for HSV-1 revealed that the inner dimensions of the capsid as determined by Schrag *et al* (1989) would necessitate a DNA packing density 2.5 times higher than that calculated for the two

phages. They determined the configuration of DNA within the capsid by cryoelectron microscopy and computerised image reconstruction. The images obtained of the core showed a uniformly dense ball of DNA with an interstrand spacing of approximately 2.6 nm. The internal diameter of the capsid was found to be 86 nm, compared to the figure of 62 nm given by Schrag et al. (1989), and the mass of DNA extended radially as far as the inner surface of the capsid. The uniform density of the DNA ball did not show a central region attributable to a protein plug, and since there were no candidate proteins for such a plug in their reconstructions, the authors proposed that the core contains no protein at all, but comprises only DNA. These observations reveal that the internal organisation of encapsidated HSV-1 DNA is essentially identical with that of phages T4 and λ (Lepault et al., 1987). Booy et al. (1991) note the work of Puvion-Dutilleul (1987), and suggest that the 'core' of cylindrical protein plug is an artefact of collapsed DNA produced during specimen preparation.

4.2 Tegument

The term tegument was first used to define the material between capsid and envelope by Roizman & Furlong (1974). The existence of tegument as a distinct virion component was not fully recognised by early observers, who, for instance, saw 'a zone of low density', present only in some virions (Nii et al., 1968a); the review of Roizman (1969) discusses the uncertain existence of an 'inner envelope'. This is due to a lack of distinctive features in the tegument - it is usually described as being 'amorphous'. Tegument thickness varies amongst the herpesviruses, and great variation is often observed between individual virions in the same preparation of HSV, but this is probably an artefact resulting from specimen damage - Vernon et al. (1982) noted that tegument morphology and thickness were dependent upon the

method of preparation for electron microscopy. The observations of Schrag et al. (1989) showed that undamaged HSV-1 virions in ice-embedded sections are roughly spherical in shape, with a fairly even distribution of tegument of approximate thickness of 447 A. Working with EHV-1, Vernon et al. (1982) showed that the tegument exists as a defined layer with a degree of structural integrity, and that it is probably attached to the capsid vertices. Shrinkage of the tegument during critical point drying suggested that the tegument has a high water content. That the tegument possesses structural integrity independent of either envelope or capsid has been shown by McLauchlan & Rixon (1992). Using NP40, they removed the envelope from L-particles (which consist of tegument and envelope but do not contain capsids), and found that the residual tegument material retained its shape and stability. It is not known which components are essential to the structural integrity of the tegument.

The tegument is often described as 'proteinaceous', but the exact protein composition was hard to determine, due to the difficulty of obtaining purified tegument material free from other virion components. In practice, proteins seen in virion protein profiles are defined as tegument proteins if they are not known to be part of either the capsid or the envelope. A recent aid in the identification of tegument proteins is the discovery of L particles (Szilagyi & Cunningham, 1991). Proteins known to be located in the tegument are commonly designated by the nomenclature originally used for virion protein profiles (Spear & Roizman, 1972; Heine et al., 1974). VP1 is the 273-kDa product of UL36, essential for release of viral DNA from capsids at nuclear pores and VP16 is the trans-activator Vmw65. The vhs protein is a component of virions and has recently been shown to be located in the tegument (McLauchlan et al., 1992). These three proteins are discussed elsewhere in this text. Further components of the tegument are the related Vmw82 and Vmw81, the 82-

and 81- kDa products of the gene UL47 (McLean et al., 1990). These proteins are abundant components of the virion, yet can be stripped from virions by NP40. They are iodinated in virions in the presence but not in the absence of NP40, indicating a location in the tegument (McLean et al., 1990). Experiments with mutants in UL47 reveal that this gene functions to enhance significantly the efficiency of Vmw65-mediated transactivation during the infection process (Zhang et al., 1991). These authors suggest also that the UL46 gene product might also be a tegument protein. By comparison with other published virion protein profiles, McLean (1990) suggested that Vmw82 and Vmw81 are equivalent to VP13 and VP14 respectively, and this was confirmed by Whittaker et al. (1991) who showed that VP13 and VP14 are products of UL47. Whittaker & Meredith (1990) report purification of VP13, VP14 and another tegument protein, VP22, by reverse-phase high performance liquid chromatography. In a further report, Meredith et al. (1991) show that VP13 and VP14 are phosphorylated in virus-infected cells, but not in preparations of purified virus, and that VP13, VP14 and VP22 contain O-linked oligosaccharide units which suggest a possible role for these proteins in transcriptional control. VP22 was recently shown to be encoded by the gene UL49 (Elliott & Meredith, 1992).

Several proteins have been assigned to the tegument by workers not using the VP nomenclature, and they remain uncorrelated with the original virion protein profiles. The protein kinase activity associated with herpes virions was located by Lemaster & Roizman (1980) to de-enveloped particles of HSV -1 and -2 consisting of capsids with attached tegument material. Stevely et al. (1985) located this activity in PRV to the tegument, and showed that both HSV-1 and PRV virions contained several distinct protein kinases, one of which was the host cell-derived casein kinase II. HSV-1 does specify at least one protein kinase, in the gene US3 (Frame et al., 1987; Purves et al., 1987), which is a homologue of a PRV-

specified protein kinase (reviewed by Leader & Katan, 1988). The US3 protein kinase post-translationally modifies the phosphoprotein encoded by the UL34 gene (Purves et al., 1991). The product of UL13, which appears to be a protein kinase which undergoes autophosphorylation, has been shown to be a component of the virion (Smith & Smith, 1989; Cunningham et al., 1992). The 10-kDa phosphorylated product of the gene US9 studied by Frame et al. (1986) is also thought to be a tegument protein - although present in virions and in NP40-solubilised extracts of envelope/tegument, this protein is not found in capsids. It does, however, associate with unenveloped capsids within the nucleus. This protein, known as 10K, undergoes extensive post-translational processing in connection with phosphorylation, but only the lower molecular weight forms are incorporated into virions. The function of 10K is not known. The gene UL11 encodes two myristylated polypeptides, with apparent molecular weights of 13-16 kDa (MacLean et al., 1989; MacLean et al., 1992). These are present in L particles, and separate partially with the membrane fraction of purified virions and partially with the NP40-insoluble fraction. They are thus are deemed to be tegument components (Maclean et al., 1992).

4.3 Envelope

Virions of HSV-1 have a diameter ranging from 1875-2370 A with a mean of 2145 A (Schrag et al., 1989), and as in all herpesviruses they are completely enclosed by a trilaminar membrane (Morgan et al., 1954; Wildy et al., 1960). This envelope is host cell-derived (see section on envelopment) but is modified by the presence of viral proteins. A characteristic of herpesvirus envelopes is the possession of numerous spikes distributed over their surface, often clearly visible in the electron microscope (Wildy et al., 1960; Schrag et al., 1989). The study of Stannard et al. (1987) showed that the various viral

glycoproteins, major polypeptide components of the envelope, are constituents of these spikes. Monoclonal antibodies coupled to colloidal gold permitted identification of glycoproteins present in the different structures projecting from the envelope. The most prominent spikes, 14 nm long with a flattened T-shaped top, contain gB. Long spikes, some up to 24 nm, contain gC, and the shortest spikes, 8-10 nm long, contain gD. The gC spikes have an even distribution whereas the gB and gD spikes appear to be clustered, although this may be an artefact of fixation procedures (cp Schrag et al., 1989). Other shapes of spike were seen in this study which did not bind the monoclonals used, and it was suggested that these spikes comprised other glycoproteins.

Nine glycoproteins have been identified in HSV-1. Four are encoded by genes in the unique long region; these are gL (UL1), gH (UL22), gB (UL27) and gC (UL44). Four are encoded by genes in the unique short region: gG (US4), gD (US6), gI (US7) and gE (US8) (McGeoch et al., 1988a; Hutchinson et al., 1992). The ninth glycoprotein is predicted to be specified by the gene US5 (McGeoch et al., 1985), and Roizman & Sears (1990) state that it has been identified and designated gJ.

Glycoproteins B, D and H were discussed in the sections on attachment and penetration. They are all essential for viral replication in tissue culture. Glycoprotein C, whilst dispensible for lytic infection, has a function in attachment. Glycoprotein B has been shown to exist as homodimers (Sarmiento & Spear, 1979; Highlander et al., 1991). Glycoprotein G is dispensable for growth in cell culture, as is the product of US5 (Longnecker & Roizman, 1987; Weber et al., 1987), as well as gE (Longnecker & Roizman, 1986) and gI (Longnecker et al., 1987). The functions of these glycoproteins are unclear, but it is thought they may be involved in alternative pathways of attachment and/or penetration, enabling the virus to make

use of a greater variety of cell receptors on different cell types. Glycoprotein L forms a complex with gH and is essential for the correct processing and surface expression of gH (Hutchinson et al., 1992).

On the basis of the published DNA sequence of HSV-1, the genes UL10, UL20, UL43 and UL53 were predicted to encode proteins with multiple hydrophobic regions which would have potential for interaction with membranes (McGeoch et al., 1988), They are predicted to cross a membrane several times. Their properties suggest a role in signal transduction or as ion channels, or an involvement in tegument-envelope interactions. UL10 and UL43 have been shown to be dispensable for growth in tissue culture (MacLean et al., 1991; Baines & Roizman, 1991). UL20 is not necessary in tissue culture for assembly, envelopment or maturation of infectious virions but in some cell lines it is essential for egress of mature virions from the infected cell (Baines et al., 1991). However, it seems that UL53 may be an essential gene (MacLean et al., 1991), since these workers were unable to obtain lacZ insertion mutants in this gene. An antiserum raised against the carboxy-terminal region of the UL10 ORF detected the gene product in infected-cell extracts but not in virions. Similar experiments respecting UL20 suggest that this gene product is a component of the virion (MacLean et al., 1991). The UL20 gene product has been shown to be membrane-associated in infected cells, and it shares some of the properties of intrinsic membrane proteins, in that it is resistant to extraction with alkali and that it aggregates on boiling with SDS (Baines et al., 1991). The gene UL45 was predicted to encode a protein with a hydrophobic N terminus, and was also thought potentially to be membrane-associated (McGeoch et al., 1988). Subsequently, the product of UL45 was shown to be present in the virion (McLean, 1990).

4.3.1 L Particles

A non-infectious herpes simplex virus-related particle

has been described by Szilagyi & Cunningham (1991) which consists of an envelope and some tegument material but no capsid or DNA. Although containing many of the usual envelope and tegument proteins, these particles, termed L particles, contain in addition at least five phosphoproteins not found in virions. One of these is the IE protein Vmw175 (McLauchlan & Rixon, 1991). It is interesting to note that Vmw175 has been reported to be a minor component of HSV tegument (Yao & Courtney, 1989; Yao & Courtney, 1991), and the VZV homologue of Vmw175, IE62, has been shown to be a major component of VZV tegument (Kinchington et al., 1992). This may in fact represent the presence of contaminating L particles in the virion preparations used for these experiments. The status of L particles is uncertain, and the presence of the additional proteins may indicate differences in the pathways of assembly of virions and L particles. L particles may have potential as a vaccine, since when purified from infectious virions they still possess many immunogenic virion proteins. A lack of DNA also means that L particles have reduced potential for oncogenicity. However, there is some evidence that a component of the HSV virion has mutagenic properties, with the implication that this may play a role in cellular transformation by mutagenising cellular genes (Clarke & Clements, 1991). Experiments with L particles have shown them to be significantly less mutagenic than wild type virus, implying that the mutagenic activity is due either to a capsid protein or to the viral DNA (Clarke, 1990). The residual mutagenic activity of the L particles was probably due to wild type virus contaminating the L particle preparation, indicating that great care would be necessary in preparing L particles for use in vaccinations. One way to reduce the background of infectious virions is to make use of the mutant ts1201. At the NPT, this mutant produces virions only at low levels, but L particles are produced in quantities similar to those generated by wild-type virus (Rixon et al., 1991). L particles, although non-infectious, have

Name	Gene	M_r (kDa) estimated by:			
		Gibson & Roizman, 1972	Zweig et al., 1979a	Cohen et al., 1980	Rixon et al., 1990
VP5	UL19	155	155	154	150
VP19C	UL38	53	50	50	54
VP21	UL26	44		40	42
VP22a	UL26.5	38.8	40	38	40
VP23	UL18	33	32	33	34
VP24	UL26	25	25	26	24
VP26	UL35		12	12	12

Table 1. The capsid proteins of HSV-1

been shown to facilitate infection by HSV-1 by supplying two tegument proteins , VP16 and the vhs protein (McLauchlan et al., 1992).

4.4 Proteins of the capsid

Studies on the composition and structure of capsids of HSV-1 have identified seven capsid proteins, although only five are associated with all capsid forms. These proteins are listed in order of descending molecular weight in Table 1. Properties of each of these proteins and the genes encoding them will be discussed in turn, before a separate consideration of structural aspects of the capsid.

4.4.1 VP5

In this thesis the nomenclature of capsid proteins follows that of their first description (Spear & Roizman, 1972; Gibson & Roizman, 1972). It is of some importance to note other names which have been used for these capsid proteins; in the case of VP5 these include ICP5 (Honess & Roizman, 1973), VP1 (Powell & Watson, 1975), ICP155 (Powell & Purifoy, 1976), Vmw155 (Marsden et al., 1976), M155 (Marsden et al., 1978), p155 (Zweig et al., 1979a), NC1 (Cohen et al., 1980) and p5 (Newcomb et al., 1989). It is generally referred to as the major capsid protein (MCP).

The gene encoding VP5 was first mapped on the genome by Morse et al. (1978) and Marsden et al. (1978) in an analysis of HSV intertypic recombinants. The approximate map locations of the 5' and 3' ends of an unspliced 6-kb mRNA originating in this region were determined by Costa et al. (1981) by S1 nuclease mapping. Costa et al. (1984) mapped this mRNA more accurately, and following in vitro selection and translation of the mRNA they were able to precipitate the 155-kDa product using an antibody raised against VP5 purified from capsids. A comparison of

tryptic peptides of the translation product with those of VP5 confirmed their identity. The DNA sequence of this ORF was presented by Davison & Scott (1986b), and was designated UL19 by McGeoch *et al*. (1988). The gene is leftward oriented with a single 1374-codon ORF, and is 3'-coterminal with the distal gene UL18 (Costa *et al*., 1981; Costa *et al*., 1984), which specifies the capsid protein VP23 (Rixon *et al*., 1990). The predicted molecular weight of the product of UL19 is 149075, which is in close agreement with the observed size of VP5 made in HSV-1-infected cells - 150 kDa (Rixon *et al*., 1990), 155 kDa (Gibson & Roizman, 1972; Killington *et al*., 1977; Zweig *et al*., 1979a) and 154 kDa (Cohen *et al*., 1980). This suggests that extensive post-translational modification does not occur. Zweig *et al*. (1979) did not detect a type-specific difference in molecular weight of the MCP, but Killington *et al*. (1977) found the M_rs of HSV types 1 and 2 to be 155 and 158-160 kDa respectively. UL19 is considered to be an essential capsid gene after the report of Weller *et al*. (1987) which described two capsid negative *ts* mutants, *ts*G3 and *ts*G8, which synthesised reduced levels of VP5. A lesion was mapped in both of these mutants to a region containing part of genes UL18 and UL19. However, UL18 is now known to be an essential capsid gene (Rixon *et al*., 1990). These authors stated that each mutant had a second lesion, in ICP36 (*ts*G3) and in ICP40 (*ts*G8), on the basis that the mutants produced reduced levels of these proteins at the non-permissive temperature. Celluzzi & Farber (1990) report that production of another capsid protein, VP19C, is affected in these two mutants. Clearly more work is necessary to clarify the status of *ts*G3 and *ts*G8. In view of the proposed role of VP5 as the constituent of the hexameric capsomers, it would appear that UL19 is indeed an essential capsid gene.

UL19 is widely regarded as an early-late gene (Costa *et al*., 1985a), and production of UL19 mRNA is greatly reduced in the presence of inhibitors of DNA synthesis

(Holland et al., 1980; Conley et al., 1981). VP5 is readily detectable in infected cells from 2 hours post-infection (Honess & Roizman, 1974; Bibor-Hardy et al., 1985a), and Harris-Hamilton & Bachenheimer (1985) detected VP5 mRNA at 2 hours p.i. The HCMV MCP mRNA is only detectable at late times p.i., and is not detectable at all in the presence of inhibitors of DNA synthesis (Rudolph et al., 1990b), and the MCP of EBV is not detectable in the presence of DNA synthesis inhibitors (Takada et al., 1983; Vroman et al., 1985).

Major capsid protein counterparts of VP5 have been identified in many other herpesviruses, and comparisons at the sequence level are available in a number of cases. The VZV MCP has predicted and apparent molecular weights of 154,971 (Davison & Scott, 1986a) and 155,000 (Zweerink & Neff, 1981; Grose et al., 1983) respectively. The gene sequence was determined by Davison & Scott (1986a) and designated gene 40, and there is a 50% amino acid homology between the MCPs of HSV-1 and VZV (Davison & Scott, 1986b). The MCP of PRV has predicted and apparent molecular weights of 146 and 142 kDa respectively, and has 58% amino acid homology with VP5 (Ladin et al., 1982; Lomniczi et al., 1987; Yamada et al., 1991). The MCP of HCMV has only a 25% amino acid homology with that of HSV-1 and has predicted and apparent molecular weights of 154,000 and 153,000 respectively (Chee et al., 1989; Gibson, 1983). In the published analysis of the genome sequence of HCMV the MCP gene is designated UL86 (Chee et al., 1990). The MCP of HHV-6 has an amino acid homology of 43.8% with its HCMV counterpart, but only 24.7% with that of HSV-1, and has predicted and apparent molecular weights of 152,000 and 135,000 respectively (Littler et al., 1990). The HHV-6 MCP gene is designated reading frame 4L by Lawrence et al. (1990). The MCP of EBV has a 27% amino acid homology with that of HSV-1 (Baer et al., 1984; Davison & Scott, 1986b), and has predicted and apparent molecular weights of 153,909 and 160,000 respectively (Baer et al., 1984; Dolyniuk et al., 1976),

and in the published DNA sequence of EBV the MCP gene is designated BcLF1 (Baer et al., 1984). Random sequencing of MDV and HVT has revealed that there are homologues of VZV gene 40 in both viruses (Buckmaster et al., 1988). A similar analysis of MHV-68 revealed a homologue of the EBV gene BcLF1 (Efstathiou et al., 1990). The MCP gene of EHV-1 is gene 42 (personal communication, Dr E.Telford). The smallest known herpesvirus MCP is that of CCV (Telford et al., 1992), which has a molecular weight of approximately 127 kDa (personal communication, Dr A.J.Davison). It is interesting to note that the DNA sequence of CCV does not reveal any gene homologous to other known herpesvirus MCP genes (Davison, 1992).

Studies of human immune sera have shown VP5 to be a dominant antigen (Eberle & Mou, 1983), as is the case with the MCPs of EBV (Mueller-Lantzsch et al., 1979) and of VZV (Zweerink & Neff, 1981; Weigle & Grose, 1984). In contrast is the MCP of HCMV, which is of limited antigenicity in the natural host (Jahn et al., 1987), and that of HHV-6, which elicits a strong response in only a limited number of patients (Shiraki et al., 1989; Littler et al., 1990). Antigenic cross-reactivity of the HSV MCP has been demonstrated with the MCPs of VZV (Shiraki et al., 1982; Vafai et al., 1990), bovine mammillitis virus (and weakly with PRV and equine abortion virus) (Yeo et al., 1981) and HCMV (Rudolph et al., 1990a). Mayyasi et al. (1967) failed to demonstrate antigenic cross-reactivity between capsids of HSV and EBV, and in the epitope-mapping study of Middeldorp & Meloen (1988), although ten dominant antigenic epitopes were identified in the EBV MCP, only one was in a region of sequence homology with the MCPs of HSV-1 and VZV.

The VP5 of HSV types 1 and 2 has been reported to be DNA-binding (Powell & Purifoy, 1976; Purifoy & Powell, 1976).

4.4.2 VP19C

A 53-kDa capsid protein was first reported by Gibson &

Roizman (1972) and was named VP19. However, they thought that a second, non-capsid protein was migrating at the same position as VP19. Heine et al. (1974) detected three comigrating proteins, the third of which they identified as the capsid protein, renaming it VP19C. Amongst other terms which have been used to denominate this protein are ICP31 (Honess & Roizman, 1973), Vmw53 (Marsden et al., 1976), p50 (Zweig et al., 1979a), NC2 (Cohen et al., 1980) and ICP32 (Braun et al., 1984a).

The first reported mapping of VP19C was that of Costa et al. (1983). Using S1 nuclease analysis they mapped four partially overlapping mRNAs to between 0.16 and 0.19 map units. Two of these were in vitro translated, and the translation products shown to react weakly with the polyclonal rabbit serum raised against VP19C by Cohen et al. (1980), and they concluded that one or both of these mRNAs encoded the capsid protein. This work was based on the assumption that the gene specifying VP19C had previously been mapped to this region by Lemaster & Roizman (1980). This, however, was a profound misinterpretation of Lemaster & Roizman's work, since they had specifically stated that the gene they had mapped was that encoding VP18.8, a phosphoprotein which comigrated with VP19C in virion protein profiles. Knipe et al. (1981) mapped ICP32 using intertypic recombinants to a region between 0.58-0.61 map units. Braun et al. (1984a) identified VP19C with ICP32 on the basis of these mapping data, noting that the genome fragment under study specified only one protein of the correct molecular weight, and showing that VP19C in capsid profiles comigrated with ICP32 in infected-cell profiles. ICP32 had also been mapped some years earlier by marker rescue to between approximately 0.49-0.52 map units by the same research group (Morse et al., 1978). None of these three map locations is correct - the first suggestion of the true map location of VP19C being made by Pertuiset et al. (1989). These workers described a ts mutant, ts2, which did not assemble capsids at the non-permissive

temperature and which had a lesion in the region between 0.553-0.565 map units, corresponding to the ORF UL38. Because the only known structural protein with a molecular weight comparable to that predicted for the UL38 gene product was VP19C, it was suggested that UL38 might specify this protein, although adherence to the mapping data for VP19C of Knipe et al. (1981) and Braun et al. (1984a) led them to reject this suggestion. The proposal of UL38 as the gene specifying VP19C was investigated further by Yei et al. (1990), who used antibodies predicted to react with the UL38 gene product to demonstrate that this gene product in infected-cell profiles is the protein ICP32. However, the claim of these authors to have established that UL38 encodes the capsid protein VP19C rested upon the earlier identification by Braun et al. (1984a) of VP19C with ICP32, and was not well-founded. Convincing evidence that the capsid protein VP19C is the product of UL38 was furnished by Rixon et al. (1990), who extracted VP19C from protein gels of purified capsids and sequenced the amino terminus of the purified protein. Comparison of the amino acid sequence obtained for VP19C with that predicted for the product of UL38 confirmed its identity.

UL38 is rightward oriented with a single 465-codon ORF. Two unspliced mRNAs, having a common 5' end, originate from UL38. The first is of 1.9 kb and the second is a 7-kb transcriptional readthrough product, and is 3'-coterminal with the distal genes UL39 and UL40 (Anderson et al., 1981). Northern blotting analysis is claimed to have shown UL38 to be regulated as a true-late gene (Anderson et al., 1980; Flanagan et al., 1991). However, small amounts of the 1.9-kb UL38 mRNA were clearly detectable in the presence of an inhibitor of DNA synthesis. Yei et al. (1990) showed that in HSV-1, some ICP32 was made in the presence of an inhibitor of DNA synthesis, but they could not detect the HSV-2 protein under equivalent conditions. UL38 was again claimed to be regulated as a true-late gene on the basis of Northern

blotting analysis by Goodart et al. (1992). However, small amounts of mRNA produced under the control of the UL38 promoter were clearly detectable in the presence of an inhibitor of DNA synthesis. The designation of UL38 as a true-late gene can also be questioned on the grounds that whilst HSV-1 capsid assembly is dependent upon expression of UL38 (Pertuiset et al., 1989), it is not dependent upon virus DNA replication (reviewed by Dargan, 1986).

The predicted molecular weight of the UL38 gene product is 50260. The 1.9-kb and 7-kb mRNAs both specify a 54-kDa protein on in vitro translation (Anderson et al., 1981). The size of VP19C as measured on protein gels of purified capsids is of the same order - 53 kDa (Gibson & Roizman, 1972), 50 kDa (Zweig et al., 1979a) and 54 kDa (Rixon et al., 1990), suggesting that extensive post-translational modification does not occur. Yei et al. (1990) state that the mobility of the HSV-2 ICP32 increases during the course of infection, possibly reflecting the use of different translational initiation sites or proteolytic processing of a precursor. They did not detect similar changes in the type 1 protein. It is interesting to note that Celluzzi & Farber (1990) state that the type 1 mutants tsG3 and tsG8 both manifest a shift in mobility of this capsid protein from 53 kDa at the permissive to 51 kDa at the non-permissive temperature.

VP19C has been shown to be covalently linked by disulphide bonds to VP5 in capsids of HSV-2 (Zweig et al., 1979a), and the type 1 and type 2 proteins have been reported to be DNA-binding (Braun et al., 1984a).

A number of genes homologous to UL38 have been identified in other herpesviruses and sequenced. The HSV-2 UL38 has 78% amino acid homology with the HSV-1 gene (Yei et al., 1990). The VZV gene 20 (Davison & Scott, 1986a) shares 34% amino acid identity (McGeoch et al., 1988), and the EBV gene BORF1 (Baer et al., 1984) shares 22% amino acid

identity. Random sequencing of MDV revealed a counterpart of VZV gene 20 (Buckmaster et al., 1988). The EHV-1 homologue is gene 22 (personal communication, Dr E.Telford). EHV-1 capsids contain a 59-kDa protein (Telford et al., 1992) (Perdue et al., 1974; Perdue et al., 1975). In the case of HCMV, a potential counterpart (UL46) was identified on a positional basis, but there was no significant homology with the other known sequences (Chee et al., 1990). This is especially interesting, given the essential structural role of VP19C in HSV-1 capsids (Pertuiset et al., 1989), and in the light of reports that neither human nor simian CMV capsids contain a protein of size comparable to VP19C (Gibson, 1981; Irmiere & Gibson, 1985). EBV capsids contain a 52-kDa protein (Dolyniuk et al., 1976), but the predicted molecular weight of gene BORF1 is 39,191 (Baer et al., 1984). Amongst those herpesviruses for which sequence data are not available, possible VP19C-counterpart capsid proteins have been reported in EHV-3 (59-kDa) (Allen & Bryans, 1976) and in PRV (63 kDa - Stevely, 1975) (62 kDa - Ladin et al., 1982). A 62-kDa protein produced by *in vitro* translation of an mRNA transcribed from a cloned fragment of the PRV genome was immunoprecipitated by antiserum raised against purified PRV capsids (Lomniczi et al., 1987). It is interesting to note that the equine CMV (EHV-2) has a capsid protein of 52 kDa (Caughman et al., 1984). The DNA sequence of CCV does not show homology with any herpesvirus structural genes (Davison 1992), and it is interesting to note that CCV capsid protein profiles do not show any protein likely to be a counterpart of VP19C (personal communication, Dr A.J.Davison). The herpesvirus capsid is a highly defined structure which presumably means that architectural constraints select against gross variations in the conformations of constituent proteins (Heine et al., 1974; Dargan, 1986), so it seems unlikely that an essential structural component in one type of herpesvirus would be dispensible in another. However, amongst those genes of known sequence, the UL38 equivalents do vary in size much more than the other known capsid genes. The

largest is VZV gene 20, having 483 codons. HSV-2 UL38 has 466, and HSV-1 UL38 has 465. EHV-1 gene 22 also has 465 codons. EBV BORF1 has 364 and HCMV UL46 has only 290. The degree of relatedness of these genes is generally low, with the exception of HSV -1 and -2, with the N-terminal regions in particular showing considerable divergence. Further understanding of the structure of herpesvirus capsids and of the role of VP19C is necessary in order to resolve this anomaly.

4.4.3 VP21

A 44-kDa capsid protein was detected by Gibson & Roizman (1972) and named VP21. Other names given to this protein have been ICP34 (Honess & Roizman, 1973), Vmw43 (Marsden *et al.*, 1976), p45 (Zweig *et al.*, 1979b) and NC3 (Cohen *et al.*, 1980). It is not an essential structural component of the capsid, not being present in all capsid forms (Gibson & Roizman, 1972).

Probable counterpart proteins have been identified in capsids of the following herpesviruses: simian CMV strain Colburn (45 kDa), but not HCMV strain AD169 (Gibson, 1981; Irmiere & Gibson, 1985), EBV (47 kDa) (Dolyniuk *et al.*, 1976), PRV (41 kDa) (Stevely, 1975), EHV-1 (46 kDa, 47 kDa) (Perdue *et al.*, 1974; Perdue *et al.*, 1975) and EHV-3 (48 kDa) (Allen & Bryans, 1976).

Several polypeptide species have been identified in HSV-1-infected cells which have molecular weights of about 43 kDa, and this has been the source of some confusion. VP21 has been tentatively identified with the viral thymidine kinase, but this suggestion is now rejected (reviewed by Dargan, 1986). An infected-cell protein with DNA-binding properties identified by Bayliss *et al.* (1975) was said to be VP21, but Braun *et al.* (1984a) could not detect DNA-binding activity in the protein present in capsids. The molecular weight of VP21 present in capsids as measured by other workers agrees well with the original observation: 45 kDa (Zweig *et al.*, 1979b),

40 kDa (Cohen et al., 1980) and 42 kDa (Rixon et al., 1990). As discussed above, VP21 was at one time thought to be the constituent protein of the virion core, but this theory is now discounted. Following the report of Zweig et al. (1979b) of immunological cross-reaction between VP21 and the capsid protein VP22a, and at the suggestion of Braun et al. (1984a), VP21 is now considered to be a higher molecular weight form of VP22a, and as such it is specified within the UL26 ORF of McGeoch et al. (1988). It will be discussed further in the following section and in the Discussion.

4.4.4 VP22a

The 38.8-kDa capsid protein first described by Gibson & Roizman (1972) was named by them VP22a. It was so named because it did not have an electrophoretically identical counterpart in virion protein profiles, but was thought to be a precursor to the 37-kDa virion protein VP22, first described by Spear & Roizman (1972). The shared staining and radiolabelling properties of VP22a and VP22 lent weight to the proposal that they are related (Gibson & Roizman, 1974). Other names used to designate VP22a include P22A (Friedmann et al., 1975), p40 (Zweig et al., 1979a), NC4 (Cohen et al., 1980) Vmw40 (Preston et al., 1983) and NC3 (Dargan, 1986). It is one of a series of related proteins, the 'ICP35 family', and is electrophoretically identical to the member of that family known as $ICP35_e$ (Braun et al., 1984b). It is also known as the 'scaffolding protein' (Newcomb & Brown, 1991) and the 'assembly protein' (Gibson, 1983; Irmiere & Gibson, 1983; Preston et al., 1992). Like VP21, it is not an essential structural component of the capsid, not being present in all capsid forms (Gibson & Roizman, 1972).

The gene encoding VP22a was mapped by analysis of intertypic recombinants to the region between 0.3-0.38 map units (Marsden et al., 1978), and using the mutant ts1201 to between 0.331-0.335 map units (Preston et al.,

1983). The published sequence of the long unique region of HSV-1 identifies the gene as UL26, which is rightward oriented and unspliced (McGeoch et al., 1988a). The UL26 ORF has recently been shown to contain a second transcriptional unit (Liu & Roizman, 1991a). The promoter and coding sequences of this second reading frame, UL26.5, lie entirely within the UL26 ORF. The initiation codon of UL26.5 is located 1099 base-pairs downstream from that of UL26, and the two reading frames are in-frame and 3'-coterminal. Two mRNAs spanning this region were detected by Holland et al. (1984). These were of 2.4- and 1.4- kb, and it would seem that these are the transcripts of UL26 and UL26.5 respectively (Liu & Roizman, 1991a). Experiments using an inhibitor of DNA synthesis led Braun et al. (1984b) to conclude that the ICP35 proteins are γ polypeptides which are not stringently dependent on viral DNA replication for their synthesis. After similar experiments Holland et al. (1984) concluded that the 2.4-kb transcript is regulated with γ kinetics, and that the 1.4-kb transcript is regulated with $\beta\gamma$ kinetics. However, their use of this terminology is confused, and the kinetics of expression of UL26 and UL26.5 remain to be clearly established.

VP22a has long been known to be one of a member of related proteins ranging in size from 45 kDa-37 kDa (Preston et al., 1983; Braun et al., 1984b), which were immunoprecipitated by a monoclonal antibody against VP22a (Heilman et al., 1979; Zweig et al., 1980). VP22a was also shown to cross-react with VP21, thus confirming that they are related (Zweig et al., 1979b). Braun et al. (1984b) detected six species $ICP35_{a-f}$, which resolved into at least 20 different species on two-dimensional gel electrophoresis. The major species $ICP35_c$ and $_d$ were processed during the course of infection to become the two lowest molecular weight forms, $ICP35_e$ (38 kDa) and $_f$ (37 kDa), said to be equivalent to VP22a and VP22 respectively (Braun et al., 1984b). One of the higher molecular weight forms is presumed to be equivalent to

VP21. However, Rixon et al. (1988) showed that a monoclonal antibody which reacted with VP22a did not react with VP22, and Elliott & Meredith (1992) confirmed that VP22 is distinct from VP22a by demonstrating that VP22 is encoded by the gene UL49. Liu & Roizman (1991a) pointed out that the predicted product of UL26 is about twice as large as the observed sizes of the ICP35 proteins, the putative products of that gene. The predicted molecular weight of the UL26 gene product is 62466 (McGeoch et al., 1988a). The estimated size of VP22a in purified capsids has been given as 38.8 kDa (Gibson & Roizman, 1972), 40 kDa (Zweig et al., 1979a; Rixon et al., 1990) and 38 kDa (Cohen et al., 1980). It was this fact which prompted the discovery of UL26.5. It has now been demonstrated that the principal products of UL26.5 are $ICP35_c$ and $_d$, which subsequently have the carboxy-terminal 20 amino acids proteolytically removed, producing $ICP35_e$ and $_f$. The protease responsible for this cleavage is the 75-80 -kDa product of UL26, which, having an identical carboxy-terminal region to that of the UL26.5 products, undergoes autocleavage of its own 20 carboxy-terminal amino acids (Liu & Roizman, 1991b; Preston et al., 1992).

VP22a is not an essential structural component of the capsid (Gibson & Roizman, 1972), but it is essential for completion of the lytic cycle. The mutant described by Preston et al. (1983), ts1201, has a lesion in the UL26 ORF, and at the non-permissive temperature it assembles capsids but does not package DNA, and does not process VP22a from the higher molecular weight forms. Although the mapping data obtained using this mutant assigned VP22a to the UL26 ORF, more recent knowledge of details of this reading frame show that the lesion does not lie within UL26.5. The lesion lies upstream of UL26.5, in the amino-terminal region of UL26, affecting the protease, and it is the defective protease which is the cause of the failure of ts1201 to process VP22a properly. The role of VP22a in assembly of capsids and in packaging of DNA

will be discussed in a later section.

Possible counterparts have been described in several other herpesviruses. Ladin et al. (1982) detected a 35-kDa protein in PRV which was processed during capsid assembly from higher molecular weight forms of 41, 42 and 44 kDa. Stevely (1975), working with the same virus, also described a 41-kDa capsid protein. (These higher molecular weight species are possible counterparts of VP21). EHV-1 has been shown to have two capsid proteins of 46 and 30 kDa which are not present in all capsid forms (Perdue et al., 1975; Perdue et al., 1976). The EHV-1 DNA sequence has a UL26 homologue in gene 35 (personal communication, Dr E.Telford). Friedrichs & Grose (1986) detected (Telford et al., 1992) cross-reacting capsid proteins in VZV of 32, 34, 36 and 38 kDa. The published sequence of the VZV genome (Davison & Scott, 1986a) enabled identification of gene 33 as a homologue, having 34% amino acid identity with UL26 (McGeoch et al., 1988). The gene BVRF2 is the EBV homologue (Baer et al., 1984), and in MHV-68 there is a homologue in reading frame 14 (Efstathiou et al., 1990). The sequence of a UL26 homologue from infectious laryngotracheitis virus has been published by Griffin (1990). A recent focus of attention has been the cytomegalovirus homologue. The simian CMV (strain Colburn) has a 37-kDa assembly protein which is processed from a 40-kDa precursor (Robson & Gibson, 1989). This processing involves the cleavage of the 32 carboxy-terminal amino acids from the precursor (Gibson et al., 1990). There is also a 45-kDa protein which is an amino-terminal extension form of the assembly protein. It also is produced during capsid assembly by processing from precursor and intermediate forms of 48 and 47 kDa respectively (Schenk et al., 1991). These workers also detected 39- and 38- kDa related proteins which are either intermediates in the transition of the 40-kDa to the 37-kDa form, or aberrant forms. It may be that a multistep processing pathway is involved. The report of Welch et al. (1991a) reveals four 3'-coterminal

transcripts within the simian CMV assembly protein gene. Four overlapping proteins are predicted, of molecular weights 64, 46, 34 and 27 kDa, and in infected-cell extracts four corresponding proteins were detected using an antiserum to the common carboxy-terminal portion. A further report (Welch et al., 1991b) demonstrates that the N-terminal 249-amino-acid portion of the assembly protein contains the proteolytic activity responsible for cleavage of the C-terminal 32 amino acids. Welch et al. (1991a) also predicted four overlapping proteins to originate from the HCMV (strain AD169) homologous gene, UL80 (Chee et al., 1990), and detected the four corresponding proteins in HCMV-infected-cell extracts. They present a brief review of the homologous sequences in other herpesviruses and from a consideration of possible promoter elements within these sequences point out that the EBV gene BVRF2 could contain two reading frames, and the VZV gene 33 as many as eight.

Anti-VP22a IgG is prominent in latently infected human patients and is the first anti-HSV IgG detectable following primary infection (Eberle et al., 1985). The HCMV homologue is a major IgM-reactive antigen (Landini et al., 1985; Landini et al., 1986), and shares an antigenic epitope with a 60-kDa membrane component of human embryonic fibroblasts (Landini et al., 1991a).

4.4.5 VP23

The 33-kDa capsid protein described by Gibson & Roizman (1972) was named by them VP23. It is also variously referred to as ICP40 (Honess & Roizman, 1973), Vmw37 (Marsden et al., 1976), P38 (Marsden et al., 1978), p32 (Zweig et al., 1979a), NC5 (Cohen et al., 1980) and ICP39 (Braun et al., 1984b).

A report of the map location of VP23 derived using intertypic recombinants was published by Lemaster & Roizman (1980). The protein they mapped was found to be phosphorylated by a viral protein kinase, and was

referred to by them as ICP39.3, which they identified with the capsid protein VP23. The map location given was between 0.66-0.76 map units. However, the report of Braun et al. (1984b) retracted this finding, showing that VP23 is properly identified with ICP39, not ICP39.3, and that ICP39 is unphosphorylated. In this respect it is important to correct an error in the review of Roizman & Sears (1990). They state that the capsid protein VP23 is ADP-ribosylated, citing the work of Preston & Notarianni (1983), which was in turn based on the identification of the phosphorylated species as VP23 by Lemaster & Roizman (1980). However, that the phosphorylated species is not VP23 was demonstrated by one of the authors of the review (Braun et al., 1984b).

The gene encoding VP23 was identified as UL18 by Rixon et al. (1990), following determination of the N-terminal amino acid sequence of VP23 prepared from purified capsids. UL18 is leftward oriented with a single 318-codon ORF (McGeoch et al., 1988). A 1.5-kb unspliced mRNA originating from this ORF has been mapped; it is 3'-coterminal with the proximal gene UL19, the major capsid protein gene (Costa et al., 1981; Costa et al., 1984). Mutants in UL18 have not been reported, but, as discussed above in the section on VP5, mapping data for the two mutants tsG3 and tsG8 (Weller et al., 1987) are insufficient to exclude UL18 as the location of the lesion. UL18 is stated to be regulated as an early-late gene by Costa et al. (1985b), but experimental evidence for this claim is not presented. The kinetics of expression of UL18 are addressed in this thesis.

The predicted molecular weight of the UL18 gene product is 34,268 (McGeoch et al., 1988). This is in close agreement with the in vitro translation product (35 kDa) (Costa et al., 1984), and with the estimated size of the protein in purified capsids: 33 kDa (Gibson & Roizman, 1972; Cohen et al., 1980), 32 kDa (Zweig et al., 1979a) and 34 kDa (Rixon et al., 1990). This suggests that

extensive post-translational modification does not occur. It migrates as two species on two-dimensional PAGE (Braun et al., 1984b), is not phosphorylated, sulphated or glycosylated (personal communication, Dr A.Cross), and the N-terminus is not modified following translation (Rixon et al., 1990).

Genes homologous to UL18 are known in several other herpesviruses. Gene 41 of VZV (Davison & Scott, 1986a) shares 43% amino acid identity with UL18 (McGeoch et al., 1988). The predicted molecular weight of the product of gene 41 is 34,387 (Davison & Scott, 1986a), and purified VZV capsids have been shown to contain proteins with molecular weights of 34.5 kDa and 31.5 kDa (Zweerink & Neff, 1981). Other known homolgues are genes UL85 of HCMV (Chee et al., 1990), 3L of HHV-6 (Lawrence et al., 1990), gene 43 of EHV-1 (personal communication, Dr E.Telford) and genes in MDV and HVT corresponding to VZV gene 41 (Buckmaster et al., 1988). Capsids of both human and simian CMV contain a 34-kDa protein (Irmiere &Gibson, 1985). The EBV homologue is gene BDLF1, predicted to encode a product of molecular weight of 33,624 (Baer et al., 1984), and purified EBV capsids have been shown to contain a protein having a molecular weight of 37 kDa (Dolyniuk et al., 1976). PRV capsids contain a 32-kDa protein (Stevely, 1975; Ladin et al., 1982). A 32-kDa protein produced by in vitro translation of an RNA transcribed from a cloned fragment of the PRV genome was immunoprecipitated by serum raised against purified PRV capsids (Lomniczi et al., 1987).

4.4.6 VP24

Little is known about the 25-kDa capsid component listed by Gibson & Roizman (1972), which was called by them VP24, and is variously referred to in the literature as ICP45 (Honess & Roizman, 1973), p25 (Zweig et al., 1979a) and NC6 (Cohen et al., 1980). The reported molecular weights of this protein in capsid profiles are 25 kDa (Gibson & Roizman, 1972; Zweig et al., 1979a), 26 kDa

(Cohen et al., 1980) and 24 kDa (Rixon et al., 1990). Possible counterparts in other herpesviruses include a 28-kDa protein in capsids of human and simian CMV (Irmiere & Gibson, 1985), a 28-kDa protein in capsids of EBV (Dolyniuk et al., 1976), a 22-kDa protein in capsids of PRV (Stevely, 1975), an 18-kDa protein in capsids of EHV-1 (Perdue et al., 1975) and an 18-kDa protein in capsids of VZV (Zweerink & Neff, 1981). A recent report based on determination of amino acid sequences of fragments of VP24 prepared from purified capsids shows that VP24 is encoded by the N-terminal region of UL26 (Davison et al., 1992). The significance of this will be considered in the Discussion.

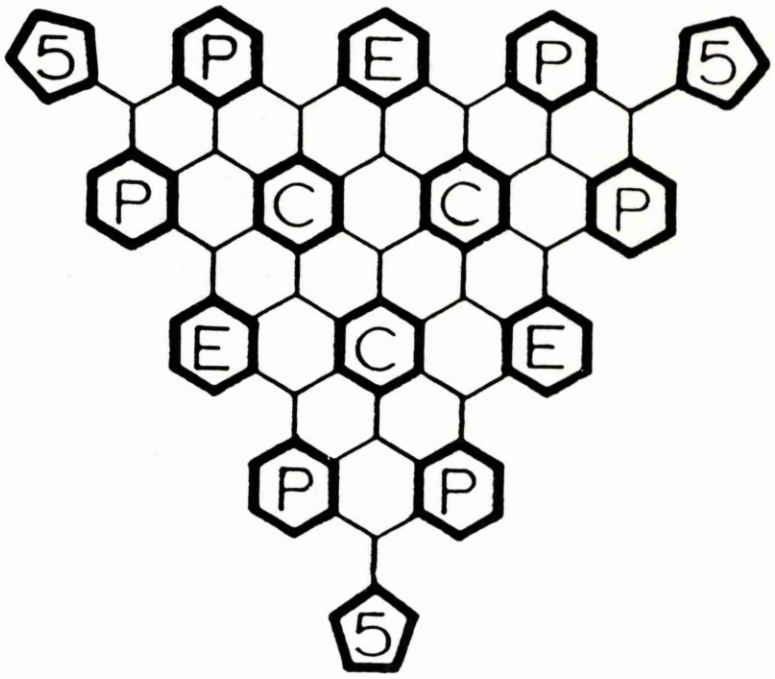
4.4.7 VP26

This protein was not detected in capsids by Gibson & Roizman (1972), nor in virions by Spear & Roizman (1972), and was first recognised as a component of HSV -1 and -2 capsids by Heilman et al. (1979), being named by them p12. Another name used of this protein is NC7 (Cohen et al., 1980). The term VP26, recently adopted for this protein by Newcomb & Brown (1989) is without precedent, but in this thesis the term VP26 will be used for consistency. The molecular weight of VP26 as determined by PAGE of purified capsids is 12 kDa (Zweig et al., 1979a; Cohen et al., 1980; Rixon et al., 1990).

A recent report suggests that VP26 may be the product of the gene UL49.5, on the basis of similarities between VP26 and the predicted polypeptide product of UL49.5 (Barker et al., 1992). However, the results of other workers show that the product of UL49.5 is probably a transmembrane protein (Barnett et al., 1992). Furthermore, VP26 was shown by J.W.Palfreyman to map between 0.44-0.52 map units (cited in Al-Kobaisi, 1989). This region of the genome contains coding sequences of several genes, including UL33 and UL35, both of which specify polypeptides of the approximate size of VP26. The gene encoding VP26 has recently been identified as UL35,

following expression of the carboxy-terminal region of UL35 as a fusion protein (McNabb & Courtney, 1992), and by determination of amino acid sequences of fragments of VP26 prepared from purified capsids (Davison et al., 1992). The study of McNabb & Courtney (1992) also found that the UL35 protein is regulated with true-late kinetics.

Gene 23 of VZV is predicted to encode a protein of 235 amino acids which has only 15% identity with the 112-amino acid predicted product of UL35 (Davison & Scott, 1986a; McGeoch et al., 1988a). The only other known homologue is gene 25 of EHV-1, which is predicted to encode a protein of 119 amino acids (personal communication, Dr E.Telford). Gene BFRF3 of EBV has no detectable homology (Telford et al., 1992) with VZV gene 23, but these genes were considered to be potential counterparts because they are aligned positionally on their respective genomes and are of similar size and are in the same orientation (Davison & Taylor, 1987). BFRF3 is predicted to encode a protein of 192 amino acids (Baer et al., 1984). As an alternative to BFRF3, Davison & Taylor (1987) also suggested that the adjacent EBV gene BFRF2 might be a counterpart of VZV gene 23, again on positional considerations, although BFRF2 is predicted to encode a protein of 656 amino acids (Baer et al., 1984). Gene UL49 of HCMV has been identified as a potential counterpart of UL35 because it is positionally conserved (Chee et al., 1990). It is predicted to encode a protein of 570 amino acids. Another, previously unidentified HCMV gene, designated UL49A, has been reported as a possible homologue of UL35 (Davison et al., 1992). UL49A is predicted to encode a protein of 109 amino acids, having an M_r of 8480. HCMV is known to have an 11-kDa capsid protein (Irmiere & Gibson, 1985), and simian CMV has a 12-kDa capsid protein (Irmiere & Gibson, 1985). Herpesvirus saimiri has a 12-kDa capsid protein (Keil et al., 1983).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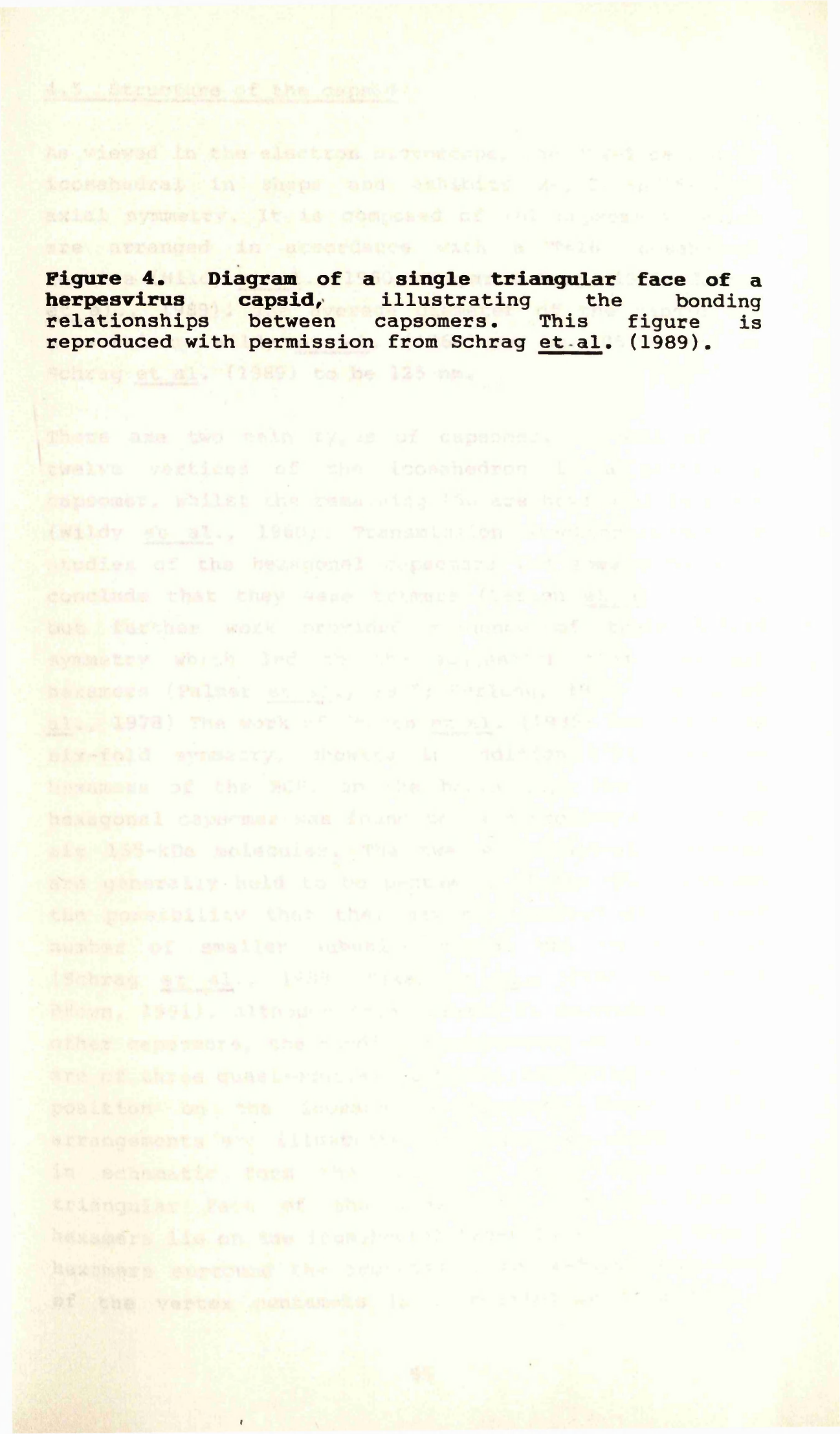


Figure 4. Diagram of a single triangular face of a herpesvirus capsid, illustrating the bonding relationships between capsomers. This figure is reproduced with permission from Schrag et al. (1989).

4.5 Structure of the capsid

As viewed in the electron microscope, the HSV-1 capsid is icosahedral in shape and exhibits 2-, 3- and 5- fold axial symmetry. It is composed of 162 capsomers, which are arranged in accordance with a T=16 icosahedral lattice (Wildy *et al.*, 1960; Caspar & Klug, 1962; Schrag *et al.*, 1989). The average diameter of the capsid was measured by Wildy *et al.* (1960) to be 105 nm and by Schrag *et al.* (1989) to be 125 nm.

There are two main types of capsomer. At each of the twelve vertices of the icosahedron is a pentagonal capsomer, whilst the remaining 150 are hexagonal in shape (Wildy *et al.*, 1960). Transmission electron microscopy studies of the hexagonal capsomers led some workers to conclude that they were trimers (Vernon *et al.*, 1974), but further work provided evidence of their 6-fold symmetry which led to the suggestion that they are hexamers (Palmer *et al.*, 1975; Furlong, 1978; Almeida *et al.*, 1978) The work of Steven *et al.* (1986) confirmed the six-fold symmetry, showing in addition that they are hexamers of the MCP, on the basis that the mass of a hexagonal capsomer was found to be approximately that of six 155-kDa molecules. The twelve pentagonal capsomers are generally held to be pentamers of the MCP, although the possibility that they may be composed of a larger number of smaller subunits cannot yet be ruled out (Schrag *et al.*, 1989; Baker *et al.*, 1990; Newcomb & Brown, 1991). Although each hexamer is surrounded by six other capsomers, the bonding arrangements of the hexamers are of three quasi-equivalent types, depending upon their position on the icosahedral lattice. These bonding arrangements are illustrated in Figure 4, which depicts in schematic form the capsomers lying on a single triangular face of the icosahedral lattice. Type E hexamers lie on the icosahedral two-fold axis, and type C hexamers surround the icosahedral three-fold axis. Each of the vertex pentamers is surrounded by five type P

hexamers (Steven et al., 1986).

The centre-to-centre distances between E, P, and C hexamers were found to be 12.2 nm by Furlong (1978) and 16.0 nm by Schrag et al. (1989), who also measured the centre-to-centre distance of P hexamers from pentamer to be 16.0 nm. Palmer et al. (1975) found the hexamer diameter to be 8-9 nm, whilst Schrag et al. (1989) found the diameter to be 11-12.5 nm and the height to be 12.5 nm. A channel runs through the centre of each pentamer and hexamer, which has a diameter of approximately 4-5 nm (Wildy et al., 1960; Palmer et al., 1975; Schrag et al., 1989). Baker et al. (1990), working with capsids of EHV-1, were able to reconstruct images of these channels in cross-section, and they found that the bore was not uniform, there being two noticeable constrictions. They suggested that a channel could function as a portal of entry of viral DNA during packaging of DNA into new capsids.

Interconnections between capsomers were observed by Vernon et al. (1974) and Palmer et al. (1975) in HSV-1 and in EHV-1. These structures, lying at a depth of about 5 nm below the outermost extent of the capsomers, each interconnect three capsomers, and they appear to be trimers of a single protein, although this is not certain (Schrag et al., 1989; Baker et al., 1990). These trimers are represented in Figure 4 as thin lines interconnecting adjacent capsomers. Beneath the level of the capsomers there appears to be a further layer of density of undetermined structure, termed the capsid floor (Newcomb & Brown, 1989; Schrag et al., 1989). The total thickness of the T=16 shell was found to be 20 nm (Schrag et al., 1989) and 14-15 nm (Booy et al., 1991) in HSV-1, and 15 nm in EHV-1 (Baker et al., 1990). The inner diameter of the capsid was found by Booy et al. (1991) to be 86 nm. Schrag et al. (1989) found the inner diameter of the T=16 shell to be 85 nm. they also found evidence for a second distinct icosahedral shell of T=4 structure

lying within the T=16 shell, but this finding was not confirmed by the study of Booy et al. (1991). The inner diameter of the EHV-1 capsid was found to be approximately 95 nm by Baker et al. (1990).

Three types of capsid have been isolated from HSV-1-infected cells and characterised, which differ in their protein and DNA content (Gibson & Roizman, 1972; Cohen et al., 1980). Type A and B capsids are isolated from nuclei, and type C capsids from the cytoplasm. B capsids contain all seven capsid proteins, and have internal structural features. They consist of a mixture of DNA-containing 'full' capsids and 'partially cored' capsids lacking DNA but containing proteinaceous internal structures (Sherman & Bachenheimer, 1988; Rixon et al., 1988). A capsids contain only five of the capsid proteins, lacking VP21 and VP22a, and having no internal structure. They are often referred to as 'empty' capsids, and, by analogy with the capsid maturation process of EHV-1 (Perdue et al., 1976), are thought to be abortive by-products of the assembly process. C capsids are nucleocapsids obtained by removal of envelope and tegument material from virions isolated from the cytoplasm of infected cells. They contain six of the capsid proteins, lacking VP22a. The situation in EHV-1 is similar, though not identical. Three types of capsid have been described, but they are all isolated from the nucleus of infected cells. 'Heavy' (H) capsids appear to be mature nucleocapsids and contain all the capsid proteins. 'Intermediate' (I) capsids contain very little DNA and only very small amounts of a 30-kDa capsid protein thought to be a counterpart of VP22a. 'Light' (L) capsids also contain very little DNA and lack the 30-kDa protein and a 46-kDa capsid protein thought to be a counterpart of VP21 (Perdue et al., 1975).

In both viruses, the structural differences between the capsid forms appear to be restricted to internal features. The icosahedral shells of HSV-1 A and C capsids

are indistinguishable by cryoelectron microscopy (Schrag et al., 1989; Booy et al., 1991), as are those of EHV-1 L and I capsids (Baker et al., 1990). Schrag et al. (1989) calculated the dry mass of empty capsids to be 4.56×10^{-16}g, and the dry mass of full capsids (excluding the mass of the core material) to be 4.58×10^{-16}g. The former figure compares well with the earlier estimate of Lampert et al. (1969), who calculated the dry mass of empty capsids to be 5.22×10^{-16}g.

4.6 Locations of capsid proteins within the capsid

VP5 was first located on the external surface of the capsid by Powell & Watson (1975), who found that capsids were agglutinated by anti-VP5 antibody. Vernon et al. (1981) found that anti-VP5 antibody coated capsids in a distribution which covered the entire capsid surface. The study of Steven et al. (1986) established the hexameric structure of the capsomers by calculating that the volume of a hexamer was sufficient to contain six copies of the MCP. Schrag et al. (1989) also calculated that the mass of each hexamer was sufficient to accomodate six copies of a 155-kDa protein. They also calculated that the mass of a pentamer was sufficient to accomodate five copies of a 160-kDa protein, and suggested that the pentamers might also be composed of VP5, as it is the only known capsid component with a mass in this range. 150 hexamers would require 900 copies of VP5, and 12 pentamers would require a further 60 copies. A figure of 960 copies of VP5 per capsid is in agreement with the previously published estimate of 690-1000 (Heine et al., 1974). VP5 accounts for 61.9% of total B capsid protein (Newcomb & Brown, 1989). Newcomb et al. (1989) estimate that EHV-1 I capsids contain 913 +/- 38 copies of the MCP.

Vernon et al. (1981) also located VP19C to the capsid surface. In this case, anti-VP19C antibody coated the regions surrounding the vertex pentamers, leading to the suggestion that the pentamers might be composed of VP19C. However, Schrag et al. (1989) found that the mass of a

pentamer was such that it was unlikely to be composed of molecules of 50 kDa, and Newcomb & Brown (1991) concluded that VP19C could not comprise the pentamers because they could remove these using guanidine hydrochloride (GuHCl) with only minimal loss of VP19C.

Newcomb & Brown (1989) also found that VP19C was somewhat exposed on the capsid surface, as it was rapidly etched from capsids by a stream of Ar^+ ion plasma. They considered that VP19C was a good candidate for the material comprising the floor or base material in which capsomers appear to be embedded. VP19C is not sufficiently exposed to be susceptible to surface iodination. Braun et al. (1984b) attempted this with both empty and full capsids, but were unable to label VP19C, although VP19C was readily labelled in disrupted capsids. A location of VP19C in the basal region of the capsomers is supported by the fact that VP19C is covalently linked to VP5 (Zweig et al., 1979), suggesting a role in anchoring of VP5. Furthermore, the report that VP19C is a DNA-binding protein (Braun et al., 1984a) suggests that, whilst anchoring VP5 on the outer side of the capsid wall, VP19C could have a role in making contact with DNA on the inside. Heine et al. (1974) estimate that there are 680-890 copies of VP19C per virion, which amounts to 9.4% of the total B capsid protein (Newcomb & Brown, 1989).

VP21 is a component of B and C capsids but not A capsids. It is a very minor component, being present in C capsids in 230-270 copies (Heine et al., 1974) and comprising only 2.1% of total B capsid protein (Newcomb & Brown, 1989). Schrag et al. (1989) thought that it was an internal component of the capsid, as did Newcomb & Brown (1989), who found that it was not easily removed from capsids by Ar^+ plasma etching.

VP22a is a component only of B capsids. However, it has been shown that B capsids consist of two populations -

'full' capsids which contain a DNA core and 'partially cored' capsids which contain a proteinaceous core (Sherman & Bachenheimer, 1988). Previously VP22a had been thought to be located on the outer surface, following the labelling of VP22a in immunoelectron microscopy experiments (Vernon et al., 1981) and during surface iodination of DNA-containing capsids (Braun et al., 1984b). However, VP22a is now known to be strongly associated with partially cored capsids and not with full capsids, and it appears that the proteinaceous core is composed of VP22a and that it is involved in the process of DNA packaging (Rixon et al., 1988; Sherman & Bachenheimer, 1988). Rixon et al. (1988) demonstrated by immunoelectron microscopy that VP22a is strongly associated with the internal surfaces of the capsid, and the same was demonstrated for the HCMV counterpart by Landini et al. (1991b). Newcomb & Brown (1989) also thought that VP22a is an internal component, because it is not easily removed from capsids by Ar^+ plasma etching. In a further report (Newcomb & Brown, 1991), they confirmed this finding by treating B capsids with GuHCl. This resulted in the removal of VP22a and of the internal proteinaceous core. Newcomb & Brown (1989) report that VP22a comprises 15.4% of total B capsid protein, but since the B capsid population consists of a mixture of full ($VP22a^-$) and partially cored (VP22a-containing) capsids, the relative amount of VP22a per partially cored capsid is uncertain.

VP23 was first shown to be exposed on the surfaces of capsids by Braun et al. (1984b), in their surface iodinations of full and empty capsids. On the basis of this observation, Schrag et al. (1989) suggested that VP23 could be the constituent of the intercapsomeric trimers. This would require 960 copies of VP23, a figure lower than the estimate of 1240-1720 given by Heine et al. (1974). The possibility exists that there is more than one structural location of VP23 in the capsid. An exposed surface location was also suggested by the study

of Newcomb & Brown (1989), who found that VP23 was easily dislodged from capsids exposed to Ar^+ ion plasma. They suggested that VP23 could comprise either the trimers or the pentamers, or be a constituent of the capsid floor. That VP23 cannot comprise the pentamers was shown by Newcomb & Brown (1991), who extracted the pentamers from capsids using GuHCl without removal of appreciable amounts of VP23. Newcomb & Brown (1989) report that VP23 comprises 10.2% of total B capsid protein.

Little is known about the location of VP24. Schrag et al. (1989) suggested that it may be located in the capsid floor. Since it was not easily eroded from capsids by Ar^+ ion plasma, Newcomb & Brown (1989) also concluded that VP24 is located internally. These workers report that VP24 comprises only 1% of total B capsid protein. There are no published estimates of the number of copies of VP24 per particle.

Again, little is known as to the location of VP26 within capsids. Schrag et al. (1989) suggested that it may be located in the relatively continuous band of protein they observed at a radius of 450-500 A. Newcomb & Brown (1991) suggest that VP26 may be located in or around the capsid vertices because removal of the vertices with GuHCl involves loss of VP26. The only published estimate for the amount of VP26 present per capsid is for 1355 +/- 145 copies of the VP26 counterpart in I capsids of EHV-1 (Newcomb et al., 1989).

4.7 Assembly of capsid

Herpesvirus capsid assembly takes place in the nucleus of infected cells (Morgan et al., 1954). VP5 has been shown by immunofluorescent staining to be heavily localised to the nucleus during infection (Powell & Watson, 1975), and was found to separate with the nuclear fraction of infected cells (Fenwick et al., 1978). Cohen et al.

(1980) were able to demonstrate by immunofluorescent staining that VP5, VP19C, VP21/VP22a, VP23 and VP26 each localise to the nucleus. (They were unable to demonstrate this for VP24 because they could not obtain sufficient amounts of protein to raise antibody.) VP5 is strongly associated with the cytoplasmic cellular cytoskeleton within five minutes of synthesis, and moves directly from the cytoplasmic to the nuclear framework without appearing in a soluble phase (Knipe & Spang, 1982; Quinlan & Knipe, 1983; Bibor-Hardy et al., 1985a). VP5, VP19C and VP23 have all been shown to be strongly associated with the nuclear framework, leading to the suggestion that capsid assembly may take place within this framework (Bibor-Hardy et al., 1982; Bibor-Hardy et al., 1985b). Little is known about the mechanics of assembly of the various capsid proteins. The only capsid protein which has been shown to associate with partially formed capsids in vitro is VP26. Purified B capsids were treated with GuHCl, which removed the pentamers and the internal core material (primarily VP22a) and also involved the loss of VP26. When the reaction mixture was purified of GuHCl, VP26 reassociated with these capsids. Since this process did not involve re-forming of the pentamers, it is concluded that VP26 is not the constituent of those elements. These experiments demonstrate that stable capsid structures can exist without the need for the vertex pentamers or any internal material, either VP22a or DNA. (Newcomb & Brown, 1991). The existence of A capsids also demonstrates that stable capsids can exist without any VP21, or any internal material.

Heilman et al. (1979) first demonstrated by immuno-fluo-rescent staining that VP22a localises to the nucleus of infected cells, and Cohen et al. (1980) showed the same for either or both of VP21 and VP22a using a cross-reacting antibody. Conflicting results have been reported concerning the subcellular locations of the unprocessed forms of VP22a. Braun et al. (1983) and Braun et al.

(1984b) reported that the precursor forms ICP35$_c$ and $_d$ fractionated with the cytoplasm, and that the processed forms VP22a and VP22 (ICP35$_e$ and $_f$) fractionated with the nucleus, and surmised that the processing event is concomitant with location to the nucleus. However, Rixon *et al*. (1988) disputed this finding in a study using *ts*1201 (which fails to process VP22a from higher molecular weight forms at the NPT). They found that unprocessed forms of VP22a did enter the nucleus and that they were incorporated into 'partially cored' capsids.

Condensations of VP22a, about 60 nm in diameter, are often seen in the centre of B capsids *in vivo* (Friedmann *et al*., 1975; Preston *et al*., 1983; Rixon *et al*., 1988). Similar structures have also been noted in EHV-1 (Perdue *et al*., 1976) and in simian CMV (Lee *et al*., 1988). They are also found in capsids *in vitro* after capsid purification (Sherman & Bachenheimer, 1988; Perdue *et al*., 1975; Newcomb *et al*., 1989). VP22a extracted from capsids *in vitro* also exists as discrete condensations of about 60 nm in diameter (Newcomb & Brown, 1991). These authors suggest that such aggregations of VP22a would be capable of acting as scaffolds around which other capsid proteins could condense to form the capsid shell.

A herpesvirus protein which acts in this fashion was first postulated by Mark & Kaplan (1971). They observed that in cells infected with PRV under conditions of arginine deprivation, viral structural proteins were synthesised normally but not transported to the nucleus. They postulated an arginine-dependent protein which would have a role in nuclear localisation. The protein would act to condense structural proteins in the nucleus, creating reduced levels of these proteins and leading to a flow of structural proteins into the nucleus. They point out that such a protein would have to be made in the nucleus, otherwise it would also condense structural proteins in the cytoplasm. Ladin *et al*. (1982) report work on a 35-kDa PRV capsid protein which is processed

from 41-, 42- and 44- kDa forms. The 35-kDa form was only detectable in the nucleus, whilst the higher molecular weight forms were restricted to the cytoplasm. They inferred that the processing event occurs either in the nucleus or in the cytoplasm immediately before transport. Since processing to the 35-kDa form did not occur in three capsid$^-$ mutants, processing to the 35-kDa protein and capsid assembly were inferred to be interdependent events. The mechanism of assembly they proposed was one whereby the processing event occurred, with capsid assembly as a concomitant. Assembly would create reduced levels of capsid proteins in the nucleus which would lead to increased transport or diffusion of capsid proteins into the nucleus. According to this model, a defect in any capsid protein would therefore not only prevent capsid assembly but would prevent processing of the assembly protein.

The report of Braun *et al*. (1984b) that unprocessed forms of VP22a were restricted to the cytoplasm whilst VP22a and VP22 were found only in the nucleus appears to present a similar situation in HSV-1. However, the situation is not identical, because in the case of *ts*1201, unprocessed forms of VP22a do locate to the nucleus and capsid assembly does occur in the absence of the processing event (Rixon *et al*., 1988). Furthermore, in the mutant *ts*2, which is defective in the essential capsid protein VP19C, processing of VP22a does occur in the absence of capsid formation, and VP22a is correctly localised to the nucleus (Preston *et al*., 1992).

4.8 DNA packaging and capsid maturation

Newly replicated herpesvirus DNA molecules consist of large head-to-tail concatemers (Ben-Porat & Tokazewski, 1977; Ben-Porat & Rixon, 1979; Jacob *et al*., 1979; Roizman, 1979), which are cleaved to single-genome molecules during the encapsidation process. Stow *et al*.

(1983) demonstrated that signals required for both cleavage and encapsidation of DNA reside within the a sequence. Specific regions within the a sequence appear to be critical for this process (Varmuza & Smiley, 1985; Deiss & Frenkel, 1986; Deiss et al., 1986; Nasseri & Mocarski, 1988). Two virus-specific proteins of >250 kDa and 140 kDa have been identified which bind specifically to sites within the a sequence and which might have a function in the cleavage/packaging process (Chou & Roizman, 1989). The cleavage and packaging events appear to be coupled, inasmuch as that in experiments reported to date, cleavage always results in packaging (Ladin et al., 1980; Deiss & Frenkel, 1986; Sherman & Bachenheimer, 1987; Addison et al., 1990; Al-Kobaisi et al., 1990).

Addition of drugs which block DNA synthesis significantly reduces the rate of synthesis of VP5 (Holland et al., 1980; Conley et al., 1981), and also reduces the rate of nuclear transportation of the residual VP5 (Knipe & Spang, 1982). However, under conditions where synthesis of viral DNA is suppressed by the use of inhibitory agents or in DNA^- ts mutants, assembly of partially cored capsids occurs normally, although many of the ts mutants produced capsids in lower-than-normal numbers (Nii et al., 1968b; Friedmann et al., 1975; Schaffer et al., 1974; Cabral & Schaffer, 1976; Atkinson et al., 1978; Dargan & Subak-Sharpe, 1983).

Evidence from electron microscopic studies suggested that DNA is packaged into preformed capsids. Strands of electron-dense material, thought to be nucleoprotein complexes, can be observed penetrating empty capsids (Nii et al., 1968a; Friedmann et al., 1975; Haguenau & Michelson-Fiske, 1975). This is confirmed by several ts mutants which are DNA^+ and assemble partially cored capsids at the NPT but which fail to package DNA (Addison et al., 1984; Addison et al., 1990; Al-Kobaisi et al., 1991). A further mutant, ts1201, assembles only partially cored capsids at the NPT, which upon downshift to the PT

and under conditions of inhibition of protein synthesis mature to become full nucleocapsids (Preston *et al.*, 1983). Sherman & Bachenheimer (1988) studied several *ts* mutants which assembled partially cored capsids at the NPT. Of particular interest was the observation that these mutants did not make any detectable A capsids at the NPT. Empty or A capsids were only made during growth at the PT, suggesting that A capsids are generated during, as an alternative to, or subsequent to, packaging of DNA. The empty or L capsids of EHV-1 likewise appear to be by-products of the maturation process rather than precursors. Pulse-chase experiments showed that partially cored or I capsids were made first, and that the appearance of L capsids correlated with the appearance of mature, full nucleocapsids or H capsids (Perdue *et al.*, 1986).

Friedmann *et al.* (1975) first proposed a mechanism of capsid assembly which involved VP22a as a scaffolding protein, on the basis of similarities with the scaffolding proteins of double-stranded DNA bacteriophages (see Discussion). The fact that VP22a when extracted from capsids exists in condensations of about 60 nm in diameter is consistent with its proposed role as a scaffold, around which other capsid proteins could condense to form a partially cored capsid (Newcomb & Brown, 1991). VP22a is lost from these capsids at or near the time that viral DNA is packaged (Sherman & Bachenheimer, 1988; Rixon *et al.*, 1988). It is not clear whether VP22a actually has a role in the packaging of DNA, or whether it is simply removed in order to allow entry of the DNA. Neither is it known whether VP5 or VP19C, capsid proteins reported to have DNA-binding activity, play any role in securing DNA during the packaging process. The genes UL25, UL28 and UL33 have all been shown to be essential for the assembly of full capsids. Mutants in these genes form partially cored capsids but fail to package DNA. The precise roles of the gene products is not known (Addison *et al.*, 1984; Addison

et al., 1990; Al-Kobaisi et al., 1991). A recently constructed UL48 deletion mutant exhibited a defect in capsid assembly at the level of DNA encapsidation. The reason for this is not clear, but this report demonstrates a second key function during HSV-1 infection of the product of UL48, the transactivating tegument protein VP16 (Weinheimer et al., 1992).

4.9 Addition of Tegument

The maturation of capsids to virions involves addition of tegument and envelope. About 25% of enveloped, cytoplasmic virions contain empty capsids, leading to the suggestion that lack of VP22a is the marker inducing virion maturation (Schrag et al., 1989). However, it should be noted that, at least in HCMV, partially cored capsids (ie containing the VP22a counterpart) are matured to become cytoplasmic virions (Irmiere & Gibson, 1983; Irmiere & Gibson, 1985). Furthermore, in the mutant ts1201, the only defect is a lack of the processed form of VP22a, but capsids do not leave the nucleus or acquire tegument (Preston et al., 1983). The formation of L particles during infections by ts1201 demonstrates that condensation of tegument material and its subsequent envelopment is not necessarily initiated by capsids (Rixon et al., 1992). Characterisation of L particles has shown that when stripped of envelope, tegument has intrinsic structural stability, and it is clearly able to condense without the need for a capsid as a scaffold (McLauchlan & Rixon, 1992). Mutants have been isolated which are deficient in the tegument genes UL11, UL41, UL46, UL47 and US9 but which do form tegument, showing that these gene products do not play an essential role in the structure or assembly of tegument (MacLean et al., 1989; MacLean et al., 1991; Read & Frenkel, 1983; Fenwick & Everett, 1990; Zhang et al., 1991; Umene, 1986; Longnecker & Roizman, 1986).

The site of tegument aquisition in HSV-1 is not known. In HHV-6 it is reported to be in a specific region of the cell termed the tegusome, which appears to be an invagination of the cytoplasm into the nucleus (Roffman et al., 1990). This report shows capsids with tegument in various stages of deposition. Stackpole (1969) observed capsids in the cytoplasm of cells infected with a frog herpesvirus, which appeared to be in the process of aquisition of a layer of tegument. Smith & de Harven (1973) observed the same in infections with HCMV. However, although this report also found unenveloped capsids of HSV-1 in the cytoplasm, these were never seen in association with tegument.

4.10 Envelopment and Egress

Confusion exists over the means by which progeny capsids become enveloped. The main reason for this is the difficulty in interpreting electron micrographs in reconstructing the pathway of envelopment. A further complication is that studies on different herpesviruses have given conflicting results, and it may be that several different pathways of envelopment occur amongst the Herpesviridae. There does appear to be general agreement that the primary site of envelopment of herpesviruses is the inner nuclear membrane. Many studies have observed nuclear capsids deriving an envelope from this membrane by budding into the perinuclear space (Nii et al., 1968a; Darlington & Moss, 1968; Stackpole, 1969; McCracken & Clarke, 1971; Wharton et al., 1981; Johnson & Spear, 1982; Poliquin et al., 1985; Whealy et al., 1991; Campadelli-Fiume et al., 1991; Cheung et al., 1991). Darlington & Moss (1968) presented evidence showing that for HSV, PRV and EHV-1, a second envelope was acquired upon passing through the outer nuclear membrane, to produce a cytoplasmic vesicle containing a virion. These vesicles then migrated to the cell membrane and the virions were released by exocytosis. In a study of a frog

herpesvirus, Stackpole (1969) found that the envelope acquired at the inner nuclear membrane was lost upon passing through the outer membrane, and that the capsid re-acquired an envelope when budding into a cytoplasmic vesicle. These vesicles then migrated to the cell membrane and released the virions by exocytosis. Similar sequences of de-envelopment and re-envelopment have been observed by other workers for HSV and PRV (Morgan et al., 1959; Nii et al., 1968a; McCracken & Clarke, 1971).

Johnson & Spear (1982) demonstrated the involvement of the Golgi apparatus in envelopment by studying HSV-1-infected cells treated with the ionophore monensin, which blocks the transit of membrane vesicles from the Golgi apparatus to the cell surface. Yield of extracellular virus was almost eliminated by treatment with monensin, whereas cell-associated virus continued to accumulate, albeit at reduced levels. Electrophoretic analyses showed that these intracellular virions contained immature forms of the envelope glycoproteins. The pathway of envelopment proposed in this report was one which began with capsids acquiring an envelope at the inner nuclear membrane. Enveloped particles would then move via cytoplasmic vesicles to the Golgi, the site of glycoprotein maturation, and from there would move to the cell membrane and be exocytosed from the cell. It is not clear whether the virions actually enter the Golgi or interact with it via Golgi-derived enzyme-containing vesicles. This model is also presented by Torrisi et al. (1992), who demonstrate the aquisition of immature glycoproteins by nascent virions in the perinuclear space, and the transition from immature to mature glycoprotein forms during transport to the extracellular space. Involvement of the endoplasmic reticulum (ER) has been demonstrated for HSV-1 and PRV (Cheung et al., 1991; Whealy et al., 1991). Transport of molecules through the ER to the Golgi and their subsequent processing are disrupted by the fungal metabolite brefeldin A (BFA). BFA causes a rapid redistribution of the Golgi apparatus by inducing a

retrograde transport of Golgi components throughout the ER. Treatment of virus-infected cells with BFA resulted in the accumulation of enveloped virus particles in the perinuclear space and the ER. BFA also interfered with glycoprotein maturation. In the case of PRV, Whealy et al. (1991) suggest that transport through the ER and transport in cytoplasmic vesicles via the Golgi may represent two alternative pathways of virus egress.

Jones & Grose (1988) reported that capsids of VZV pass into the cytoplasm without any process of envelopment, and that an envelope is acquired by budding into a cytoplasmic vesicle, which then undergoes processing by the Golgi. In their study of HHV-6, Roffman et al. (1990) proposed that tegumented capsids pass from tegusomes into the cytoplasm at points where the membranes of the tegusome and nucleus have fused. Acquisition of envelope was again proposed to be by budding into cytoplasmic vesicles. Campadelli-Fiume et al. (1991) concluded that the unenveloped capsids observ-able in the cytoplasm during infections with HSV-1 represent an abortive stage in the pathway of envelopment, which occurs when the virion envelope fuses with the membrane of the vesicle in which it is being transported. This report was based on studies of a virus carrying a mutation in gD, which had a reduced restriction on the ability of the virion envelope to fuse with cytoplasmic membranes. This mutant accumulated unenveloped capsids in the cytoplasm and had a greatly reduced yield of extracellular virus.

The gene product of UL20 appears to be essential for egress of virus. A virus inactivated in this gene produces mature infectious virions but fails to transport them to the cell membrane. Instead there is an accumulation of enveloped virions in the perinuclear space (Baines et al., 1991). The UL20 gene product is membrane-associated, but the mechanism of its action in egress is not clear. Some cell lines are able to complement its function (Baines et al., 1991).

MATERIALS AND METHODS

2A MATERIALS

1 Chemicals

Chemicals were supplied by BDH Chemicals UK, Koch-Light Laboratories, Sigma (London) Ltd, May & Baker Ltd, Pharmacia LKB Ltd, Du Pont Ltd, Bio Rad Laboratories Ltd, James Burroughs Ltd and Fluka Chemicals Ltd.

2 Radiochemicals

All radiochemicals were obtained from NEN Dupont and Amersham International plc.

3 Enzymes

Restriction endonucleases, DNA-modifying enzymes, and enzyme buffers were obtained from Bethesda Research Laboratories, New England Biolabs, Northumbria Biologicals Ltd, Boehringer Mannheim GmbH and Promega Biotech. RNase and lysosyme were supplied by Sigma.

4 Oligonucleotides

Synthetic restriction enzyme linker oligonucleotides were obtained from New England Biolabs. Oligonucleotides A, B, C and D, used in the cloning of UL19, were synthesised by Dr J.McLauchlan using a Biosearch 8600 DNA Synthesiser.

5 Cell Lines

The BHK cells used were from the BHK-21 clone 13 fibroblastic cell line derived from baby hamster kidney cells (MacPherson & Stoker, 1962). The CV-1 cells used were from a Green Monkey kidney epithelial cell line

(Jensen et al., 1964; Kit et al., 1965). The TK^- cells used were from the TK^-143 human osteomyeloma cell line (Rhim et al., 1975; Panicali & Paoletti, 1982). A continuous line of Spodoptera frugiperda cells (Brown & Faulkner, 1977) was routinely used for the growth of wild-type and recombinant AcNPV.

6 Tissue Culture Media

BHK cells were grown in ETC_{10}, consisting of Glasgow-modified Eagle's medium (GMEM; Busby et al., 1964) supplied by Gibco Ltd, supplemented with 10% tryptose phosphate broth, 10% calf serum, 100 units/ml penicillin and 100 ug/ml streptomycin.

CV-1 and TK^- cells were grown in Dulbecco's-modified Eagle's medium (MEM) supplied by Gibco Ltd, supplemented with 5% foetal calf serum, 4-mM L-glutamine, 100 units/ml penicillin and 100 ug/ml streptomycin. TK^- cells were always grown in the presence of 25 ug/ml of BUdR, to prevent the outgrowth of TK^+ reversion mutant cells.

S.frugiperda cells were grown in TC100/5 medium, which consisted of TC100 medium (Gibco) supplemented with 5% foetal calf serum, 100 units/ml penicllllin and 100 ug/ml streptomycin.

7 Viruses

The wild-type HSV-1 virus was strain 17 syn^+ (Brown et al., 1973). The phosphonoacetic acid-resistant mutant PAA^r-1 was derived from HSV-1 strain 17 syn^+ (Hay & Subak-Sharpe, 1976); the mutation has been mapped within the DNA polymerase gene (Crumpacker et al., 1980). The wild-type vaccinia virus was the Western Reserve strain (Parker et al., 1941). The wild-type baculovirus AcNPV was the MP strain of Autographa californica nuclear

polyhedrosis virus (Hink & Vail, 1973). The recombinant baculovirus AcRP23lacZ contains the lacZ gene (Possee & Howard, 1987).

8 Plasmids

pl11 contains the HSV-1 IE-3 gene under the control of the SV40 early promoter and enhancer (Everett, 1986).

pl75 contains the HSV-1 IE-3 gene under the control of the SV40 early promoter and enhancer (Everett, 1986).

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

pG35a consists of an HSV-1 BamHI k joint-spanning region containing two a sequences, cloned into the BamHI site of pAT153 (Twigg & Sherratt, 1980), and was constructed by Stow et al. (1983).

pgDCAT contains the promoter of the HSV-1 gene encoding gD (US6) fused to the CAT gene in the vector pBLW2 (Everett, 1986; Gaffney et al., 1985).

pGX79 contains the HSV-1 DNA restriction fragment HindIII k inserted into the HindIII site of the vector pAT153 (Matz et al., 1983).

pGX128 contains the HSV-1 DNA restriction fragment KpnI i in the vector pAT153, and was constructed by Davison & Wilkie (1983). KpnI i was inserted into the PstI site of pAT153 following addition of homopolymer tracts of deoxycytidine residues to the KpnI fragment and deoxyguanosine residues to the PstI fragment.

pGX142 contains the HSV-1 DNA restriction fragment KpnI t in the vector pAT153, and was constructed by Davison & Wilkie (1983) in the same manner as pGX128.

pGX237 contains a truncated HSV-1 UL26 gene ORF in the vector pFJ10 (Rixon & McLauchlan, 1990), and was constructed by Preston et al. (1992). It is described further in the Results.

pGX239 contains the HSV-1 UL26.5 gene ORF in the vector pFJ10, and was constructed by Preston et al. (1992). It is described further in the Results.

pMJ601 and pMJ602 are the vaccinia transfer vectors constructed by Davison & Moss (1990), and are described further in the Results.

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

9 Antibodies

1060 is a monoclonal antibody against the HSV-1 capsid protein VP23, the product of UL18. It was produced by Dr J.Palfreyman, and initially characterised by Dr A.M.Cross. Further characterisation is reported in this thesis.

5010 is a monoclonal antibody against the HSV-1 capsid protein VP22a, the product of UL26.5, and was produced and characterised by Dr A.M.Cross (Rixon et al., 1988).

10555 is a rabbit antiserum raised against HSV-1 strain 17-infected Rabbit Kidney cells (personal communication, Dr H.S.Marsden).

The goat anti-mouse antibody used in the immuno-

fluorescence experiments was an anti-mouse IgG (Southern Biotechnology), and was the kind gift of Dr I.Sommer. The goat anti-rabbit antibody was an anti-rabbit IgG (Jackson), and was the kind gift of Dr I.Sommer.

10 Bacterial Strains

All plasmids used in this study were propagated in Es-cherichia coli K12 strain DH5α (F^-, endA1, hsdR17, supE44, thi-1, recA, gyrA96, relA1, del(argF-lac zya)U169, φ80dlacZ M15) (Hanahan, 1983). Library-efficiency E.coli DH5α competent cells were supplied by Bethesda Research Laboratories.

11 Bacterial Culture Media

L Broth	10 g/l NaCl, 10 g/l bactopeptone and 5 g/l yeast extract.
L Broth Agar	L Broth plus 1.5% w/v agar

Ampicillin, where appropriate, was added to L Broth or L Broth Agar at 50 ug/ml.

12 Commonly Used Solutions

EEB 10-mM NaAc, 2-mM EDTA, 80-mM Tris pH7.8

Fix methanol:H_2O:acetic acid, 50:50:7

5x gel loading buffer: 5x TBE, 50% glycerol, bromophenol blue, xylene cyanol.

2x HeBs: 260-mM NaCl, 9.8-mM KCl, 1.6-mM Na_2HPO_4, 11-mM D-glucose, 42-mM HEPES.

PBSA: 170-mM NaCl, 3.4-mM KCl, 10-mM Na_2HPO_4, 2-mM KH_2PO_4, (pH7.2).

PBS: PBSA plus $CaCl_2.H_2O$ and $MgCl_2.6H_2O$ both at 1 g/l.

SDS-PAGE buffers:

boiling mix	30% SGB, 30& glycerol, 15% 2ME, 6% SDS, 0.03% bromophenol blue.
sample buffer	one-third dilution of boiling mix.
4x RGB	1.5-M Tris pH8.9, 0.4% SDS.
4x SGB	0.488-M Tris pH6.7, 0.4% SDS.
tank buffer	52-mM Tris, 53-mM glycine, 0.1% SDS.
Solution 1	50-mM glucose, 10-mM EDTA, 25-mM Tris pH8.0.
Solution 2	0.2-M NaOH, 1% w/v SDS.
Solution 3	3-M KAc, 2-M acetic acid.
TBE:	125-mM Tris, 40-mM Boric Acid, 2.7-mM EDTA, not pH'd.
TE:	10-mM Tris HCl, 1-mM EDTA, pH8.0.
Trypsin:	0.25% w/v trypsin dissolved in tris-saline.
Versene:	0.6-mM EDTA dissolved in PBSA containing 0.0002% phenol red.

2B METHODS

2B.1 Construction and Preparation of Plasmids

1.1 Restriction Enzyme Digests

Diagnostic restriction enzyme digests were generally carried out in 20-ul volumes of the appropriate buffer as specified by the manufacturers. The number of units of enzyme added was dependent on the activity of the enzyme and the amount of DNA being digested. Reaction mixtures were generally incubated at 37°C for 1 h. Preparative restriction enzyme digestions used increased amounts of DNA and enzyme under similar conditions in 100-ul volumes.

1.2 Separation of DNA Fragments by Non-Denaturing Gel Electrophoresis

200-ml horizontal slab gels (260mm x 160mm x 5mm) containing 0.8% (w/v) agarose were electrophoresed submerged in 1x TBE plus 0.5 ug/ml ethidium bromide at up

to 12 V/cm. Samples were loaded in 0.2 volumes of 5x gel loading buffer. DNA was examined and photographed with short-wave u.v. transillumination. Long wave u.v. was used to reduce DNA damage when preparative gels were being examined. 50-ml agarose gels (100mm x 70mm x 7mm) were used for analysis of mini-prep DNA. These gels were electrophoresed in TBE at 100 V for approximately 1 h.

1.3 Purification of DNA Fragments from Gels

An agarose slice containing the required DNA fragment was removed from the gel and placed in dialysis tubing. The DNA was electroeluted from the agarose in 1x EEB at 50 V for 1 h. The EEB containing the DNA was removed from the dialysis tubing, extracted with phenol/chloroform and precipitated with 2 volumes of ethanol at -20°C for 1 h.

1.4 Purification of Synthetic Oligonucleotides

The 16-mer oligonucleotides A and B, used in the cloning of UL19, were purified using Oligonucleotide Purification Cartridges (Applied Biosystems), according to the manufacturer's instructions. The 60-mer oligonucleotides C and D, also used in the cloning of UL19, were purified in the following manner. The DNA was eluted from the synthesiser in 1 ml of ammonia. Following incubation at 55°C for 5 h, the ammonia was removed by evaporation on a rotary drier. The DNA was resuspended in 50 ul of H_2O and half removed to a fresh tube and stored at -20°C. The remainder was mixed with an equal volume of 90% formamide in 1x TBE then boiled for 5 min and loaded onto a denaturing 15% polyacrylamide gel (acrylamide:bisacrylamide, 24:1) containing 8-M urea (this concentration of gel was suitable for the purification of 15-100-mer oligonucleotides). Formamide dye mix (2 ul) was loaded into wells adjacent to those containing the oligonucleotide sample as a migration

marker and the gel electrophoresed in 1x TBE at 300 V for 4 h, until the bromophenol blue had almost reached the bottom of the gel. The gel was then transferred onto clingfilm and the DNA viewed over a silica gel thin-layer chromatography plate with an angled short-wave u.v. lamp. The DNA in the gel absorbed u.v. light thus casting a shadow on the fluorescent chromatography plate. The required band was excised and the DNA eluted into 0.5 ml of elution buffer (0.5-M NH_4OAc, 10-mM $MgCl_2$, 0.1% SDS and 1-mM EDTA) overnight at 37°C with constant agitation. The DNA solution was sequentially extracted with phenol/chloroform (1:1) and and chloroform, then ethanol precipitated. The DNA was pellet-ed, dried, resuspended in H_2O, and its concentration measured by spectrophotometry (OD_{260} 1.0 = 20 ug single-stranded DNA/ml.

1.5 DNA Ligation

DNA ligations were performed in 20 ul 1x ligase buffer, with 0.5-mM ATP, 50-500 ng DNA and 1 unit of T4 DNA ligase, at 15°C for 2 h. To prevent self-annealing of the vector, it was usually treated with calf intestinal phosphatase during the restriction enzyme digestion. Staggered-cut termini of DNA fragments which required to be converted to blunt ends were treated with T4 DNA polymerase. Reactions contained 0.5 to 1.0 ug DNA, 33-mM Tris HCl (pH7.8), 66-mM potassium acetate, 10-mM magnesium acetate, 100 ug/ml BSA, 222-uM dCTP, dGTP, dATP, dTTP plus 4 units of T4 polymerase, and were incubated at 15°C for 3 h. Phosphorylated oligonucleotide linkers were inserted into plasmids by ligation with linear molecules using the same procedure as for vector/fragment ligations. A 50-fold molar excess of linker over plasmid DNA was used.

1.6 Transformation of Competent E.coli

1 ug of ligation mix was added to 20 ul of competent DH5α cells and incubated on ice for 30 min. The mixture was then heat-shocked at 42°C for 40 s and added to 80 ul of SOC medium (2% bactopeptone, 0.5% yeast extract, 10-mM NaCl, 2.5-mM KCl, 10-mM $MgCl_2$, 10-mM $MgSO_4$, 20-mM glucose). Cultures were agitated at 37°C for 1 h, and then spread on L Broth Agar plates containing ampicillin. Plates were incubated overnight.

1.7 Mini-Prep Analysis of Transformed E.coli Colonies

Mini-prep DNA was prepared by the alkaline lysis procedure. Colonies were picked from agar plates into 10 ml of L Broth containing ampicillin and shaken at 37°C overnight. The following day, cells from 1.5 ml of the culture were pellet-ed at 5,000 r.p.m. for 1 min in a benchtop microfuge. The pellet was resuspended in 100 ul of solution 1 containing lysozyme and incubated at RT for 5 min. 200 ul of Solution 2 was added, and after vortexing, the sample was incubated on ice for 10 min. 150 ul of Solution 3 was added, and after vortexing, the sample was incubated on ice for a further 10 min. This mixture was centrifuged at 13,000 r.p.m. in an MSE Micro Centaur benchtop centrifuge for 5 min, and the pellet discarded. The supernatant was precipitated with an equal volume of isopropanol at -20°C for 20 min. After centrifugation at 13,000 r.p.m. for 5 min, the pellet was washed in ethanol, dried, and resuspended in 50 ul of H_2O. Mini-prep DNA was stored at -20°C and 5 ul was used for restriction enzyme analysis. 0.2 ul of 1 mg/ml RNase was added to digestions of mini-prep DNA.

1.8 Large-Scale Preparation of Plasmid DNA

Transformed bacteria were picked and inoculated into

10 ml of L Broth plus ampicillin and incubated at 37°C overnight. 0.5 ml of this culture was used to inoculate 300 ml of L Broth plus ampicillin, and the culture was shaken at 37°C overnight. The culture was then pelleted by centrifugation at 7,000 r.p.m. for 10 min in a Sorvall RC-5B centrifuge (GS3 rotor). After decanting the supernatant, the pellet was resuspended in 7.5 ml of Solution 1 containing 1 mg/ml of lysozyme, and incubated at RT for 5 min. Then 14 ml of Solution 2 was added, and the sample was vortexed and incubated on ice for 10 min. 15 ml of Solution 3 was added, and the sample was left on ice for a further 10 min. The sample was then centrifuged at 17,000 r.p.m. for 30 min in a Sorvall RC-5B centrifuge (SS34) rotor to pellet the cell debris and cellular DNA. The supernatant containing the plasmid DNA was precipitated with an equal volume of isopropanol at -20°C. DNA was pelleted by centrifugation at 3,000 r.p.m. for 15 min in a Sorvall RT6000B benchtop centrifuge, washed in ethanol, dried, and resuspended in a total volume of 1 ml of H_2O.

The DNA solution was prepared for caesium banding by dissolving 1.15 g of CsCl and adding 50 ul of 10 mg/ml EtBr per ml of DNA solution. This was transferred to a Beckman TLV-100 ultracentrifuge tube and topped up with stock solution containing similar amounts of CsCl and EtBr. The tube was heat-sealed and centrifuged for 4 h at 100,000 r.p.m. at 20°C in a Beckman TL100 ultracentrifuge. DNA was visualised by daylight or long-wave u.v. transilluminator and the lower supercoiled plasmid DNA recovered with a large bore needle and syringe. The DNA was extracted three times with isopropanol equilibrated with saturated CsCl solution to remove EtBr, diluted three fold with H_2O, and precipitated with ethanol. Once resuspended in TE, the DNA was treated with 50 ug RNase (1 h at 65°C) before extraction with phenol/chloroform and ethanol precipitation. The DNA was lyophilised, resuspended in H_2O and its concentration determined by spectrophotometry (OD_{260} 1.0 = 50ug DNA/ml).

1.9 DNA Sequencing

pBJ196 DNA was sequenced by the dideoxy chain termination method (Sanger et al., 1977), using the Pharmacia T7 sequencing kit consisting of dideoxy sequencing reactions using T7 DNA polymerase. This was carried out according to the manufacturer's instructions.

The products of sequencing of pBJ196 were resolved on a 6% denaturing polyacrylamide gel prepared from Sequagel™ (National Diagnostics) sequencing gel solutions, according to the manufacturer's instructions. This solution was poured into a mould (230mm x 450mm x 0.35mm), and, after polymerisation, was prerun at 40 W for 30 min prior to loading of samples. Electrophoresis was performed in 0.5x TBE at 40 W for approximately 2 h.

2B.2 Growth and Titration of Virus Stocks

2.1 Tissue Culture

BHK cells were routinely passaged in 850-cm^2 plastic roller bottles seeded with approximately 2 x 10^7 cells at 37°C in 100 ml ETC_{10} and an atmosphere of 5% CO_2 in air. Confluent monolayers were harvested in 20 ml ETC_{10} after two versene washes and brief trypsinisation with trypsin/versene (1:4). Cells were resuspended and remained viable for at least 5 days stored at 4°C.

CV-1 cells were routinely passaged in 150-cm^2 plastic tissue culture flasks at 37°C in an atmosphere of 5% CO_2 in air. Monolayers could be maintained at 31°C for up to one week. Confluent monolayers were harvested in 10 ml MEM after two versene washes and trypsinisation with trypsin/versene. Cells were resuspended and remained viable for at least 5 days stored at 4°C.

TK^- cells were routinely passaged every 2-3 days in 150-

cm^2 flasks at 37°C in an atmosphere of 5% CO_2 in air. These cells did not survive at 31°C. Confluent monolayers were harvested in 10 ml MEM after two versene washes and a very brief trypsinisation with trypsin/versene. Cells did not survive at 4°C.

S.frugiperda cells were routinely passaged every 2-3 days in 150-cm^2 tissue culture flasks in TC100/5 at 28°C in air. Confluent monolayers were dislodged by vigorous shaking into 10 ml of TC100/5. When stored at 4°C cells remained viable for at least 24 h.

2.2 Preparation of Stocks of Infectious Virus

Stocks of wild-type and mutant HSV-1 were prepared using 80%-confluent monolayers of BHK cells. These were seeded with 4×10^4 p.f.u. of virus per 850-cm^2 roller bottle in 40 ml ETC_{10} (a m.o.i. of 0.002 p.f.u./cell) and incubated for 4-5 days at 31°C until the cells exhibited obvious c.p.e. (31°C rather than 37°C was found to be better for growth of high-titre viral stocks). The cells were shaken into medium and pelleted at 1,000 r.p.m. for 5 min at 4°C using a Sorvall RT6000B benchtop centrifuge. Cell-associated virus (CAV) was prepared by sonicating the pellet in 2 ml ETC_{10}. Cell-released virus (CRV) was pelleted from the supernatant at 9,000 r.p.m. for 2 h at 4°C using a Sorvall RC-5B centrifuge (GS-3 rotor), gently resuspended in 2 ml ETC_{10} and sonicated. Stocks were stored at -70°C.

Stocks of wild-type and recombinant vaccinia virus were prepared using confluent monolayers of CV-1 cells in 150-cm^2 flasks. These were seeded at a m.o.i. of 0.01 in 50 ml of MEM. After approximately 5 days of incubation at 37°C, the cells were shaken into the medium and centrifuged at 1,000 r.p.m. for 5 min at 4°C. The pellet was resuspended in 1 ml of MEM and stored at -20°C. Stocks of vaccinia virus were sonicated before use.

Stocks of wild-type and recombinant AcNPV were propagated in S.frugiperda cells. Monolayers in 150-cm^2 tissue culture flasks were infected at a m.o.i. of 4 p.f.u. per cell, incubated at RT for 1 h and then overlaid with 30 ml of TC100/5 and incubated at 28°C for 2-4 days until the cells exhibited an obvious c.p.e. The cells were shaken into the medium and pelleted at 1,000 r.p.m. for 10 min at 4°C. CRV in the supernatant was aliquoted and stored at -70°C.

2.3 Titration of Virus Stocks

Stocks of wild-type and mutant HSV-1 were titrated on BHK cell monolayers in 50-mm plates. Cells were infected with serial 10-fold dilutions of virus in 0.1 ml of PBS/10% newborn calf serum. After absorption for 1 h, plates were overlaid with 4 ml 0.6% Noble agar medium (100 ml 1.3x Eagle's A, 20 ml Eagle's B without phenol red, 30 ml 3.2% Noble agar, 5 ml newborn calf serum) to prevent secondary plaque formation, and incubated for 2-3 days at 37°C prior to fixing with Cidex (2.5% glutaraldehyde) and staining with Giemsa stain. The plates were washed after 15 min and plaques counted using a plate microscope.

Stocks of wild-type and recombinant vaccinia virus were titrated on CV-1 cell monolayers in 35-mm plates. After removal of growth medium these were inoculated with 10-fold serial dilutions of virus in MEM and incubated at 37°C for 1 h. The inoculum was removed and the cells overlaid with 2 ml of MEM-PR (MEM lacking phenol red and containing 1% (w/v) low-melting point agarose). After the agarose had set, the cells were incubated at 37°C for 2-3 days and then fixed with Cidex and stained with Giemsa stain. Plaques were counted by eye.

Stocks of wild-type and recombinant AcNPV were titrated on S.frugiperda cell monolayers in 35-mm plates. After removal of growth medium these were inoculated with 10-

fold serial dilutions of virus in TC100/5 and incubated at RT for 1 h. The inoculum was removed and the cells overlaid with 2 ml of a warm (37°C) mixture containing equal volumes of 3% molten low-melting point agarose (Sea-Plaque) and TC100/5, followed (when set) by 1 ml of liquid TC100/5. The infected cells were incubated at 28°C for 3-4 days and then stained for 24 h with 0.5 ml of a solution of 1 part 0.4% Neutral Red plus 24 parts TC100/5. Plaques were counted by eye.

2B.3 Growth, Labelling and Purification of HSV Particles

3.1 Virions

Virions were labelled and purified essentially by the method of Szilagyi & Cunningham (1991). Monolayers of BHK cells in roller bottles were infected with HSV-1 strain 17 at a m.o.i. of 1 p.f.u./10^5 cells and incubated at 31°C for 3 days. The growth medium was replaced with 40 ml of fresh medium, and incubation continued for a further 48 h. To label the viral proteins, 26 MBq of ^{35}S-methionine was added per bottle 7 h after the medium was changed. Once the cells exhibited obvious c.p.e., they were shaken into the medium, and pelleted by low-speed centrifugation (1,000 g for 10 min at 4°C), and the virus particles in the clarified medium were pelleted by centrifugation at 23,000 g for 2 h at 4°C. The pellet was then gently resuspended in 1 ml of modified medium (culture medium without phenol red or calf serum) and layered onto a 35-ml preformed gradient of 5 to 15% Ficoll 400 (Sigma) suspended in this medium. After centrifugation in a swing-out rotor (26,000 g for 2 h at 4°C in an AH627 cellulose nitrate tube), the lower particle band was withdrawn by side puncture. These virions were pelleted by centrifugation (80,000 r.p.m. for 2 h at 4°C in an AH627 tube), gently resuspended in 200 ul of modified medium and either used immediately or stored at -70°C.

3.2 Capsids

HSV-1 intranuclear capsids were purified essentially according to the method of Irmiere & Gibson (1985) and Rixon et al. (1990). Cells were infected and labelled as described above for virions. When cells exhibited obvious c.p.e., they were shaken into the medium and collected by low-speed centrifugation (1,000 r.p.m. for 10 min at 4°C in a Sorvall RT6000B centrifuge). This cell pellet was fractionated in 10 ml of 0.5% NP40 in PBS for 5 min at 4°C. Nuclei were recovered by centrifugation at 1,000 r.p.m. for 10 min at 4°C. The nuclear pellet was subjected to three cycles of freezing on solid CO_2 and thawing at 37°C with vortexing. Nuclear debris was pelleted and the supernatant subjected to centrifugation through a 10 to 40% (w/w) sucrose gradient (Gibson & Roizman, 1972) in a Sorvall TST41 swing-out rotor at 38,000 r.p.m. for 20 min. This usually resolved two bands of capsids (see Figure 5). The gradient was then either separated into sequential fractions and samples of each fraction subjected to PAGE analysis (see Figure 7), or the two bands of capsids were removed by side puncture and pelleted by centrifugation at 70,000 r.p.m. for 30 min in a TLA100 rotor. The pellet was resuspended in 1 ml of PBS and recentrifuged. This pellet was resuspended in 0.5 ml PBS. Capsid preparations were stored at -20°C.

2B.4 PAGE Analysis of Viral Proteins

SDS-PAGE was performed essentially as described by Marsden et al. (1976). Stock solutions of 30% acrylamide with 5% or 2.5% bisacrylamide were prepared in H_2O. 1.5-mm thick gels were prepared in vertical glass plate sandwiches. For single concentration gels, a resolving gel of acrylamide with bisacrylamide cross-linker was prepared in 1x RGB, having a ratio of acrylamide:bisacrylamide of 39:1, on top of which was polymerised a

stacking gel of 5% acrylamide in 1x SGB. Some gels contained DATD as cross-linker, in a ratio of acrylamide:DATD of 57:1. The 5-12% gradient gel used to produce Figure 7 contained bisacrylamide as cross-linker, in a ratio of acrylamide:bisacrylamide of 19:1.

Protein samples were boiled for 5 min in sample buffer, immediately loaded onto the gel and electrophoresed in tank buffer at 10 mA overnight. Gels were fixed by shaking for 45 min in Fix, and dried at 80°C under vacuum onto Whatman filter paper. Dried gels were exposed to autoradiographic film at -70°C. The gel shown in Figure 43 was stained with a solution of 0.2% Coomassie brilliant blue in methanol:water:acetic acid (50:50:7) for 1 h, washed for several hours in a solution of methanol:water:acetic acid (500:8800:140), and dried at 80°C under vacuum onto Whatman filter paper.

2B.5 Generation of recombinant vaccinia viruses

This was performed essentially as described by Davison and Moss (1989). Approximately 1 ug of plasmid DNA was resuspended in 100 ul of Hepes-buffered saline (0.14-M NaCl, 5-mM KCl, 1-mM $Na_2HPO_4.2H_2O$, 0.1% w/v glucose, 20-mM Hepes pH 7.05). Carrier DNA was not used. 5 ul of 2 M-$CaCl_2$ were added, and the mixture was incubated at RT for 30 min. This suspension was added to a 17-mm well containing a drained confluent monolayer of CV-1 cells that had been infected 2 h previously with vaccinia virus at a multiplicity of 0.05. After 30 min at RT, the monolayer was overlaid with 1 ml of MEM, and was incubated at 37°C for 3.5 h. The medium was aspirated, overlaid with 0.5 ml of fresh MEM and incubated at 37°C for 2 days. Infected cells were scraped into the medium, placed in vials, and disrupted by sonication. Recombinant viruses, containing lacZ inserted into the TK gene, were plaque-purified thrice on 30-mm dishes containing confluent monolayers of TK^- cells as follows. After

absorbing 0.1 ml of appropriate dilutions of virus for 1 h, the inoculum was aspirated and monolayers were overlaid with 2 ml of MEM-PR (MEM lacking phenol red and containing 1% w/v low melting point agarose) containing 25 ug BUdR/ml. After allowing the agarose to set at 4°C, the dishes were incubated at 37°C for 2 days. The dishes were then overlaid with 1 ml MEM-PR containing 0.3 mg X-gal/ml, and incubated overnight at 37°C. Well-separated blue plaques were picked and sonicated in 0.5 ml of medium before the next round of plaque purification. A purified plaque of each recombinant was used to infect a 25-cm^2 flask of TK^- cells in the presence of 25 ug BUdR/ml. After incubation at 37°C for 2 days, infected cells were scraped, pelleted and sonicated in 0.5 ml of MEM. A 150-cm^2 flask of CV-1 cells was then infected with a 0.25-ml inoculum and incubated at 37°C for 3 days. The cells were scraped, pellet-ed and sonicated in 0.5 ml of MEM, and the resulting virus stock was stored at -20°C.

2B.6 Preparation of Infected-Cell Extracts

Whole-cell extracts of recombinant-vaccinia- and HSV-1-infected cells were prepared in the following manner. 17-mm wells containing monolayers of CV-1 or BHK cells were infected with 5 p.f.u. per cell of virus in 100 ul of MEM. Following a 1 h period of adsorption at 37°C, the inoculum was removed and replaced with 1 ml of MEM. Following a further period of incubation of 2 h, the medium was removed and replaced with 0.5 ml of MEM containing 1/5 the normal level of methionine and supplemented with 20 uCi per ml of ^{35}S-methionine. The cells were incubated at 37°C for a further 21 h. At 24 h p.i. the medium was removed and replaced with 200 ul of extraction buffer (0.1-M Tris pH8, 10% v/v glycerol, 0.5% NP40, 0.5% sodium deoxycholate, 0.2-mM PMSF [Showalter *et al.*, 1981]), and incubation was continued at 4°C for 1 h. The sample was then removed into a 1.5-ml reaction vial and stored at -20°C until use.

2B.7 Immunoprecipitation

Infected-cell extracts were prepared as in section 2B.6. These were clarified by centrifugation at 7,000 r.p.m. for 5 min at 4°C. 50 ul of antibody (a 1/50 dilution of 1060 or 5010, or a 1/100 dilution of ascitic fluid), 20 ul of infected-cell extract and 10 ul of rabbit anti-mouse Ig were incubated overnight at 4°C. Antigen-antibody complexes were precipitated by the addition of 100 ul of a 10% suspension of heat-killed formalin-fixed Staphylococcus aureus (gifted by Dr A.M.Cross) and further incubated for 1 h at 4°C. After centrifugation of the mixture at 14,000 r.p.m. for 10 min, the pellet was resuspended and washed three times by centrifugation in buffer containing 0.15-M NaCl, 0.1-M Tris pH7.4, and 0.25% NP40. Bound antigen was released by treatment with 0.2-M glycine pH2.5 for 30 min at 4°C. The bacteria were removed by centrifugation at 1,400 r.p.m. and supernatants were analysed by SDS-PAGE.

2B.8 Cell Fractionation

For the preparation of cytoplasmic and nuclear fractions a method based on that of Piette et al. (1985) was employed. CV-1 cells grown in 30-mm dishes were infected at a m.o.i. of 5 p.f.u./cell and incubated from 1 - 18 h post-infection in one-tenth methionine MEM containing 50 uCi ^{35}S-methionine/ml. Cells were washed in cold phoshate-buffered saline (PBS), scraped into 1 ml of fresh PBS, centrifuged at 2000 r.p.m. for 5 min at 4°C, and resuspended in 500 ul lysis buffer (10-mM Hepes pH8, 50-mM NaCl, 0.5-M sucrose, 1-mM EDTA, 0.5% Triton X-100, 1-mM PMSF and 7-mM 2ME). Following 10 strokes with a Dounce homogeniser, on ice, the nuclear fraction was collected by centrifugation at 3000 rpm for 10 min at 4°C and resuspended in 25 ul of lysis buffer; the supernatant was retained as the cytoplasmic fraction.

2B.9 Immunofluorescence

Cells grown on 13-mm cover slips were infected with virus or transfected with plasmid DNA. Following fixation in cold methanol at -20°C for 10 min, they were washed in staining solution (Hank's balanced salts solution plus 4.76 g/l HEPES, Sigma H-3375, 5.2g/l HEPES, Sigma H-7006, 4% v/v newborn calf serum and 0.05% Sodium Azide). Antibody (50 ul), diluted in staining solution, was placed on each cover slip, and incubated at room temperature for 30 min. The cover slip was then rinsed three times in fresh staining solution before incubation with the FITC-conjugated goat anti-mouse secondary antibody for 30 min at room temperature. The cells were again fixed in cold methanol and examined under UV illumination.

2B.10 Transfection of plasmid DNA

The method followed was essentially that of Shen et al. (1982). DNAs to be transfected (1 ug each of pl11, pl75 and pBJ182 or pGDCAT) were resuspended in 57 ul H_2O and 66 ul 2X HEBS (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid, 35-mM; NaCl, 273-mM; KCl, 10-mM; Na_2HPO_4, 1.4-mM; D-glucose, 11-mM; pH 7.05); 9 ul 2-M $CaCl_2$ was added, and the DNA was allowed to precipitate for 30 min at room temperature. 30-mm dishes containing 75%-confluent monolayers of BHK cells growing on 13-mm glass coverslips were aspirated, the Calcium-DNA coprecipitate was added to the monolayer, and incubated with occasional rocking at 37°C. After 45 min, 2 ml of plating medium (20 ml of medium from the original cell cultures to be transfected plus 17.75 ml of fresh growth medium, 2.0 ml of 2X HEBS, and 0.25 ml of 2-M $CaCl_2$) was added to the monolayers, and incubation continued at 37°C. At 3 to 4 h after plating, the medium was aspirated, and the cells were washed with 2 ml of fresh plating medium. 1 ml of 25% DMSO in 1X HEBS was added,

and the plates incubated for 4 min with occasional rocking. The DMSO was removed, and the cells were washed twice with 2 ml of plating medium and incubated in 2 ml of plating medium at 38.5°C, for a further 68h.

2B.11 Electron Microscopy

Cells were harvested by scraping into PBS. Duplicate samples were pelleted into BEEM capsules (Agar Aids) and fixed for 1 h with 2.5% glutaraldehyde in PBS. Samples were then embedded in either Epon 812 resin (Agar Aids) for morphological examination, or in Lowicryl K4M (TAAB Laboratories) for immunolocalisation studies. Samples for Epon embedding were post-fixed for 1 h with 1% OsO_4 and washed in PBS before being dehydrated through a series of increasing ethanol concentrations (30%, 50%, 70%, 90% v/v in PBS, then two changes in 100% ethanol). The pellets were then infiltrated with 50% v/v Epon resin in ethanol, followed by two changes of Epon resin alone. The pellet, embedded in the final change of Epon resin, was incubated at 65°C for three days to polymerise the resin.

Samples for Lowicryl embedding were dehydrated through a graded ethanol series without OsO_4 post-fixation. The samples were placed in 30% ethanol at 4°C for 30 min, then in 50% ethanol for 60 min, and shifted to -20°C. All subsequent dehydration and infiltration steps were performed at -20°C. The samples were placed sequentially in 70%, 90% and 100% ethanol for 1-h periods and infiltrated with Lowicryl K4M resin by 1-h exposures to a 1:1 mixture of resin and ethanol followed by two changes of pure resin. Polymerisation was performed using a 360-nm wavelength u.v. lamp (Taab Laboratories) for 24 h at -20°C and a further two days at RT.

Pelleted and embedded cells were cut with a diamond knife on an ultra-microtome (Ultracut E, Reichart-Jung), and thin sections were collected on parlodion-coated copper

grids (Epon sections) or uncoated nickel grids (Lowicryl sections). Epon-embedded sections were stained with saturated uranyl acetate in 50% v/v ethanol for 1 h, rinsed with deionised H_2O and counter-stained with lead citrate for 1 h. Sections were examined using a JEOL 100S electron microscope.

2B.12 Electron Microscopic Immunolocalisation

All procedures were carried out at RT. Grids containing sections of Lowicryl-embedded cells were submerged briefly in PBS containing 0.05% Tween 20. This solution was also used for all antibody dilutions and for washing the sections after treatment with antibody. The sections were transferred into a 1/50 dilution of 5010 for 45 min, then jet-washed and placed into a 1/50 dilution of a goat anti-mouse antibody tagged with 10-nm gold particles (Janssen Life Sciences). After a further 45 min, they were jet-washed, rinsed in distilled H_2O and dried. Contrast was enhanced by exposing sections to OsO_4 vapour for 1 h.

2B.13 Western Blotting

Western blotting was essentially by the method described by Towbin et al. (1979). Proteins were separated on a 9% SDS-polyacrylamide gel and transferred to a nitrocellulose filter using a Bio-Rad Trans-Blot apparatus. VP23 immobilised on the nitrocellulose was detected by incubating the filter for 1 h at 37°C in a 1/100 dilution of 1060. Bound antibody was detected using the Promega Protoblot horseradish peroxidase system. This employs an anti-mouse immunoglobulin-horseradish peroxidase conjugate and a 4 chloro-1 napthol substrate to develop a deep purple colour where bound antibody is detected. The manufacturer's instructions were followed closely.

2B.14 Labelling of DNA by Nick Translation

This was performed using ^{32}P as described by Rigby et al. (1977). 1.0 ug of pG35a DNA was incubated in a reaction mix containing 40-mM dATP, 40-mM dTTP, 2.5 ul of 10x nick translation buffer (5-M Tris HCl pH7.5, 1-M $MgCl_2$, 1-M DTT, 5 mg/ml BSA) and 2 x 10^{-4} ug DNase at RT for 3 min. The mix was placed on ice and diluted to 25 ul by the addition of 30 uCi of both alpha-^{32}P-dCTP and alpha-^{32}P-dGTP, 3 units of E.coli DNA polymerase I plus distilled water. The reaction was incubated at 15°C for 1 h. ^{32}P-labelled DNA was then separated from unincorporated triphosphates by running through a 10-ml medium Sephadex G50 column in a 10-ml pipette plugged with siliconised glass wool. Columns were loaded with the sample with bromophenol blue dye, and the fastest-eluting fractions taken as purified DNA.

2B.15 Southwestern Assay of DNA-binding

Samples containing proteins to be analysed for DNA-binding ability were separated by SDS-PAGE on a 10.5% polyacrylamide slab gel cross-linked with DATD. These proteins were electrophoretically transferred to a nitrocellulose filter using a Bio-Rad Trans-Blot apparatus. The nitrocellulose filter was then processed for binding potential by pretreating in binding buffer (10-mM Tris pH7.2, 50-mM NaCl, 1-mM EDTA, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.2% BSA [Russell & Precious, 1982]) containing 2.5% NP40 detergent, by shaking for 1 h on a moving stage in a closed plastic box. The detergent was removed by washing the sheet in several changes of binding buffer followed by overnight incubation at RT. The filter was then immersed in 10 ml of binding buffer containing nick-translated pG35a DNA at a level of 100,000 c.p.m./ml, and was incubated at RT for 2 h with shaking. Excess label was removed by washing twice for 30 min in binding buffer. The filter was then

dried and exposed to film for autoradiography.

2B.16 Electrophoresis Under Nonreducing and Reducing Conditions

16.1 Electrophoresis in Cylindrical Gels

Samples of CV-1 cells coinfected with vMJ535 and vMJ534 were electrophoresed in cylindrical gels under non-reducing conditions, according to the method of Zweig et al. (1979a). The gel medium consisted of 2.5% acrylamide, 0.938% bisacrylamide, 0.5% agarose, 0.2% SDS, 40-mM Tris pH7.4, 20-mM sodium acetate and 2-mM EDTA. Gels were cast in 6-cm glass tubes with a 2-mm bore, using solutions prewarmed to 40°C (Liu et al., 1977). Electrophoresis was conducted in an apparatus specially constructed for this purpose, in electrolyte buffer consisting of 0.2% SDS, 40-mM Tris pH7.4, 20-mM sodium acetate and 2-mM EDTA. The sample buffer consisted of 2% SDS, 10% glycerol, 40-mM Tris pH7.4, 20-mM sodium acetate, 2-mM EDTA and 0.005% bromophenol blue. The sample buffer contained no reducing agent, and samples were not boiled before electrophoresis.

16.2 Two-Dimensional Electrophoresis

Electrophoresis was conducted in a cylindrical gel as described above. The gel was then incubated in an equilibration buffer containing 0.1% SDS, 0.0625-M Tris pH6.8, 2-mM EDTA, 10% glycerol, 0.005% bromophenol blue and 40-mM 2ME for 10 min at 100°C. The gel was then laid across the top of the stacking gel of a 9% polyacrylamide slab gel. The slab gel was between two glass plates, one having a bevelled edge to accomodate the cylindrical gel. The cylindrical gel was sealed in place using a melted agarose solution. Following electrophoresis, the slab gel was prepared for autoradiography as described previously.

2B.17 Expression of VP5 in a Recombinant Baculovirus

In order to assess the expression of VP5 by AcUL19, S.frugiperda monolayers in 17-mm wells were infected with wild-type AcNPV, AcUL19, or AcRP23lacZ at a multiplicity of 10 p.f.u./cell, and incubated for 48 h at 28°C. Cells were harvested in 200 ul of extraction buffer (0.1-M Tris pH8, 10% v/v glycerol, 0.5% NP40, 0.5% sodium deoxycholate, 0.2-mM PMSF [Showalter et al., 1981]), and samples were analysed on a 7.5% SDS polyacrylamide gel. Proteins were visualised by staining with Coomassie brilliant blue. For electron microscopy, AcUL19- and AcNPV-infected cells were harvested and embedded in Epon 812 resin as described previously.

RESULTS

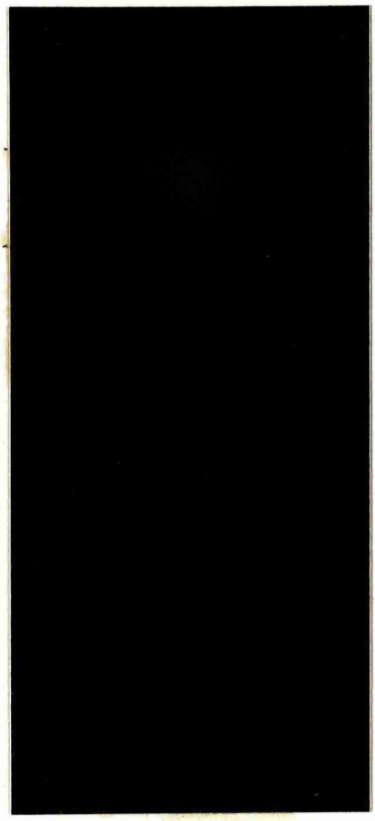

A Capsids
B Capsids

Figure 5. Velocity gradient centrifugation of HSV-1 intranuclear capsids. Centrifugation of 0.5 ml of nuclear extract at 38,000 r.p.m. for 20 minutes through a 12-ml 10 to 40% sucrose gradient separated the A and the B capsids as indicated.

1 AIMS OF PROJECT

The aim of this research was to investigate aspects of capsid structure and assembly in HSV-1. To this end four capsid protein genes were successfully cloned and expressed in a recombinant vaccinia system, and several properties of the products of these genes were investigated. Ultimately it is hoped that all the capsid protein genes can be cloned and expressed in a system which will allow assembly of capsids from cloned gene products. This would enable experiments which would yield valuable information on the pathway of assembly and on capsid architecture. Preliminary studies focussed on the capsid protein composition of HSV-1 capsids.

2 CAPSID PROTEIN PROFILES

Capsid protein profiles have been reported for several herpesviruses. A consensus exists as to the number and identities of the proteins comprising the HSV-1 capsid (Gibson & Roizman, 1972; Zweig *et al.*, 1979a; Cohen *et al.*, 1980; Rixon *et al.*, 1990). The experiments reported in this section confirm these findings, and analyse the distribution of capsid proteins in sucrose velocity gradients. The method of capsid purification used in these experiments was that of Irmiere & Gibson (1985) and Rixon *et al.* (1990), and is detailed in the Materials and Methods.

BHK C13 cells infected with HSV-1 strain 17 were harvested, and intranuclear capsids were purified. Samples of nuclear extract were centrifuged through 10 to 40% sucrose velocity gradients, yielding two light-scattering bands containing A and B capsids (Figure 5).

Separate bands could be withdrawn from the centrifuge tube by side puncture and aspiration. The upper band, containing A capsids, was always much less dense than the

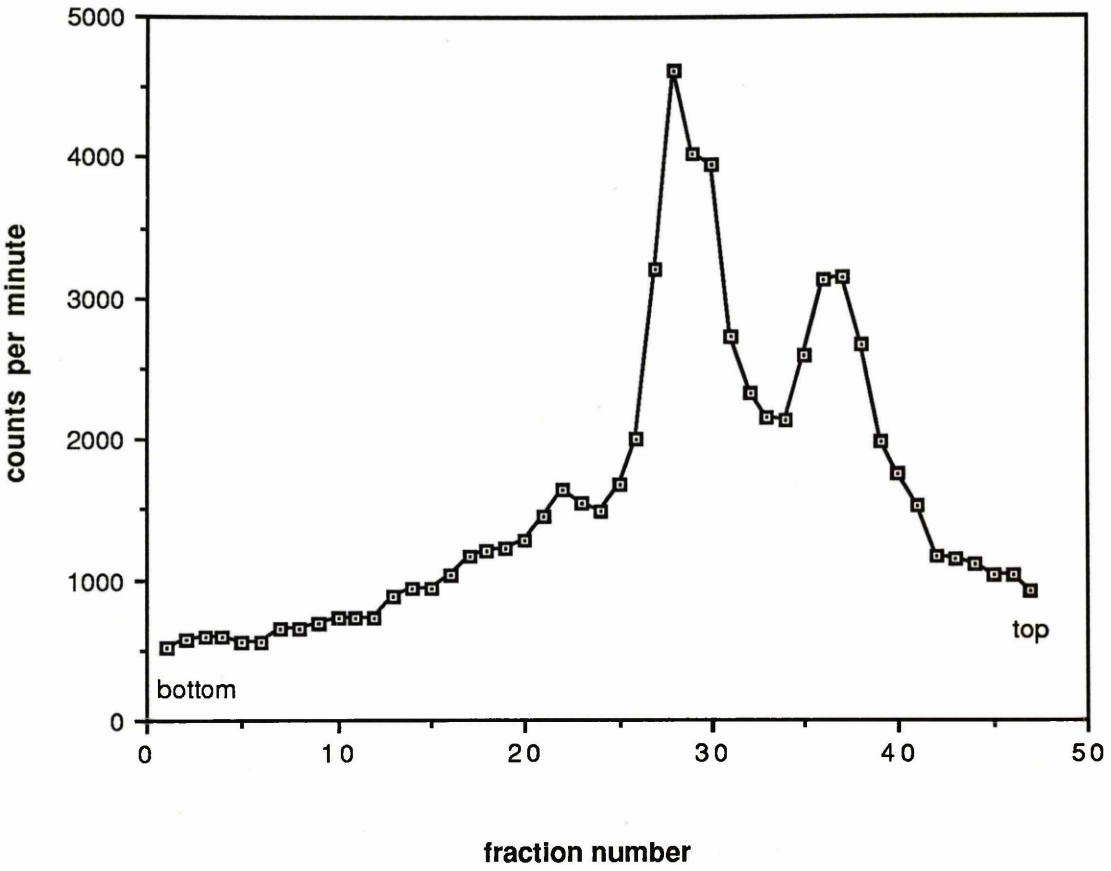

5000
4000
3000
2000
1000
0
counts per minute
bottom
top
0
10
20
30
40
50
fraction number

Figure 6. Graph of scintillation counts of fractions of a sucrose gradient of intranuclear capsids. Forty-seven successive 225-ul fractions were collected from a 12.5-ml sucrose gradient containing visibly separate bands of ^{35}S-labelled B and A capsids. 40-ul aliquots of each fraction were spotted onto filter paper discs and the amount of ^{35}S (expressed here as counts per minute) contained in each sample was measured by scintillation counting. The results are represented here in graphical form. The proportion of ^{35}S-methionine present in each fraction of the gradient is represented by the height of the line. Fraction number 1 represents the lower region of the gradient, below the band of B capsids, and fraction number 47 represents the upper region of the gradient, above the band of A capsids. Two peaks can be seen on the graph; the higher peak represents the B capsids, and the lower peak the A capsids.

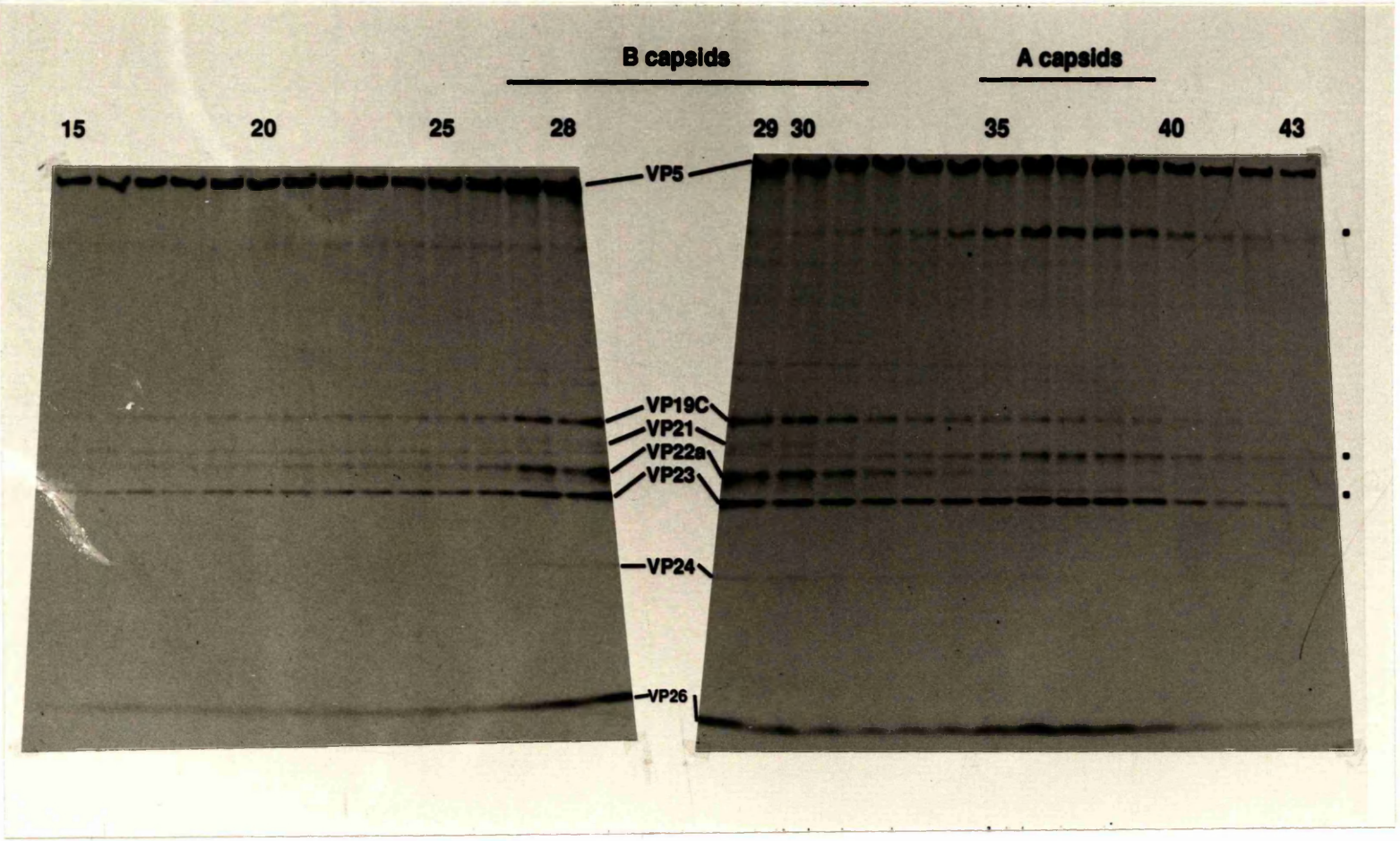

B capsids
A capsids
15
20
25
28
29
30
35
40
43
VP5
VP19C
VP21
VP22a
VP23
VP24
VP26

Figure 7. PAGE analysis of fractions of a sucrose gradient of intranuclear capsids. 30-ul aliquots of fraction numbers 15 to 43 (details given in Table 2, Figure 6, and text) were analysed in successive lanes over two 5 to 12% SDS-polyacrylamide slab gels. The cross-linker was bis-acrylamide. Samples were labelled with ^{35}S-methionine, and were visualised by autoradiography. The lanes are numbered according to the number of the gradient fraction run in that lane. Fractions 27 to 31 contain peak levels of B capsid proteins, and correspond to the region of the gradient which contained the light-scattering band of B capsids. Fractions 35 to 39 contain peak levels of A capsid proteins, and correspond to the region of the gradient which contained the light-scattering band of A capsids. Each of the seven capsid proteins is indicated. Three dots at the right hand side of the figure indicate the positions of other proteins which cosediment with the peak at the position of A capsids.

lower band, and often did not contain sufficient amounts of protein to be useful for analysis by PAGE. The lower band, containing B capsids, did more often contain sufficient yield of protein. This difference in band density has also been reported by other workers (Gibson & Roizman, 1972; Allen & Bryans, 1976).

The distribution of capsids throughout the sucrose gradients was investigated by puncturing the base of a centrifuge tube containing a suitable gradient, and collecting successive fractions as the gradient dripped out of the tube. A 0.8 x 40 mm needle (21G) was used, and each fraction consisted of 3 successive drops (approximate volume 75 ul per drop) from a total gradient volume of 12.5 ml. In one such experiment, 47 successive fractions were obtained from a gradient containing capsids labelled with ^{35}S-methionine. The amount of label present in each fraction was determined by scintillation counting of 40-ul aliquots, and the results are presented in graphical form in Figure 6. Two peaks of radioactivity can be seen, which correspond to the lower (B capsid) band and the upper (A capsid) band.

30-ul aliquots of fractions 15 to 43 were analysed by PAGE over two 5 to 12% gradient gels (Figure 7). The intensity of each protein varies across the gels, and peaks can be seen which correspond to the B and A capsid bands. In lane 29, representing the peak of the B capsid band, all seven capsid proteins can be seen. In lane 36, representing the peak of the A capsid band, VP21 is absent, although a small amount of VP22a is present. It is interesting to note that most of the capsid proteins are present throughout the entire gradient. VP5, VP23 and VP26 each show two peaks corresponding to the B and A capsid bands. VP19C, VP21, VP22a and VP24 each peak at the position of B capsids. VP19C does not show a distinct peak at the position of A capsids in this experiment. VP24 is a minor capsid protein and is very faint on these gels. The fact that small amounts of VP22a can be seen in

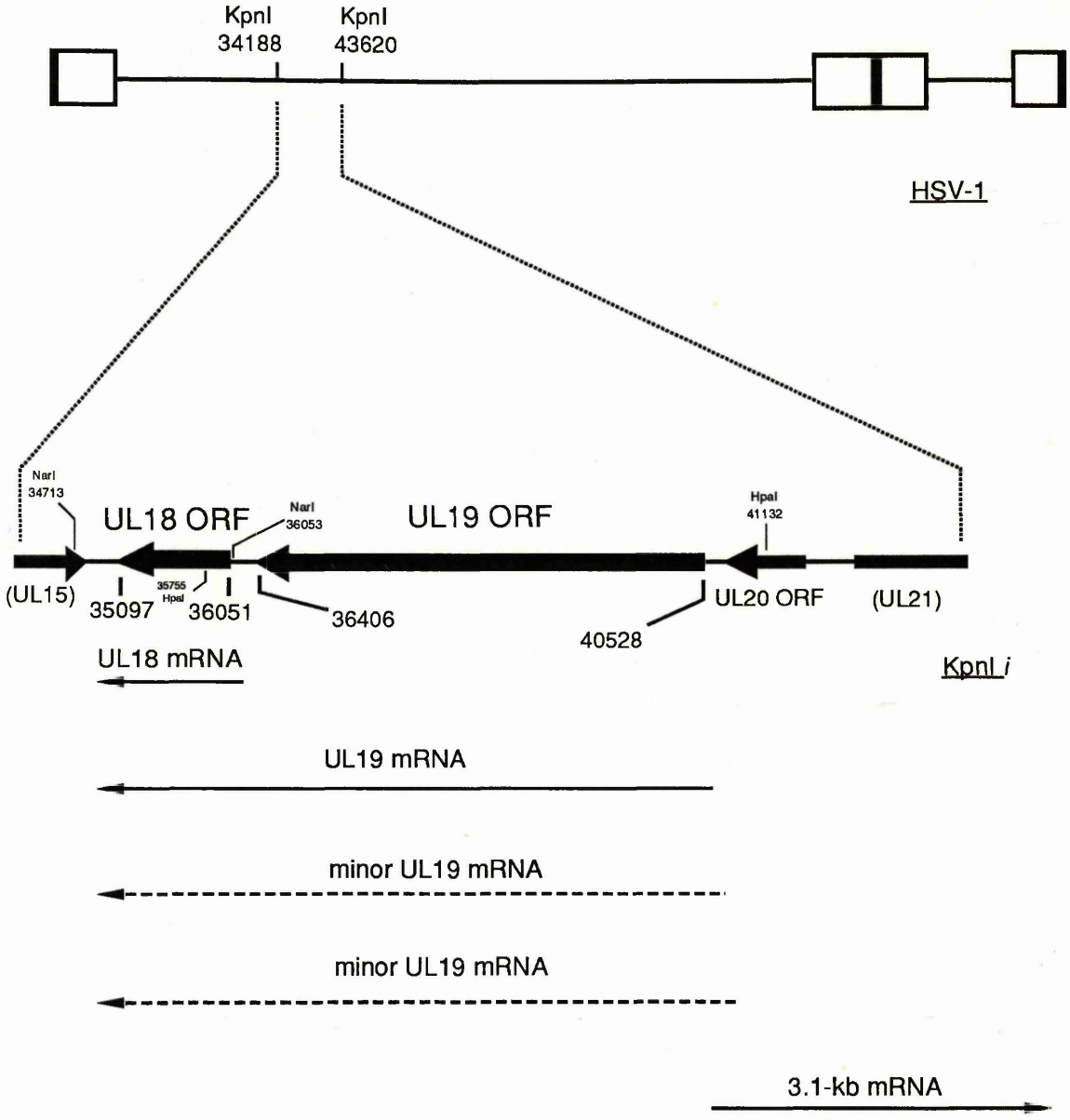


Figure 8. The locus of UL18 and UL19

the A capsid band probably indicates the presence of contaminating B capsids in the A capsid band. Presumably some A capsids separate at the position of B capsids, and vice versa.

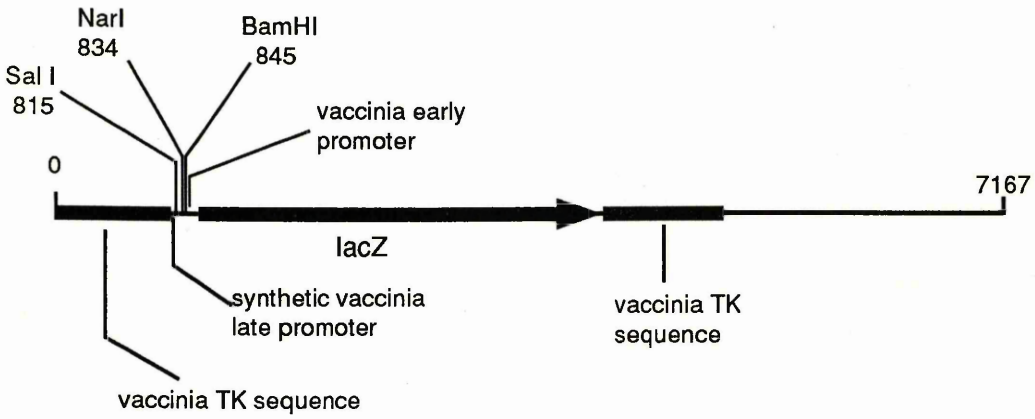
Three further proteins are of interest. The positions of these proteins are marked with a dot at the right hand side of the autoradiograph. These are not known capsid proteins, yet each shows a peak of concentration at the position of A capsids. This would not be expected of a contaminating protein. The first of these proteins lies between VP5 and VP19C. In preparations of HSV-1 virions, a protein of this size accumulates on storage (personal communication, Dr F.J.Rixon), and it is possible that this protein is a breakdown product of VP5. The second of these proteins lies between VP21 and VP22a, and the third lies between VP22a and VP23. The identities of these two proteins are not known. However, they might also represent breakdown products of VP5, or of another capsid protein.

3 CLONING OF CAPSID GENES

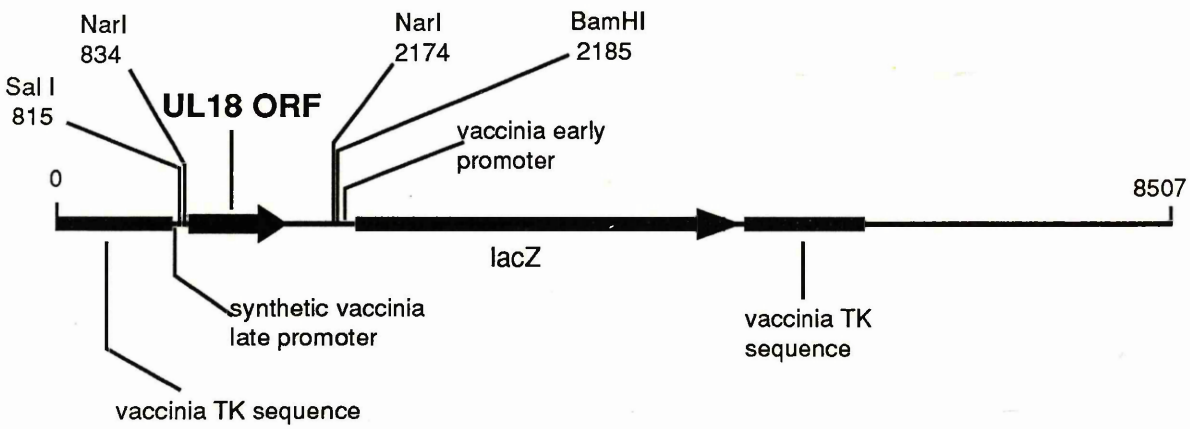
3.1 The locus of UL18 and UL19

The two capsid protein genes UL18 and UL19 are contained within the HSV-1 KpnI i fragment (Marsden et al., 1978) which extends from position 34188 to position 43620 according to the numbering of the HSV-1 sequence (McGeoch et al., 1988a). This corresponds to a map location of approximately 0.224-0.286 map units (Figure 8). The UL18 and UL19 reading frames are transcribed from right to left on the prototype arrangement of HSV-1 DNA. The translation initiation codon of UL18 is at position 36051 and the TAA stop codon is at 35097 (McGeoch et al., 1988a). The UL18 mRNA is unspliced and is 1.5 kb in length (Costa et al., 1984). It initiates at approximately position 36250 (Costa et al., 1984) and

terminates at one of two AATAAA polyadenylation sequences which begin at 35032 and 35028 respectively (Costa et al., 1984; McGeoch et al., 1988a). The sequence reported by McGeoch et al. (1988a) contains a potential regulatory element of sequence CATAAAA commencing at 36268. The initiation codon of UL19 is at position 40528 and the TAA stop codon is at 36406 (McGeoch et al., 1988a). The major UL19 mRNA is unspliced and is 6 kb in length (Costa et al., 1984). The 5' terminus is at position 40768, and promoter elements which are recognisable upstream are a TATATAA sequence beginning at 40796, and two potential CAAT sequences, beginning at 40850 (GGCCATCTT), and at 40870 (GGCCAATCTT) (Costa et al., 1984; Dennis & Smiley, 1984; McGeoch et al., 1988a). Although there is an AATAAA sequence beginning at position 36405, mapping data indicate that the UL19 mRNA is 3' coterminal with that of UL18, and therefore that it must make use of one or both of the polyadenylation sites at 35032 and 35028. However, it has not been ruled out that a minor proportion of UL19 transcripts terminates close to the stop codon. Two minor UL19 mRNAs originate upstream of the major species at positions 40798 and 40858 respectively (Costa et al., 1984). A rightward-transcribed 3.1-kb mRNA of unknown function initiates from the region of the leader sequences of the 6-kb transcript, but this has not been mapped precisely (Costa et al., 1981; Costa et al., 1984). UL20 also lies within KpnI i. The C-terminal coding sequences of UL20 partially overlap the promoter region of UL19. There is no polyadenylation site close downstream from the UL20 coding region and it seems that the mRNA may be 3' coterminal with UL19 and UL18. If this is so, the transcript would be expected to be about 6.8 kb in size, and Costa et al. (1981) detected a 7-kb mRNA of undetermined orientation which at least partially overlapped the 6-kb transcript as well as the region of UL20. The C-terminal portion of exon 2 of UL15 lies at the left-hand end of KpnI i, and the N-terminal 96% of UL21 lies at the right-hand end. KpnI i is contained on the plasmid pGX128.



pMJ601



pMJ521

Figure 9. The structures of pMJ601 and pMJ521

3.2 Vaccinia transfer vectors

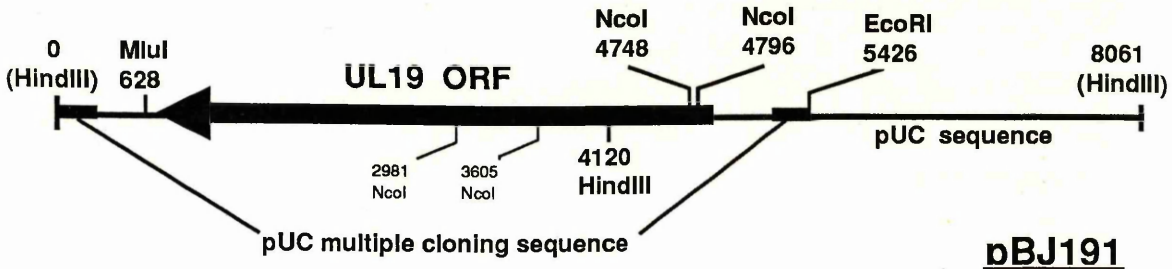
In order to express the capsid genes in vaccinia the coding sequences first had to be placed under the control of vaccinia transcriptional promoter and terminator signals. To do this use was made of the vaccinia expression plasmids pMJ601 and pMJ602 (Davison & Moss, 1990), which contain a synthetic vaccinia late promoter and a vaccinia polyadenylation signal, optimised to give maximal expression, separated by a multiple cloning sequence. This cassette is flanked by vaccinia thymidine kinase (TK) sequences to allow recombination into the viral TK gene. These two vectors also contain the β-galactosidase gene under the control of the vaccinia 7.5-kDa early promoter to assist in easy identification of recombinant viruses by their blue plaque colour.

3.3 Cloning of UL18 into the vaccinia transfer vector

NarI cuts twelve times within KpnI i. Two of these sites flank the UL18 ORF. NarI cuts two residues upstream from the UL18 initiation codon, within the sequences encoding the untranslated leader of UL18 mRNA, at 36053, and 381 residues downstream from the stop codon, at 34713 (see Figure 8), releasing the UL18 coding sequences on a 1340-base-pair fragment. This fragment was purified from pGX128 and ligated into the NarI site in the multiple cloning sequence of pMJ601, and a clone (pMJ521) was selected having the UL18 ORF under the control of the synthetic vaccinia late promoter. The structures of pMJ521 and the parental plasmid pMJ601 are illustrated in Figure 9.

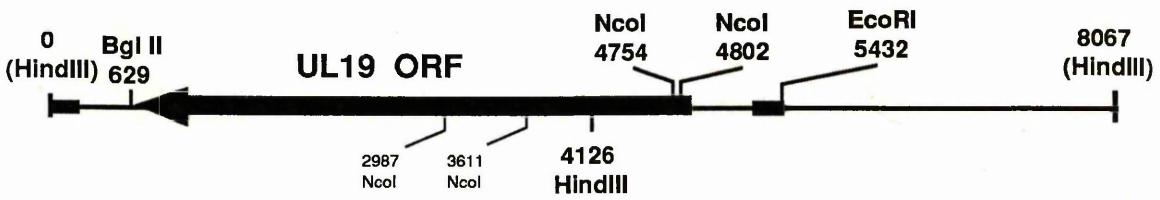
3.4 Cloning of UL19 into the vaccinia transfer vector

Unlike UL18, there were no convenient restriction enzyme



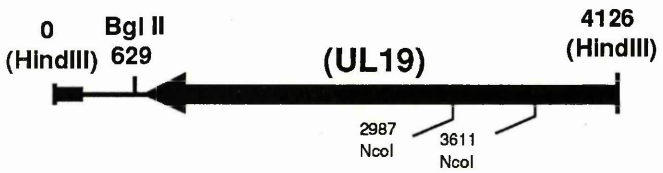
pBJ191

insert Bgl II linker



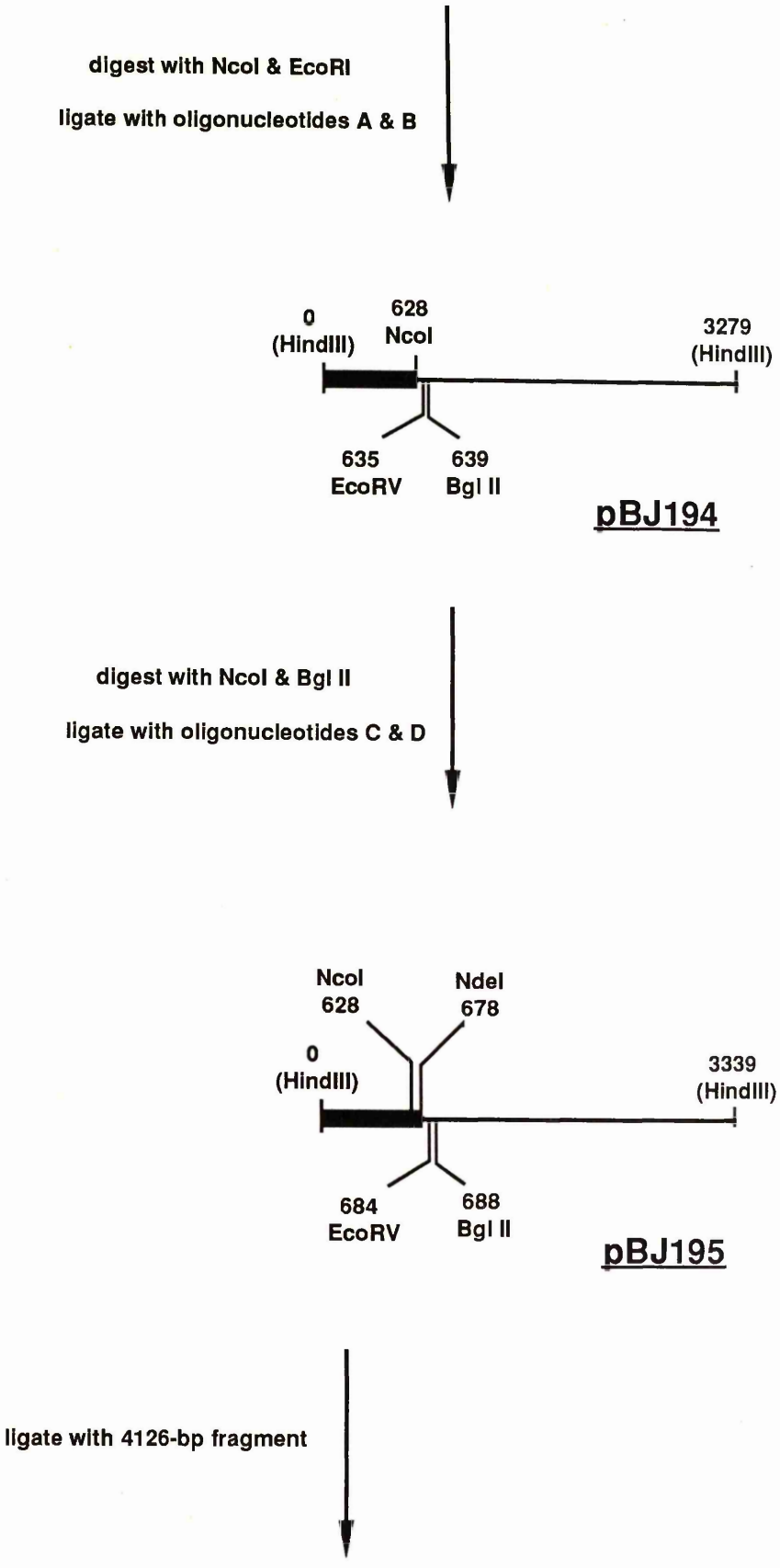
pBJ192

digest with HindIII



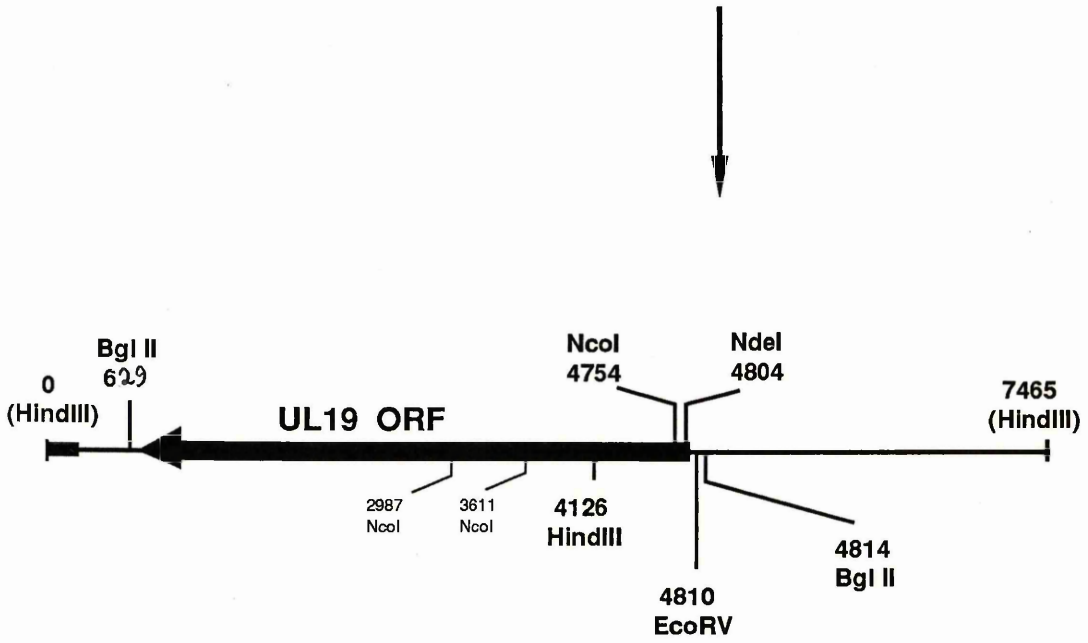
4126-bp fragment

pBJ193



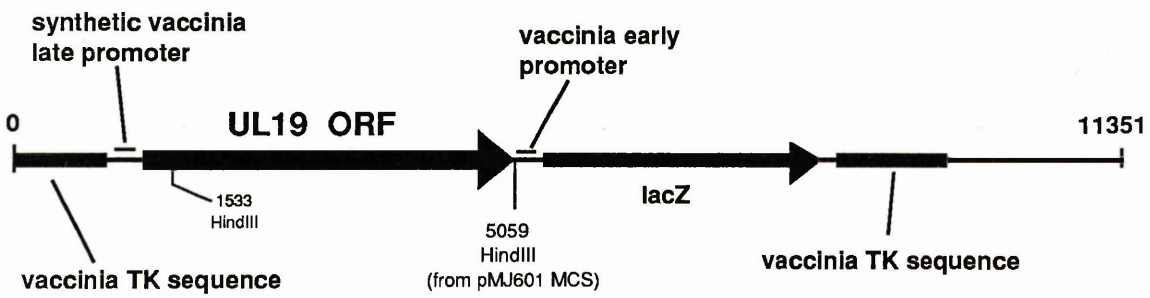
(continued overleaf)

Figure 10 A. The cloning of UL19.



pBJ196

remove 4185-bp Bgl II fragment containing UL19 and clone into BamHI site of pMJ601 (see Figure 9)



pMJ535

Figure 10 A (continued). The cloning of UL19.

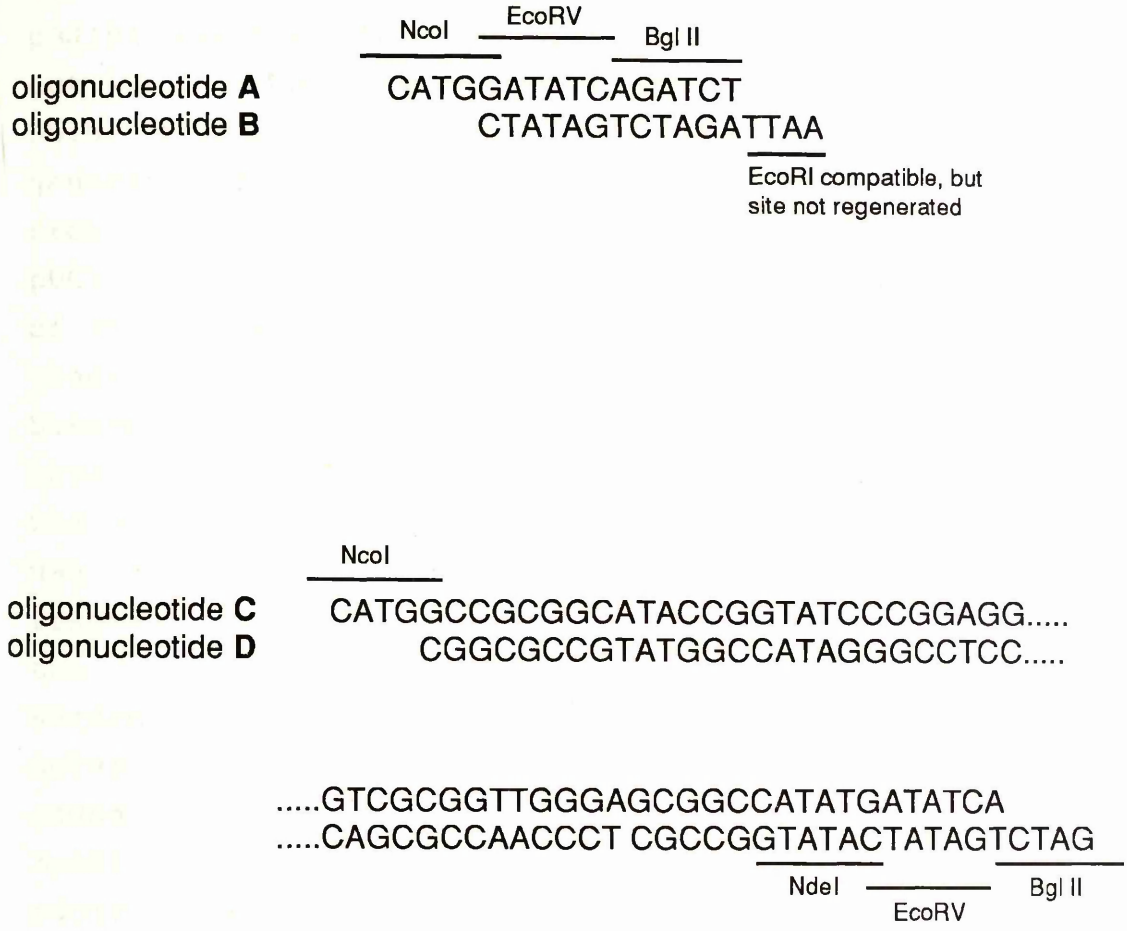


Figure 10 B. Synthetic oligonucleotides used in the cloning of UL19.

sites flanking the UL19 ORF. There—fore a much more complicated cloning strategy had to be adopted. The steps taken in the cloning of UL19 are illustrated in Figure 10A. HpaI cuts within KpnI i at 35755 and at 41132 (see Figure 8), releasing a 5377-base-pair fragment which contains the UL19 coding sequences. The 5377-base-pair HpaI fragment of pGX128 containing UL19 was ligated into the BamHI site (which had been rendered blunt-ended by treatment with T4 polymerase) of pUC19 to give pBJ191. pBJ191 was then digested with MluI which makes a single cut 45 residues downstream from the TAA stop codon of UL19, and a BglII linker was inserted by ligation to generate pBJ192. A HindIII site 676 residues downstream from the initiation codon of UL19, and another in the pUC19 multiple cloning sequence, enabled the separation of this construct into two fragments, of 3941 and 4126 base-pairs. This step was necessary because the 4126-base-pair fragment contained two NcoI sites which would have interfered with subsequent stages of the procedure. The 4126-base-pair fragment was retained for subsequent use in reconstructing the gene. The 3941-base-pair fragment was self-ligated to form a plasmid containing the 5' 679 nucleotides of UL19 as well as the pUC19 sequences (pBJ193). pBJ193 was digested with NcoI (which makes two cuts, one overlapping the UL19 ATG initiation codon and the other 48 base-pairs downstream), and with EcoRI which cuts 630 residues upstream of the ATG in the pUC19 multiple cloning sequence. This generated three fragments, of 3263, 630 and 48 residues. The 3263-base-pair fragment was purified and ligated with a 16-base-pair fragment prepared from two 16-mer partially complementary synthetic oligonucleotides (A and B, Figure 10B), to give pBJ194. The oligonucleotides were designed such that the synthetic fragment generated an NcoI site, introducing immediately upstream two further cloning sites, EcoRV and BglII. The EcoRI site of the pUC19 multiple cloning sequence was not regenerated. This plasmid now had unique NcoI, EcoRV and BglII restriction sites. It had been intended to reconstruct the UL19 ORF

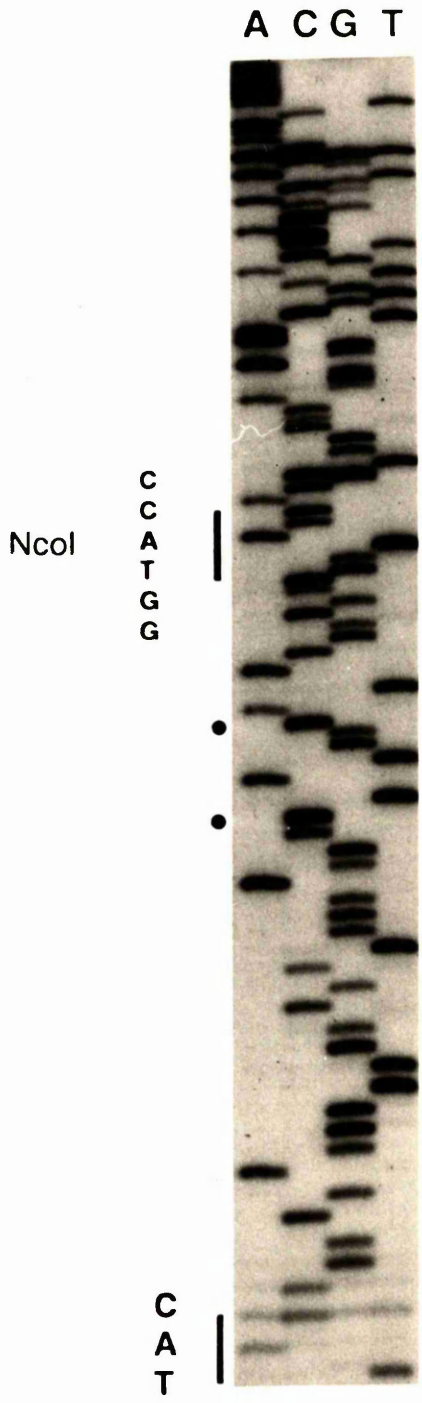

A C G T
NcoI
C C A T G G
C A T

Figure 11. Dideoxy sequencing of pBJ196. The reconstructed portion of UL19 in pBJ196 was checked to confirm the fidelity of the sequence. The position of the ATG initiation codon of UL19 is indicated (as CAT), corresponding to position 4805 on pBJ196. The position of the NcoI site at position 4754 on pBJ196 is indicated. Two dots indicate the positions of two C-C compressions. The region extending from the ATG to the NcoI site represents the reconstructed portion of UL19. Taking the compressions into account, the sequence of this region reads TAC CGG CGA GGG TTG GCG CTG GGA GGC CCT ATG GCC ATA CGG CGC CGG TAC C. This corresponds exactly to the sequence of this portion of UL19 published by McGeoch *et al*. (1988a).

by inserting the 48-base-pair NcoI-NcoI fragment of pBJ193 into the NcoI site of pBJ194. However, at this point it was deemed desirable to introduce an NdeI site into the UL19 ORF at the initiation codon, and new oligonucleotides were designed for this purpose. This was to facilitate cloning of the UL19 ORF into the T7 RNA polymerase expression vector pET3a (Studier et al., 1990). Accordingly, pBJ194 was digested with NcoI and BglII and religated using a 60-base-pair fragment prepared from two 60-mer partially complementary synthetic oligonucleotides C and D (Figure 10B), to give pBJ195. This synthetic fragment regenerated the 48-base-pair 5' coding portion of UL19, converting the initiation codon NcoI site to an NdeI site, and retaining the EcoRV and BglII cloning sites previously introduced. To reconstruct the intact UL19 open reading frame, pBJ195 was linearised at the single HindIII site and the 4126-base-pair HindIII fragment containing the remainder of the UL19 coding sequence was inserted to generate pBJ196. Digestion of pBJ196 with BglII released the UL19 ORF on a 4184-base-pair fragment, which was then cloned into the BamHI site in the multiple cloning sequence of pMJ601. A clone (pMJ535) was selected having the UL19 ORF under the control of the synthetic vaccinia late promoter.

3.5 Sequencing of UL19

Due to the complexity of this cloning procedure it was felt important to check the fidelity of the sequence at the 5' end of the UL19 ORF. The sequence of the reconstucted 5' portion of UL19 was checked in pBJ196 by the dideoxy chain termination method. Figure 11 shows an autoradiograph from which may be deduced the sequence of the relevant part of pBJ196. Reading from the bottom of the autoradiograph the sequence TAC is indicated, which corresponds to the ATG initiation codon of UL19 on the opposite strand. The sequence GGTACC is indicated, which represents the NcoI restriction site at position 4754 of

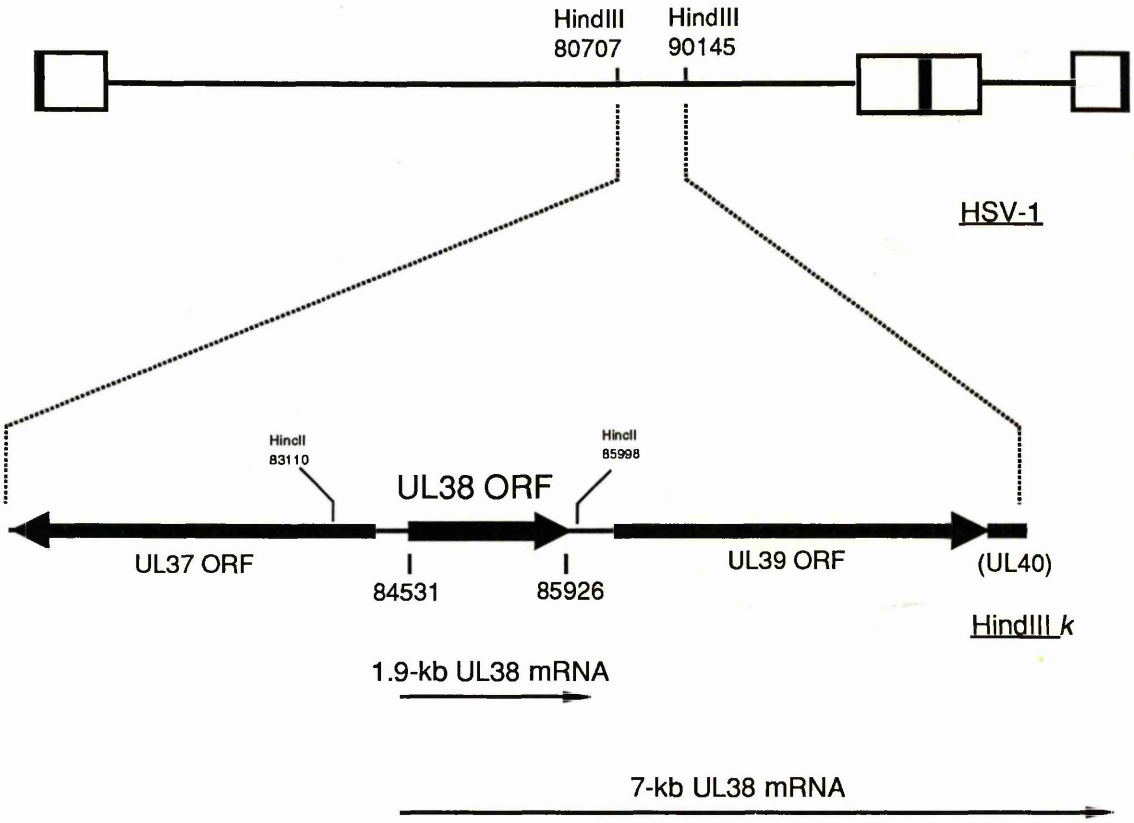


Figure 12. The locus of UL38

pMJ196 (Figure 10A). Two dots indicate the positions of two bands in the C track which are of increased density and appear to represent C-C compressions. Taking these compressions into account, the sequence of the section from the ATG to the NcoI site corresponds exactly to the sequence of this portion of UL19 published by McGeoch et al. (1988a).

3.6 The locus of UL38

The capsid protein gene UL38 is contained within the HSV-1 HindIII k fragment (Wilkie, 1976; Skare & Summers, 1977) which extends from position 80707 to position 90145 according to the numbering of the sequence as determined by McGeoch et al. (1988a). This corresponds to a map location of approximately 0.530-0.592 map units (Figure 12). The UL38 reading frame is transcribed from left to right on the prototype arrangement of HSV-1 DNA, and is unspliced. The translation initiation codon of UL38 is at position 84531, and the TGA termination codon is at position 85926 (McGeoch et al., 1988a). Two mRNAs of 1.9 kb and 7 kb have been mapped to the region of UL38 (Anderson et al., 1981). The UL38 promoter has recently been characterised. The mRNAs initiate at position 84382, and only 45 bases of DNA sequence 5' of the UL38 mRNA cap site is required to direct reporter gene expression in recombinant virus. Recogniseable promoter and regulatory elements are a TATA box beginning at position 84373, a potential CAAT box at 84351 and a potential Sp1-binding site of sequence GGGCGG at 84343 (Flanagan et al., 1991). There is a probable polyadenylation site of sequence AATAAA which begins at position 86016, and another, also of sequence AATAAA, which begins outwith HindIII k after the 3' terminus of UL40 at 90983 (McGeoch et al., 1988a). These sites appear to represent the 3' termini of the 1.9- and 7- kb mRNAs respectively. The polyadenylation site at 90983 marks the 3' termini of UL39 and UL40 (McLauchlan & Clements, 1983). Also contained in

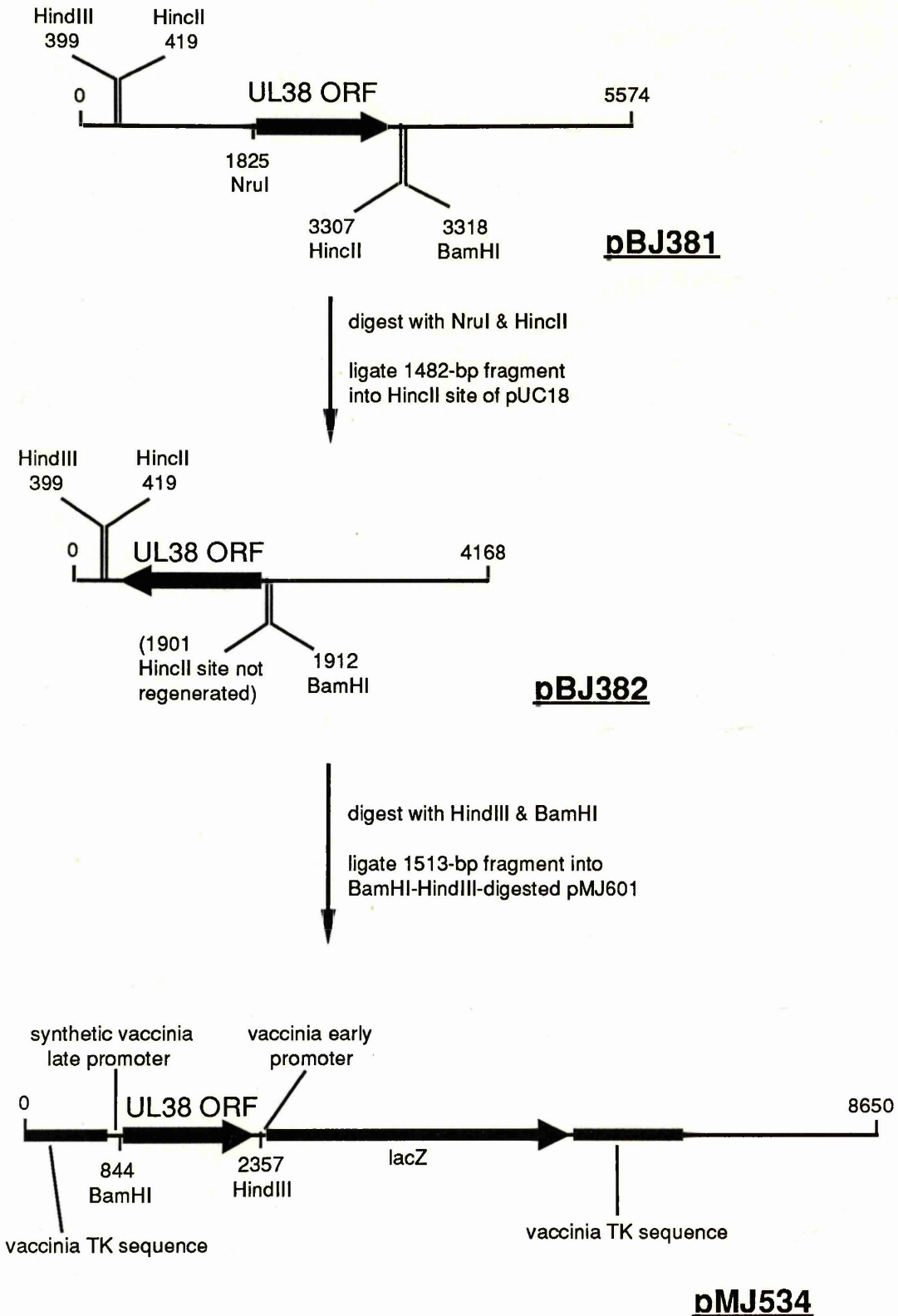


Figure 13. The cloning of UL38

HindIII k are UL37, UL39, and the N-terminal portion of UL40. HindIII k is contained on the plasmid pGX79.

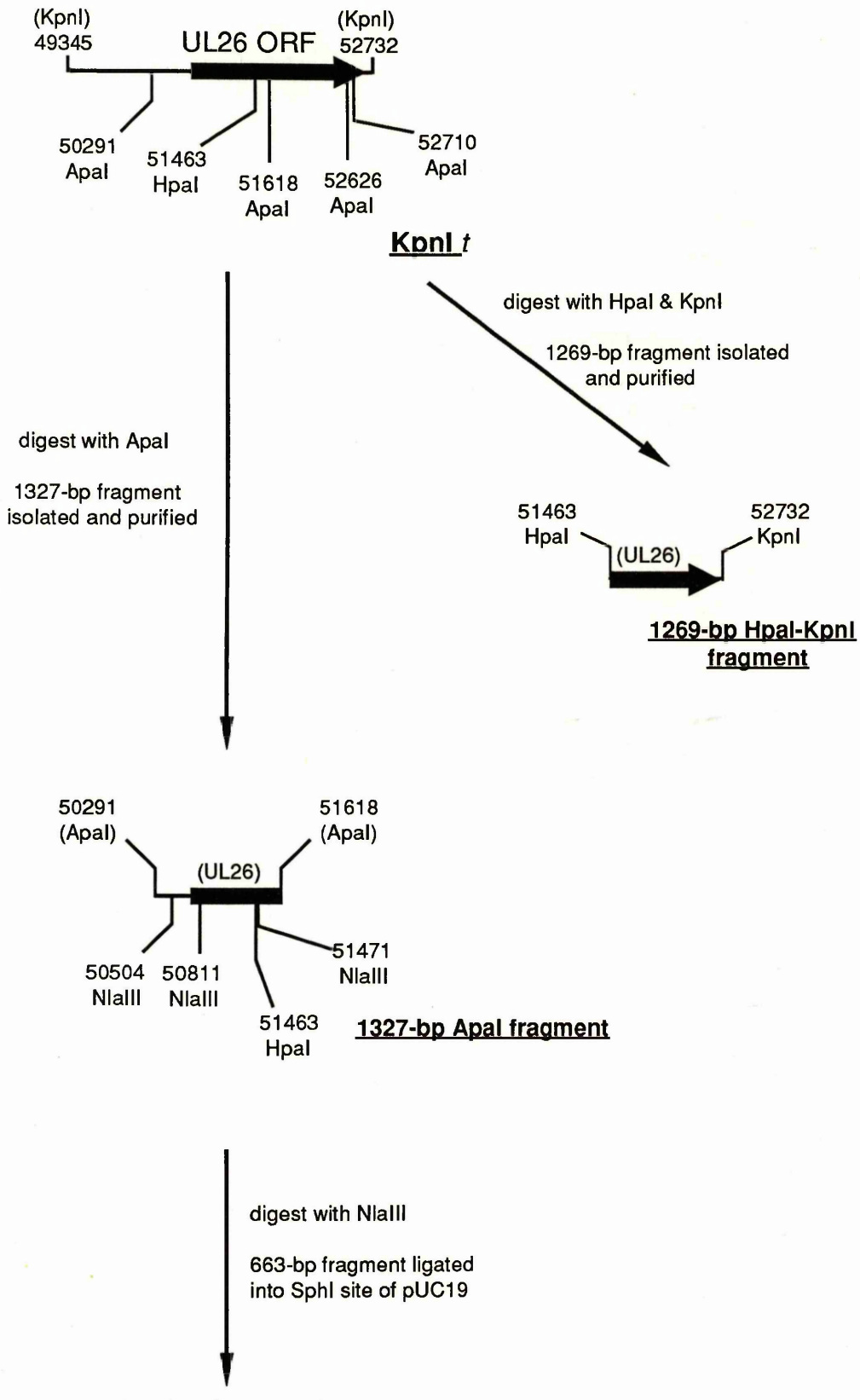
3.7 Cloning of UL38 into vaccinia transfer vector

The procedure followed for cloning UL38 is shown in Figure 13. HincII cuts eight times within HindIII k. A 2888-base-pair HincII fragment extending from 83110 to 85998 contains the UL38 coding sequences (see Figure 12). This fragment was isolated and purified from pGX79, and was ligated into the HincII site of pUC18, to give pBJ381. pBJ381 was then digested with NruI and HincII. NruI cuts once within the 2888-base-pair HincII fragment, at 84515. Thus the NruI-HincII double digest released a 1482-base-pair fragment containing UL38. NruI cuts 15 residues upstream from the UL38 initiation codon in the untranslated leader sequences, and HincII cuts 70 residues downstream from the stop codon. The 1482-base-pair fragment was ligated into the HincII site of pUC18. A clone, pBJ382, was selected in which the UL38 ATG was adjacent to the BamHI site of the pUC18 multiple cloning sequence. UL38 was then cloned as a 1513-base-pair BamHI-HindIII fragment into BamHI-HindIII-digested pMJ601, to give pMJ534.

3.8 The locus of UL26 and UL26.5

The UL26 and UL26.5 genes are contained within the HSV-1 KpnI t fragment (Preston et al., 1978), which extends from position 49345 to position 52732 according to the numbering of the sequence as determined by McGeoch et al. (1988a). This corresponds to a map location of approximately 0.324-0.346 map units (Figure 14). The UL26 and UL26.5 genes are both transcribed from left to right on the prototype arrangement of the HSV-1 genome and are unspliced. The UL26.5 ORF is entirely contained within and is in frame with that of UL26, and the two ORFs are

3' coterminal (Liu & Roizman, 1991a; Preston et al., 1992). Two 3'-coterminal mRNAs of 2.4 kb and 1.4 kb were mapped to this region by Holland et al. (1984) which appear to be the transcripts of UL26 and UL26.5 respectively (Liu & Roizman, 1991a). The preferred translation initiation codon of UL26 is at position 50809 (Preston et al., 1992), and the TGA termination codon is at 52714. A second in-frame ATG codon is located 27 nucleotides downstream from the first initiation codon at position 50836 (McGeoch et al., 1988a), which is functional as an initiation codon in the absence of the first ATG (Preston et al., 1992). The transcription initiation site is approximately 180 nucleotides upstream of the ATG; however, no sequences corresponding to a TATA box or other regulatory elements are discernable in the vicinity (Liu & Roizman, 1991a). The 3' terminus of the UL26 mRNA lies outwith KpnI t at a polyadenylation site of sequence AATAAA, beginning at position 52756 (McGeoch et al., 1988a; Liu & Roizman, 1991a). The translation initiation codon of UL26.5 is at position 51727, and the termination codon is common with UL26, at position 52714 (Liu & Roizman, 1991a; Preston et al., 1992). The UL26.5 mRNA initiates approximately 99 nucleotides upstream of the ATG. A region consisting of 168 nucleotides upstream of the transcription initiation site, delineated by a HpaI restriction site at position 51463, contains all the promoter and regulatory elements necessary for efficient expression of the UL26.5 protein in transfected cells superinfected with HSV-1 or HSV-2 (Liu & Roizman, 1991a). The sequence reported by McGeoch et al. (1988a) contains potential regulatory elements in this region at positions 51604 (TATAA), 51572 (GGGCGG) and 51561 (CAAAAT). The 3' terminus of the UL26.5 mRNA is common with that of UL26 (Liu & Roizman, 1991a). KpnI t also contains the C-terminal 70% of UL25. Three other mRNAs were detected by Holland et al. (1984) which were 3'-coterminal with those of UL26 and UL26.5. These were of 5.6 and 5.2 kb, and of 4.4 kb, and they appear to be transcripts of UL24 and UL25 respectively. KpnI t is contained within pGX142.


(KpnI)
49345
UL26 ORF
(KpnI)
52732
50291
ApaI
51463
HpaI
51618
ApaI
52626
ApaI
52710
ApaI
KpnI t
digest with HpaI & KpnI
1269-bp fragment isolated
and purified
digest with ApaI
1327-bp fragment
isolated and purified
51463
HpaI
(UL26)
52732
KpnI
1269-bp HpaI-KpnI
fragment
50291
(ApaI)
51618
(ApaI)
(UL26)
50504
NlaIII
50811
NlaIII
51471
NlaIII
51463
HpaI
1327-bp ApaI fragment
digest with NlaIII
663-bp fragment ligated
into SphI site of pUC19
(continued next page)

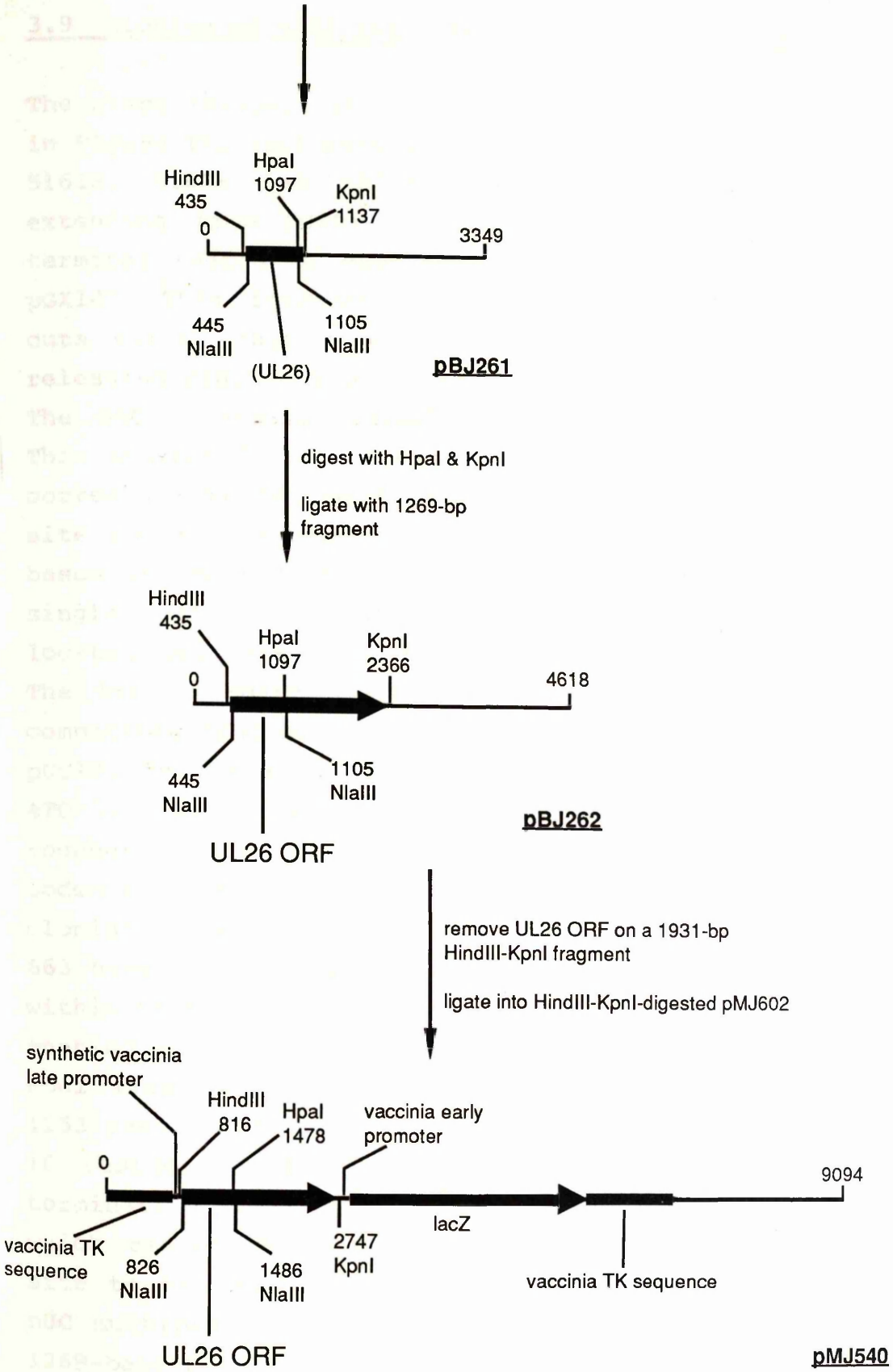


Figure 15. The cloning of UL26

3.9 Cloning of UL26 into vaccinia transfer vector

The steps followed in the cloning of UL26 are summarised in Figure 15. ApaI cuts within KpnI t at positions 50291, 51618, 52626 and 52710. The 1327-base-pair fragment extending from 50291 to 51618 which contains the N-terminal region of UL26 was isolated and purified from pGX142. This fragment was digested with NlaIII, which cuts within this fragment at 50504, 50811 and 51471, releasing fragments of 213, 307, 660 and 147 base-pairs. The 660 base-pair fragment was isolated and purified. This consisted of nucleotides 4 to 664 of the UL26 ORF, corresponding to positions 50812 to 51471. The NlaIII site cutting at position 50811 removed the first three bases of the UL26 ORF, ie the ATG initiation codon. The single HpaI site of the UL26 ORF at position 51463 was located near the 3' end of the 660-base-pair fragment. The 660-base-pair NlaIII fragment was cloned into the compatible SphI site in the multiple cloning sequence of pUC19. Thus the NlaIII sites were regenerated, and the ATG of UL26 was restored. The SphI sites were not regenerated. A clone was selected having the initiation codon adjacent to the HindIII site in the pUC19 multiple cloning sequence. This clone, pBJ261, contains the first 663 base-pairs of the UL26 ORF. The HpaI site which cuts within UL26 at position 51463 and the KpnI site at 52732 enabled separation from pGX142 of a 1269-base-pair HpaI-KpnI fragment. This fragment contained the 3'-terminal 1253 residues of the UL26 ORF and extended to include the 16 residues immediately following the end of the TGA termination codon. pBJ261 was digested with HpaI and KpnI which removed 8 bases of UL26 sequence, from the HpaI site to the NlaIII site, and a further 32 bases of the pUC multiple cloning sequence, up to the KpnI site. The 1269-base-pair fragment was ligated into the HpaI-KpnI-digested pBJ261. The resultant plasmid, pBJ262, contained the entire UL26 ORF, beginning with the initiation codon and ending at the KpnI site 16 bases 3' of the end of the termination codon, and corresponding to positions 50809

to 52732. The KpnI site was regenerated and was unique, and a unique HindIII site in pBJ262 8 base-pairs upstream of the UL26 ATG enabled removal of the UL26 coding sequences on a HindIII-KpnI fragment of 1931 base-pairs. This was ligated into the multiple cloning sequence of HindIII-KpnI-digested pMJ602 to give pMJ540.

3.10 Cloning of UL26 lacking the initial ATG into vaccinia transfer vector

The plasmid pGX237 contains a 1956-base-pair HindIII fragment inserted into pFJ10 (Preston et al., 1992). This insert includes a 1911-base-pair section of the UL26 ORF, which extends from position 50826, upstream of the second ATG at position 50836 to the KpnI site 20 bases 3' of the termination codon, at 52736. Thus the UL26 initiation codon at position 50809 is absent. This clone was used by Preston et al. (1992) and was referred to by them as containing the UL26 ORF, although it actually contains an N-terminal truncated version. It was included in this study in an attempt to repeat in the recombinant vaccinia system the results obtained by these workers by expressing this clone under IE gene regulation. This clone was of further interest in that it presented an opportunity to investigate the respective roles of the first and second ATG initiation codons of the UL26 ORF. Digestion of pGX237 with HindIII and KpnI yielded the UL26 sequences on a fragment of 1917 base-pairs. This was ligated into the multiple cloning sequence of HindIII-KpnI-digested pMJ602 to give pMJ541. However, since attempts at obtaining recombinant vaccinia viruses using pMJ541 were unsuccessful, further details of this plasmid are not presented.

3.11 Cloning of UL26.5 into vaccinia transfer vector

The plasmid pGX239 contains a 1110-base-pair HindIII

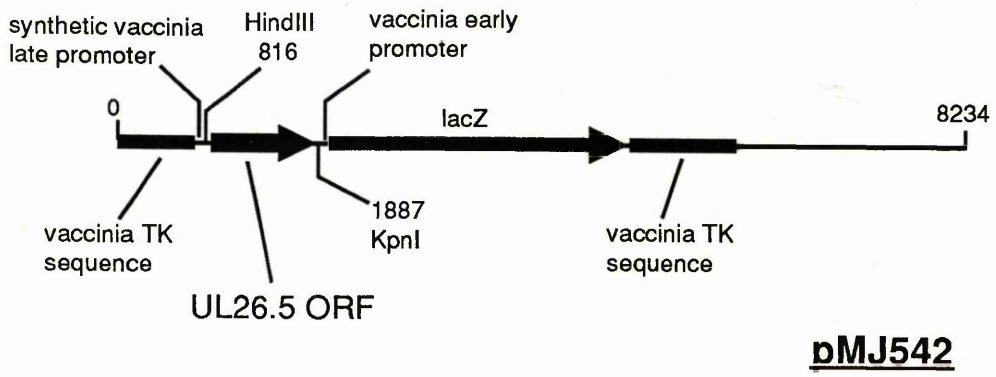
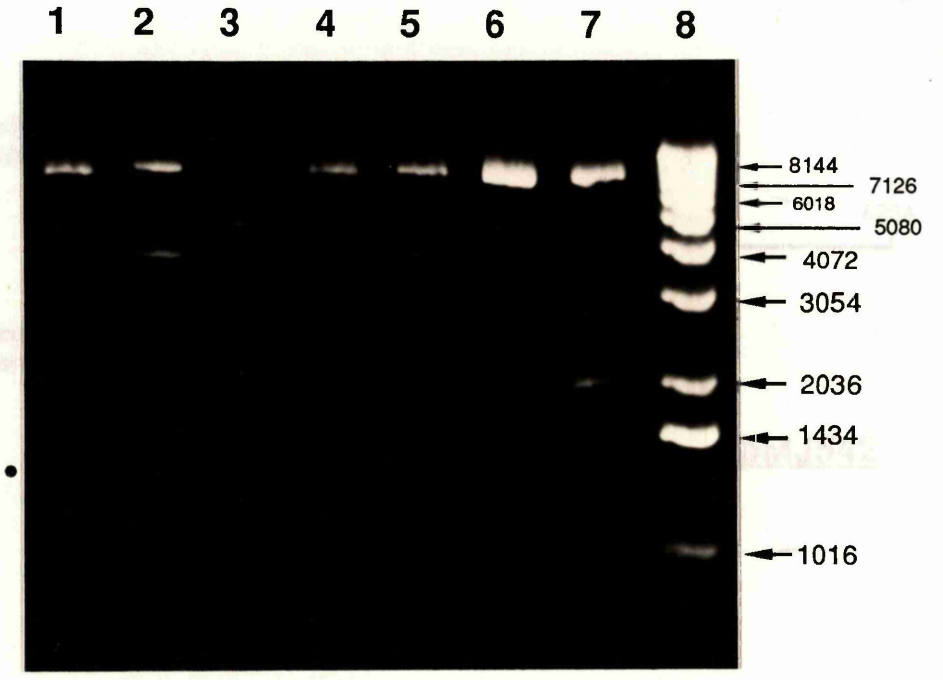


Figure 16. The structure of pMJ542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8144
7126
6018
5080
4072
3054
2036
1434
1016

Figure 17. Restriction enzyme digests demonstrating the structures of vaccinia transfer vectors containing HSV-1 capsid genes. Plasmid DNA was digested with the enzymes indicated and analysed on a 1% agarose gel. Lane 1: pMJ521 digested with NarI. (A dot at the left hand side of the photograph indicates the position of the 1340-bp NarI fragment of pMJ521.) Lane 2: pMJ535 digested with HindIII. Lane 3: pBJ196 digested with BglII. Lane 4: pMJ534 digested with BamHI and HindIII. Lane 5: pMJ540 digested with HindIII and KpnI. Lane 6: pMJ541 digested with HindIII and KpnI. Lane 7: pMJ542 digested with HindIII and KpnI. Lane 8 contains a ladder of size markers. The size (in base pairs) of each fragment in lane 8 is indicated at the right hand side of the photograph.

fragment inserted into pFJ10 (Preston et al., 1992). This insert includes a 1006-base-pair section which contains the entire UL26.5 reading frame extending from the initiation codon at position 51727 to the KpnI site 16 bases 3' of the termination codon, at 52732. This 1006-base-pair section is bounded by a HindIII site 65 base-pairs upstream of the UL26.5 initiation codon. Digestion of pGX239 with HindIII and KpnI yielded the UL26.5 sequences on a fragment of 1071 base-pairs. This was ligated into the multiple cloning sequence of pMJ602 to give pMJ542. The structure of pMJ542 is shown in Figure 16.

3.12 Restriction digests demonstrating the structures of vaccinia transfer vectors containing HSV-1 capsid genes

In order to confirm the structures of the plasmids to be used as transfer vectors in the construction of recombinant vaccinia viruses, each plasmid was analysed by restriction enzyme digestion. Fragments produced by these digestions were separated on an agarose gel and visualised under UV illumination following staining with ethidium bromide (Figure 17). pMJ521 was digested with NarI, which released the UL18 ORF contained on a 1340-base-pair fragment from the parental transfer vector pMJ601 (7167 bp) (see Figure 9). pMJ535 was digested with HindIII, which released two fragments, of 7825 bp and 3526 bp. In order to demonstrate the BglII fragment which was used to transfer the UL19 ORF into pMJ535, pBJ196 was digested with BglII, which released the UL19 ORF contained on a 4184-bp fragment, and a second fragment of 3281 bp (see Figure 10A). pMJ534 was digested with BamHI and HindIII, which released the UL38 ORF contained on a 1513-bp fragment from the parental transfer vector pMJ601 (7137 bp) (see Figure 13). pMJ540 was digested with HindIII and KpnI, which released the UL26 ORF contained on a 1931-bp fragment from the parental transfer vector pMJ602 (7163 bp) (see Figure 15). pMJ541 was digested

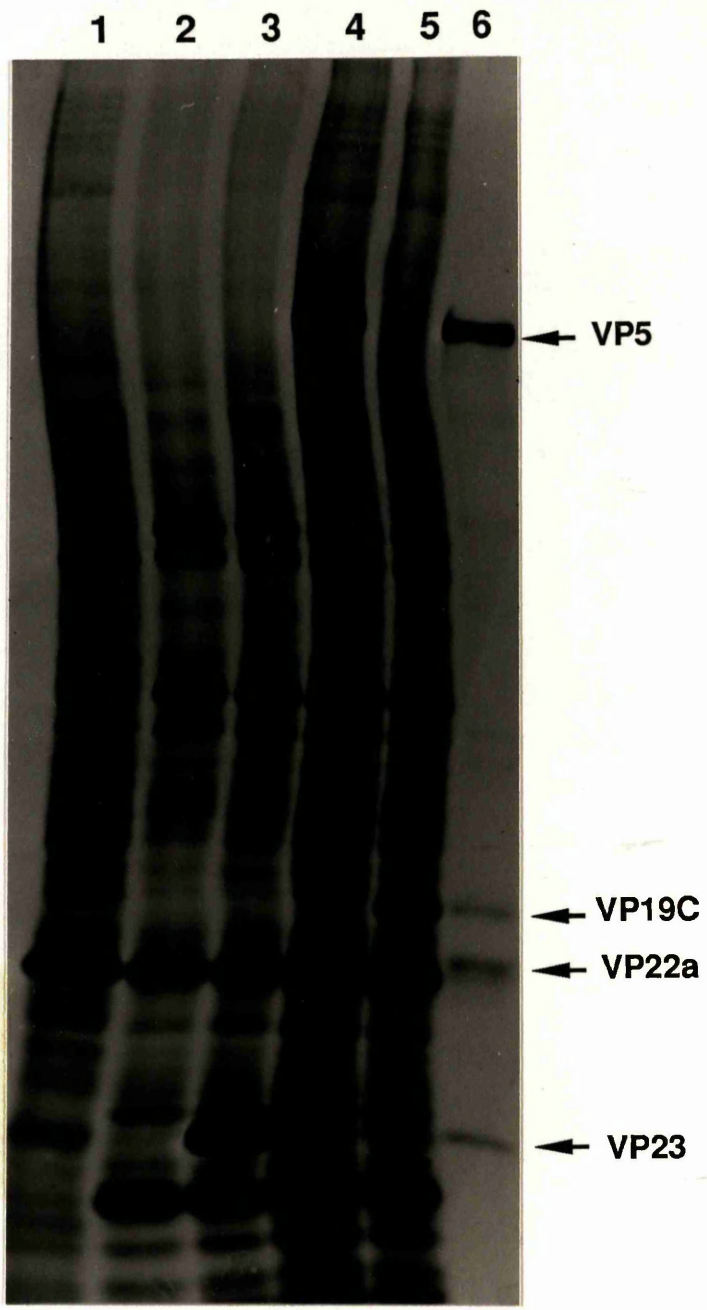
Recombinant Virus	HSV-1 Gene
vMJ521	UL18
vMJ535	UL19
vMJ534	UL38
vMJ542	UL26.5

Table 2. Recombinant vaccinia viruses containing HSV-1 capsid protein genes

with HindIII and KpnI, which released the truncated UL26 ORF contained on a 1917-bp fragment from the parental transfer vector pMJ602 (7163 bp). pMJ542 was digested with HindIII and KpnI, which released the UL26.5 ORF contained on a 1071-bp fragment from the parental transfer vector pMJ602 (7163 bp) (see Figure 16).

4 INTRODUCTION OF CAPSID GENES INTO VACCINIA

Following transfection of pMJ521 (UL18), pMJ535 (UL19), pMJ534 (UL38), pMJ540 (UL26), pMJ541 (truncated UL26) and pMJ542 (UL26.5), and superinfection with wild-type vaccinia virus, progeny viruses were selected for TK minus phenotype. Blue plaques were isolated and purified to homogeneity through at least two cycles of plaque purification. The efficiency of recombination appeared to be very low, in that very few blue plaques were ever obtained. In the cases of pMJ535 and pMJ542, the blue plaques formed were obscured by high levels of wild-type vaccinia virus which had survived the BUdR selection. These blue plaques were very small, consisting of only one or two cells, and could only be seen using a microscope. After several rounds of purification in the presence of BUdR, these tiny plaques yielded blue plaques which were visible to the naked eye. Despite repeated attempts, blue plaques could not be obtained following transfection with pMJ540 or pMJ541. This may be due simply to the low efficiency of recombination or because in these cases the HSV proteins expressed were toxic to vaccinia virus. Several plaque isolates of each recombinant were selected and grown to high titre. One of each was selected for further study; these were designated vMJ521 (UL18), vMJ535 (UL19), vMJ534 (UL38) and vMJ542 (UL26.5). These viruses are listed in Table 2 for ease of reference whilst reading this thesis.


1
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3
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5
6
VP5
VP19C
VP22a
VP23

Figure 18. Expression of HSV-1 capsid proteins by recombinant vaccinia viruses. CV-1 cells were infected with 5 p.f.u. per cell of WR strain vaccinia virus (lane 2), vMJ521 (lane 3), vMJ535 (lane 4) or vMJ534 (lane 5), and labelled from 3 to 24 hours p.i. with ^{35}S-methionine. Mock-infected cells (lane 1) were similarly labelled. Cells were harvested and 30 ul of whole cell extract was analysed on a 10.5% SDS-polyacrylamide slab gel. The cross-linker was DATD. Lane 6 contains purified HSV-1 B capsids. Arrows indicate the positions of the named capsid proteins.

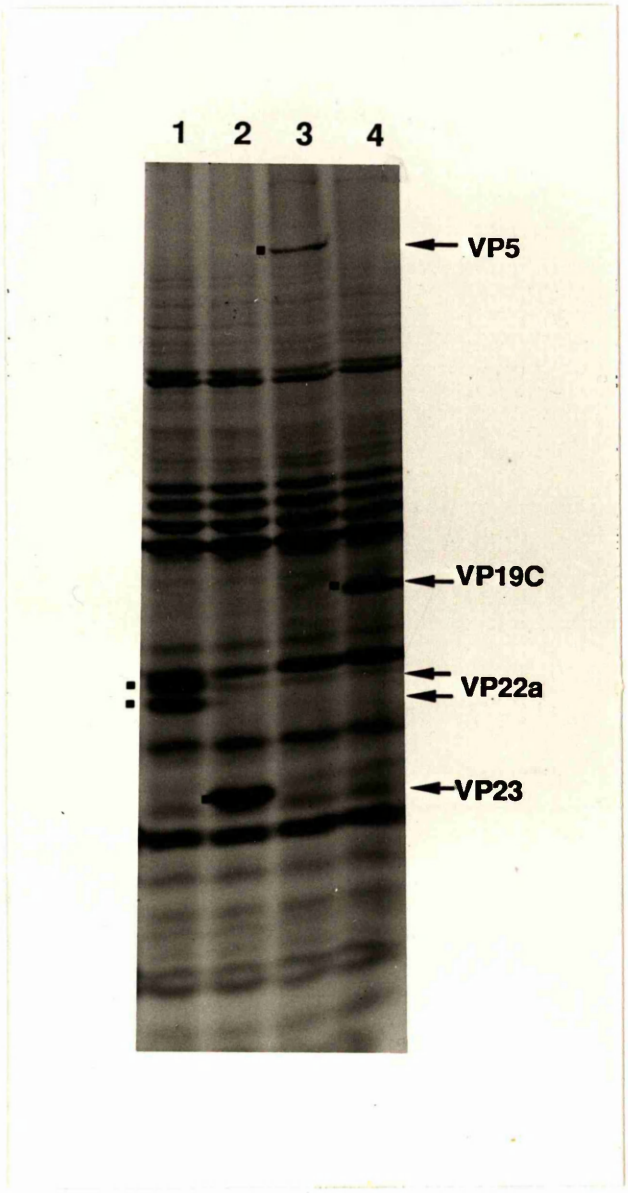

1
2
3
4
VP5
VP19C
VP22a
VP23

Figure 19. Expression of HSV-1 capsid proteins by recombinant vaccinia viruses. CV-1 cells were infected with 5 p.f.u. per cell of vMJ542 (lane 1), vMJ521 (lane 2), vMJ535 (lane 3) or vMJ534 (lane 4), and labelled from 3 to 24 hours p.i. with ^{35}S-methionine. Cells were harvested and 30 ul of whole cell extract was analysed on a 9% SDS-polyacrylamide slab gel. The cross-linker was bis-acrylamide. Proteins of interest are marked at the left hand side of each lane with a dot (▪). Arrows indicate the positions of protein bands which correspond to the named capsid proteins.

5 EXPRESSION OF CAPSID PROTEINS

To examine whether the recombinant vaccinia viruses expressed the HSV-1 proteins, confluent monolayers of CV-1 cells were infected with 5 pfu per cell of vMJ521, vMJ535, vMJ534 or vMJ542 and labelled with ^{35}S-methionine from 3 to 24 hours after infection. The cells were harvested at 24 h p.i., and whole-cell extracts were analysed by PAGE. Figure 18 shows an analysis of cells infected with vMJ521, vMJ535 and vMJ534 (lanes 3, 4 and 5 respectively). Control lanes show protein profiles of mock-infected cells, WR vaccinia-infected cells and purified HSV-1 B capsids (lanes 1, 2 and 6 respectively). The four capsid proteins visible in lane 6 are indicated. In each case the recombinant virus expresses large amounts of a novel protein not present in the parental vaccinia-infected cells. Each protein appears to comigrate with the appropriate capsid protein, being of apparent M_r 33 kDa (VP23), 155 kDa (VP5) and 53 kDa (VP19C).

Figure 19 shows the results of a similar experiment which includes a protein profile of vMJ542-infected cells. Lanes 2, 3 and 4 again each contain an abundant novel protein. Each novel protein is labelled with the name of the capsid protein with which it was shown to migrate in Figure 17, and which is known to be the product of the capsid gene used to construct the respective vaccinia virus. The sample in lane 1 consisted of cells infected with vMJ542. In this lane two novel bands can be seen, of approximate size 40 kDa. These are labelled as VP22a inasmuch as they are products of UL26.5.

The products of UL26.5 are known to be a highly processed family of proteins. In order to examine whether the products of UL26.5 expressed by vMJ542 were similarly processed and also to confirm that the novel products of vMJ542 are forms of VP22a, an immunoprecipitation experiment was carried out using the monoclonal antibody

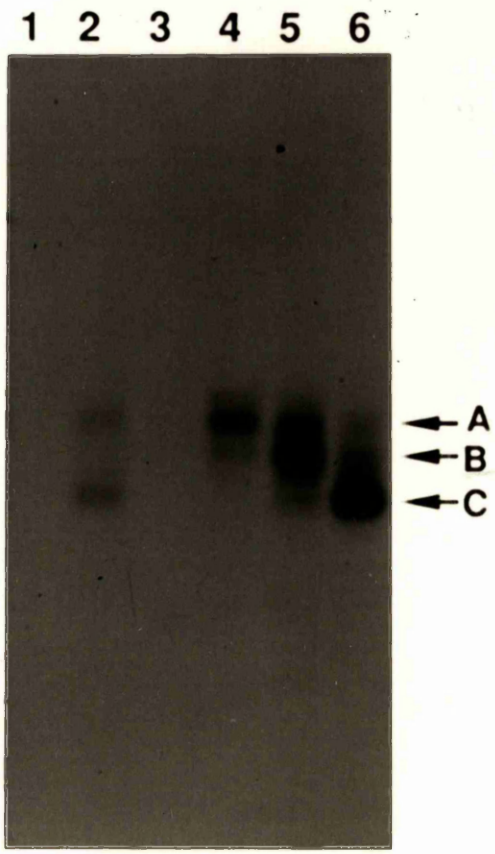
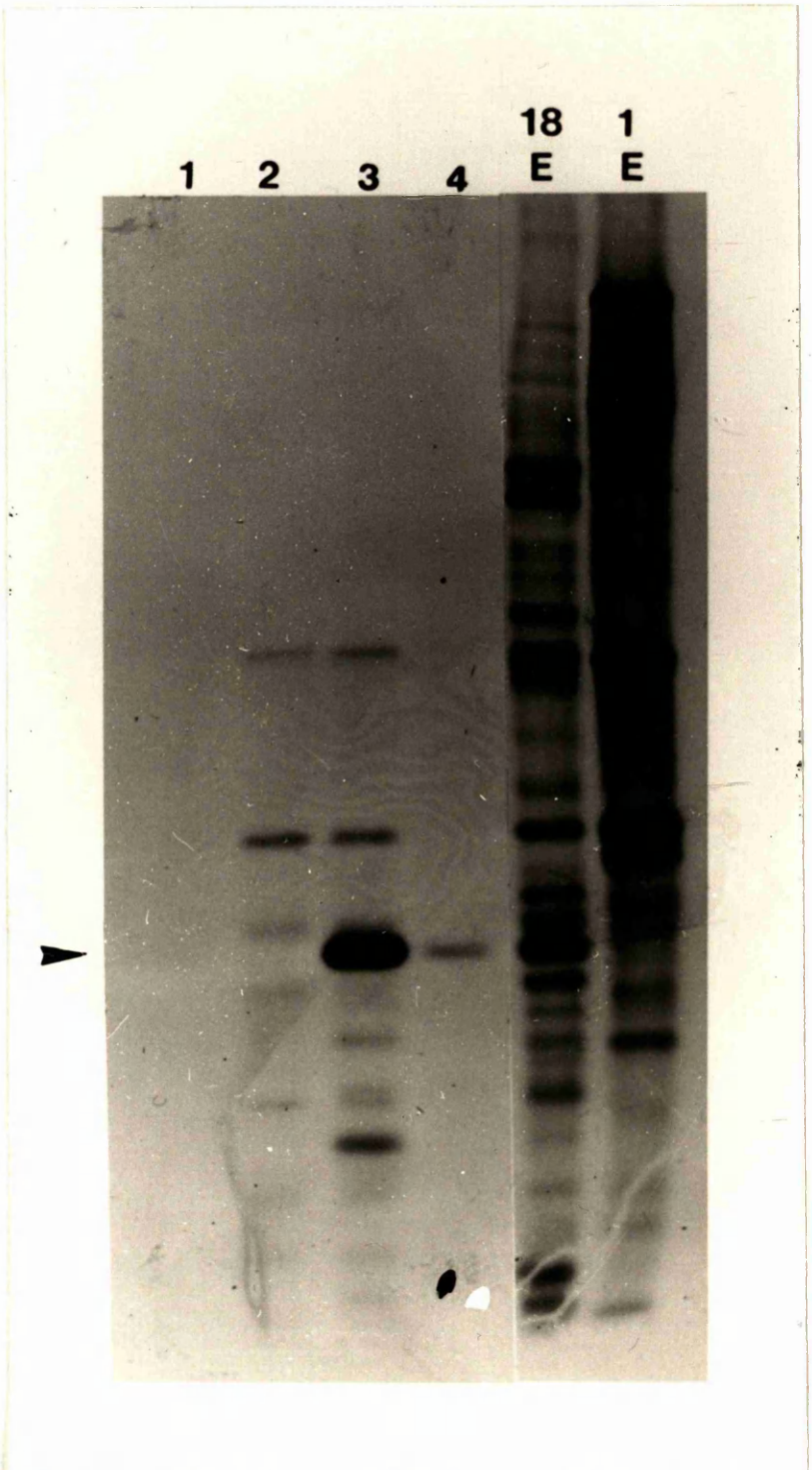

1 2 3 4 5 6
A
B
C

Figure 20. Immunoprecipitation of the products of UL26.5 expressed from HSV-1 and from vMJ542 by monoclonal antibody 5010. Autoradiograph of the immunoprecipitation of unprocessed and processed forms of VP22a from cells infected with HSV-1 (lanes 2, 5 and 6), vMJ542 (lane 4), WR vaccinia (lane 3), or mock-infected cells (lane 1). Cells were CV-1 (lanes 1 to 4), or BHK (lanes 5 and 6). Cells were labelled from 1 to 18 h p.i. with ^{35}S-methionine. The cells in lanes 5 and 6 were pulse labelled at 5 h p.i. After 30 minutes, the medium was removed, and the cells were harvested either immediately (lane 5) or after a chase of 5 h (lane 6). Following immunoprecipitation, samples were analysed on a 9% SDS-polyacrylamide slab gel. The cross-linker was bis-acrylamide. Arrows indicate the positions of three forms of VP22a.

5010, which recognises the unprocessed and the processed forms of VP22a (Rixon et al., 1988). The results of this experiment are shown in Figure 20. CV-1 or BHK cells were infected at a multiplicity of 20 p.f.u. per cell with HSV-1 (lanes 2, 5 and 6), WR vaccinia (lane 3), vMJ542 (lane 4), or were mock-infected (lane 1). The proteins present in lane 2 were precipitated from CV-1 cells infected with HSV-1 and harvested at 18 h p.i. Both unprocessed (position A) and processed (position C) forms of VP22a can be seen in lane 2. Lanes 5 and 6 show the immunoprecipitates from BHK cells infected with HSV-1, pulse-labelled at 5 h p.i. for 30 minutes, and then harvested immediately (lane 5) or after a chase of 5 h (lane 6). The transition during the period of chase from unprocessed forms of VP22a (positions A and B, lane 5) to the processed forms (position C, track 6) can be seen, as was previously demonstrated in a similar experiment by Rixon et al. (1988). In this experiment the processed forms of VP22a are seen as a single band (position C), whereas other reports have shown them to consist of a closely comigrating doublet (Rixon et al., 1988; Preston et al., 1992). Lane 4 contains the immunoprecipitates from CV-1 cells infected with vMJ542 and harvested after 18 h. It can be seen that vMJ542 synthesises abundant amounts of the higher molecular weight unprocessed form of VP22a (position A) and smaller amounts of the lower molecular weight form (position B). Thus vMJ542 expresses unprocessed forms of VP22a.

6A CHARACTERISATION OF MONOCLONAL ANTIBODY 1060

Monoclonal antibody 1060 was originally produced in this Institute by Dr J.Palfreyman and was shown to react with an HSV-1 protein of apparent M_r 33 kDa on SDS-PAGE. The antibody reacted also with HSV-2 precipitating a protein that migrated slightly faster on SDS-PAGE than the HSV-1 protein. The difference in mobility allowed the gene to be mapped using a series of intertypic recombinants.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18
E
1
E

Figure 21. Immunoprecipitation of the product of UL18 expressed by HSV-1 and by vMJ521 using monoclonal antibody 1060. Autoradiogram of the immunoprecipitation of the product of UL18 from CV-1 cells infected with 5 p.f.u. per cell of HSV-1 (lanes 1 and 4) or vMJ521 (lanes 2 and 3). Virus-infected cells were labelled with ^{35}S-methionine from 3 to 24 h p.i., when cells were harvested. Immunoprecipitation was carried out using 1060 (lanes 3 and 4) or control ascites (lanes 1 and 2). 1060 precipitated a band of approximate molecular weight of 33 kDa (arrowed) from both vMJ521- and HSV-1- infected cells (lanes 3 and 4 respectively). Lane 18E is a whole-cell extract of vMJ521-infected CV-1 cells, and contains a strong band migrating at the position of the protein precipitated by 1060. Lane 1E contains a whole-cell extract of HSV-1-infected CV-1 cells. Following immunoprecipitation, samples were analysed on a 9% SDS-polyacrylamide slab gel. The cross-linker was bis-acrylamide.

Mapping localised the gene to the long unique region of the HSV-1 genome between 0.195 and 0.276 map units (personal communication, Dr A.M.Cross). This region contains the genes UL16 to UL20 and parts of the genes UL15 and UL21. Of these genes UL16 and UL18 are predicted to encode proteins of similar sizes to that recognised by 1060 (McGeoch et al., 1988a).

To determine whether 1060 was recognising the product of UL18, it was tested in immunoprecipitation experiments against an extract from vMJ521-infected CV-1 cells. 1060 specifically precipitated a strong protein band that comigrated with the vMJ521 M_r 33-kDa band. The protein precipitated from vMJ521-infected cells also comigrated with that precipitated from HSV-1-infected cells, thereby confirming that 1060 recognised the product of UL18 (Figure 21).

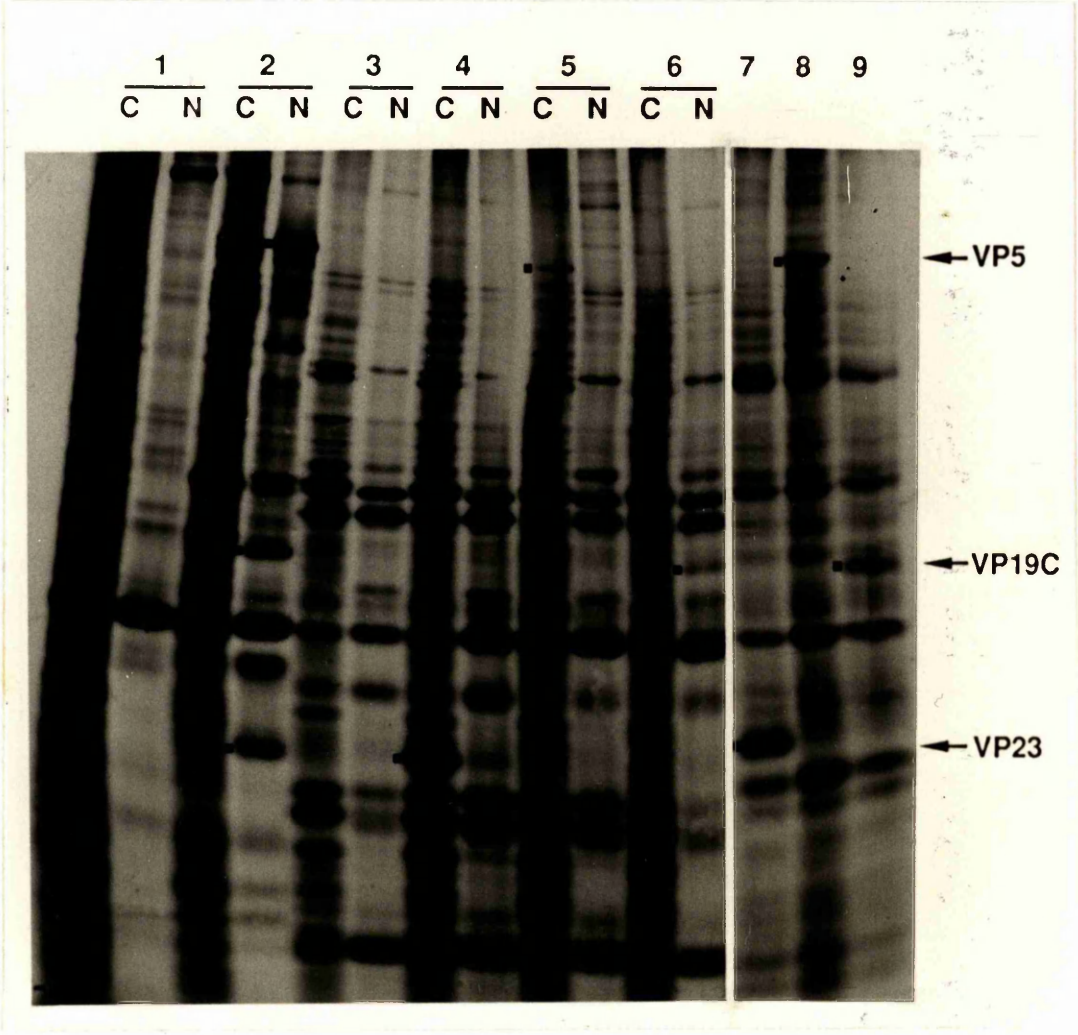
UL18 encodes an abundant capsid protein, and 1060 antibody reacted on Western blots with a preparation of purified virions. However, 1060 repeatedly failed to react with purified virions by immunoprecipitation, although an antibody to glycoprotein B reacted with the same virion preparations. Prior to immunoprecipitation the virions were solubilised in a buffer containing 0.5% nonidet P40 and 0.5% deoxycholate. This removes the virion envelope and much of the tegument, but does not disrupt the capsid. In these conditions the epitope recognised by 1060 appears to be inaccessible to antibody (personal communication, Dr A.M.Cross).

6B MARKER RESCUE OF tsG8

As described in the Introduction ts mutants belonging to two complementation groups which fail to assemble capsids at NPTs are believed to map to capsid protein genes. Ts2 has been mapped definitively to UL38 (Pertuiset et al., 1989). However, the group of mutants exemplified by tsG8

*ts*G8 DNA	rescue fragment	titre		marker rescue efficiency
		31 C	39 C	
+	-	1.2×10^7	$<1 \times 10^2$	8.3×10^{-5}
+	UL19	1×10^7	1.6×10^5	16
+	UL18	2×10^7	1×10^2	5×10^{-5}

Table 3 Marker rescue of *ts*G8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C N C N C N C N C N C N
VP5
VP19C
VP23

Figure 22. Separation of cells infected with vaccinia recombinants into cytoplasmic and nuclear fractions. Samples were prepared from CV-1 cells infected with 5 p.f.u. per cell of the following viruses: sample 1 - mock-infected; sample 2 - HSV-1; sample 3 - WR vaccinia; sample 4 - vMJ521; sample 5 - vMJ535; sample 6 - vMJ534; sample 7 - vMJ521; sample 8 - vMJ535; sample 9 - vMJ534. Cells were labelled with ^{35}S-methionine from 3 to 18 h p.i., and then harvested. Samples 1 to 6 were separated into cytoplasmic (C) and nuclear (N) fractions, while samples 7 to 9 were used as whole-cell extracts. Aliquots were analysed on a 9% SDS-polyacrylamide slab gel. The cross-linker was bis-acrylamide. Samples 1 to 6 were analysed in lanes 1 to 6 respectively, and 30 ul (from a total sample volume of 500 ul) of each cytoplasmic fraction was used (lanes 'C'), and 12 ul (from a total sample volume of 25 ul) of nuclear fraction (lanes 'N'). Samples 7 to 9 were analysed in lanes 7 to 9 respectively, and 20 ul (from a total sample volume of 200 ul) of whole-cell extract was used. Arrows indicate the positions of proteins which are the products of the HSV-1 capsid genes under investigation. These proteins are also marked at the left hand side of each lane with a dot (▪).

and tsG3 were mapped using a DNA fragment which included both the UL18 and UL19 ORFs (Weller et al., 1987). It was concluded from the phenotype of these mutants that the lesion was in UL19, since UL18 was not recognised as a capsid protein gene at that time.

To address this question the lesion in tsG8 was mapped using the cloned UL19 and UL18 ORFs contained in pBJ196 and pBJ183 respectively (pBJ183 was generated by subcloning the SalI/BamHI fragment of pMJ521 into SalI/BamHI-digested pUC19). Table 3 shows the results obtained following cotransfection of tsG8 DNA (provided by Dr F.J.Rixon) with either pBJ196 or pBJ183. The resultant titres (in p.f.u./ml) of infectious virus at the permissive (31°C) and non-permissive (39°C) temperatures are shown. The results are also expressed as marker rescue efficiencies, which were calculated using the formula [(p.f.u./ml 39°C)/(p.f.u./ml 31°C)] x 10^3. TsG8 was rescued efficiently by pBJ196 thereby confirming that the lesion maps to UL19.

7 SUBCELLULAR LOCALISATION OF THE CAPSID PROTEINS

7.1 Cell Fractionation

HSV-1 capsids are assembled in the nucleus of infected cells. To examine whether the HSV-1 capsid proteins expressed from the vaccinia vectors were efficiently transported to the nucleus, cells infected with vMJ521, vMJ535 and vMJ534 were separated into cytoplasmic and nuclear fractions. VP5 has previously been shown in cell fractionation experiments to locate to the nucleus of HSV-1-infected cells (Fenwick et al., 1978). Figure 22 shows that 18 h after infection with HSV-1 VP5, VP19C and VP23 are abundant in the nucleus. All three proteins are also present in the cytoplasmic fraction. This probably reflects the presence of mature virions which are found in the cytoplasm and on the cell surface at this time

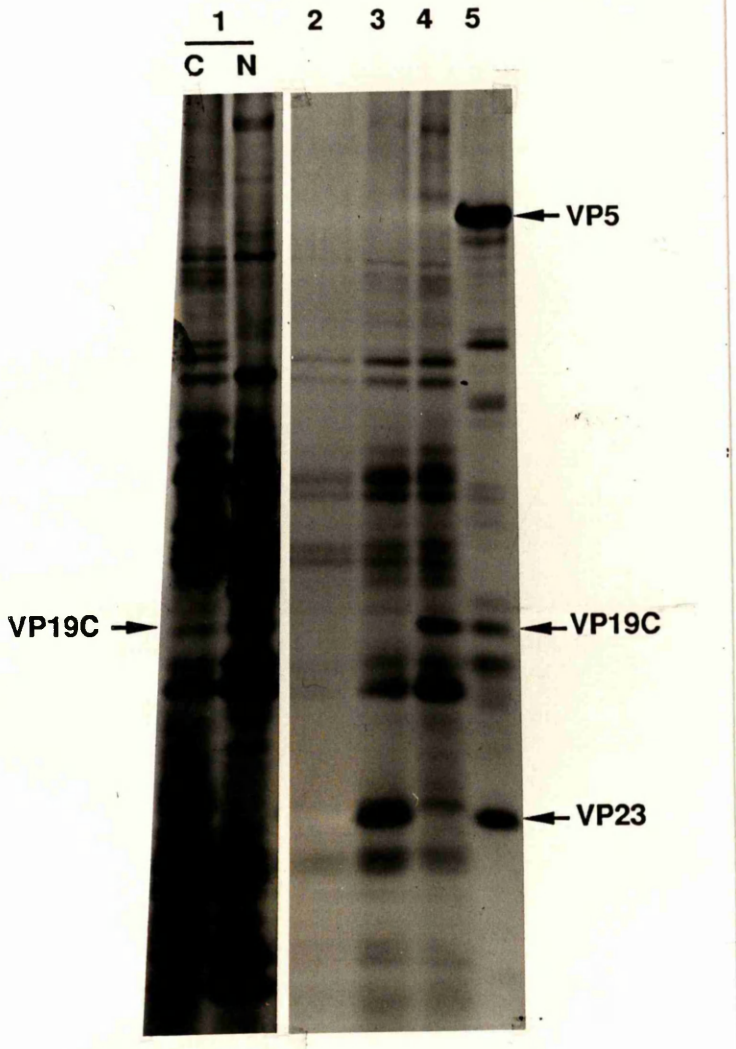

1
C N
2
3
4
5
VP5
VP19C
VP19C
VP23

Figure 23. Fractionation of cells infected with vMJ534, showing nuclear localisation of VP19C. Samples were prepared from CV-1 cells infected with 5 p.f.u. per cell of the following viruses: sample 1 - vMJ534; sample 2 - WR vaccinia; sample 3 - vMJ521; sample 4 - vMJ534. Cells were labelled with ^{35}S-methionine from 3 to 18 h p.i., and then harvested. Sample 1 was separated into cytoplasmic (C) and nuclear (N) fractions, and samples were analysed on a 9% SDS-polyacrylamide slab gel. The cross-linker was bis-acrylamide. Sample 1 was analysed in lane 1, and 30 ul (from a total sample volume of 500 ul) of the cytoplasmic fraction was used (lane 'C'), and 12 ul (from a total sample volume of 25 ul) of nuclear fraction (lane 'N'). Samples 2 to 4 were analysed in lanes 2 to 4 respectively, and 20 ul (from a total sample volume of 200 ul) of whole-cell extract (lanes 2, 3 and 4) was used. Lane 5 contains a sample of HSV-1 B capsids. (This sample contains a number of contaminating, non-capsid proteins. Arrows indicate the positions of HSV-1 capsid proteins.)

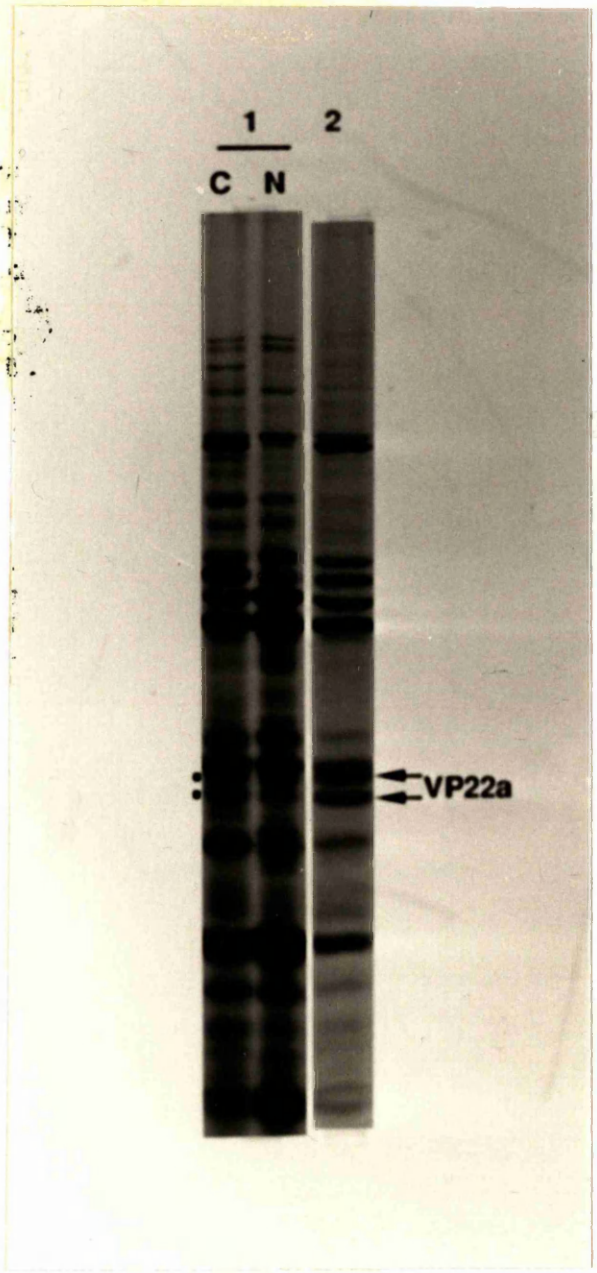

1
2
C
N
VP22a

Figure 24. Separation of cells infected with vMJ542 into cytoplasmic and nuclear fractions. The samples used in lanes 1 and 2 were prepared from CV-1 cells infected with 5 p.f.u. per cell of vMJ542. Cells were labelled with ^{35}S-methionine from 3 to 18 h p.i., and then harvested. The sample used in lane 1 was separated into cytoplasmic (C) and nuclear (N) fractions. The sample used in lane 2 was a whole-cell extract. Aliquots were analysed on a 9% SDS-polyacrylamide slab gel. The cross-linker was bis-acrylamide. 30 ul (from a total sample volume of 500 ul) of the cytoplasmic fraction was used (lane 'C'), and 12 ul (from a total sample volume of 25 ul) of nuclear fraction (lane 'N'). 20 ul (from a total sample volume of 200 ul) of whole-cell extract (lane 2) was used. Arrows indicate the positions of proteins which are the products of UL26.5. These proteins are also marked at the left hand side of lane 1C with a dot (•).

after infection. By contrast, in recombinant-vaccinia-infected cells VP23 and VP5 are almost exclusively cytoplasmic. VP19C, however, remains predominantly nuclear. This is interesting in that VP19C appears to possess intrinsic ability to localise to the nucleus, whereas VP5 and VP23 do not. These results were consistently obtained over several experiments. Another experiment, shown in Figure 23, confirms the finding for VP19C. VP19C expressed by vMJ534 is again found predominantly in the nuclear fraction.

Figure 24 shows the results of a fractionation experiment carried out on cells infected with vMJ542. Here the two bands labelled as VP22a appear to be fairly equally divided between the tracks containing the cytoplasmic and the nuclear fractions. However, it must be noted that a greater proportion of the nuclear sample is used in these experiments than of the cytoplasmic sample. This is because the fractionation procedure results in a cytoplasmic sample of 500 ul, and a nuclear sample of 25 ul. On these gels, in order to achieve comparable amounts of radiolabel in each lane, 30 ul of cytoplasmic sample was used, and 12 ul of nuclear sample, i.e. an 8 times greater proportion of nuclear sample is present on this gel than cytoplasmic sample. Thus it would appear from this experiment that in cells infected with vMJ542, the products of UL26.5 are located primarily in the cytoplasm. However, it does appear that these proteins do possess intrinsic ability to locate to the nucleus.

7.2 Cell fractionation in infections with more than one recombinant

To determine whether nuclear transport of VP5 and VP23 required the presence of more than one capsid protein, coinfection experiments were performed. Simultaneous infection of cells with any pair of the recombinants vMJ521, vMJ535 and vMJ534, or a triple infection using

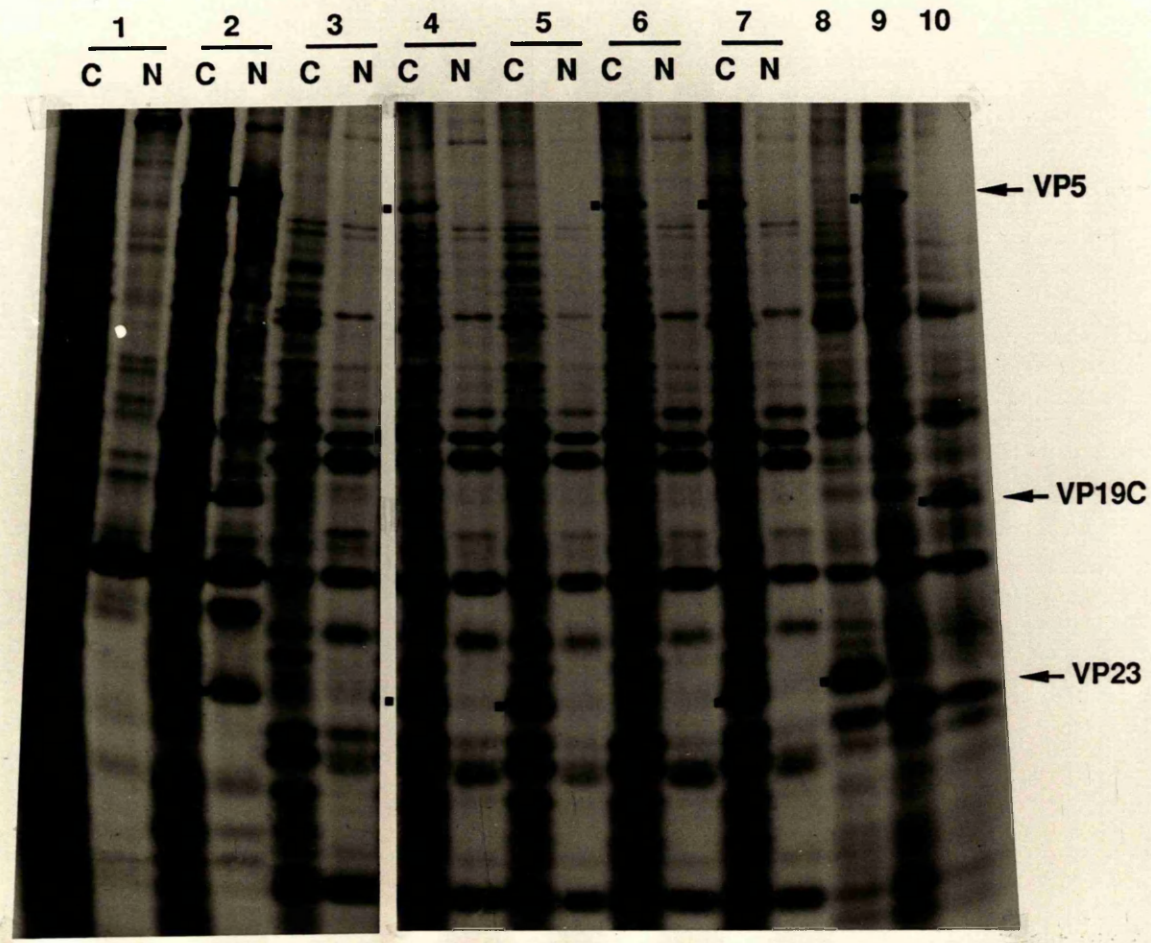

1
2
3
4
5
6
7
8
9
10
C N C N C N C N C N C N C N
VP5
VP19C
VP23

Figure 25. Fractionation of cells infected with more than one recombinant vaccinia virus. Samples were prepared from CV-1 cells infected with 5 p.f.u. per cell of each of the following viruses: sample 1 - mock-infected; sample 2 - HSV-1; sample 3 - WR vaccinia; sample 4 - vMJ521 and vMJ535; sample 5 - vMJ521 and vMJ534; sample 6 - vMJ535 and vMJ534; sample 7 - vMJ521, vMJ535 and vMJ534; sample 8 - vMJ521; sample 9 - vMJ535; sample 10 - vMJ534. Cells were labelled with ^{35}S-methionine from 3 to 18 h p.i., and then harvested. Samples 1 to 7 were separated into cytoplasmic (C) and nuclear (N) fractions, and aliquots were analysed on a 9% SDS-polyacrylamide slab gel. The cross-linker was bis-acrylamide. Samples 1 to 7 were analysed in lanes 1 to 7 respectively, and 30 ul (from a total sample volume of 500 ul) of each cytoplasmic fraction was used (lanes 'C'), and 12 ul (from a total sample volume of 25 ul) of nuclear fraction (lanes 'N'). Samples 8 to 10 were analysed in lanes 8 to 10 respectively, and 20 ul (from a total sample volume of 200 ul) of whole-cell extract was used. Arrows indicate the positions of proteins which are the products of the HSV-1 capsid genes under investigation. These proteins are also marked at the left hand side of each lane with a dot (•). VP19C is not well-resolved in lanes 5, 6 or 7.

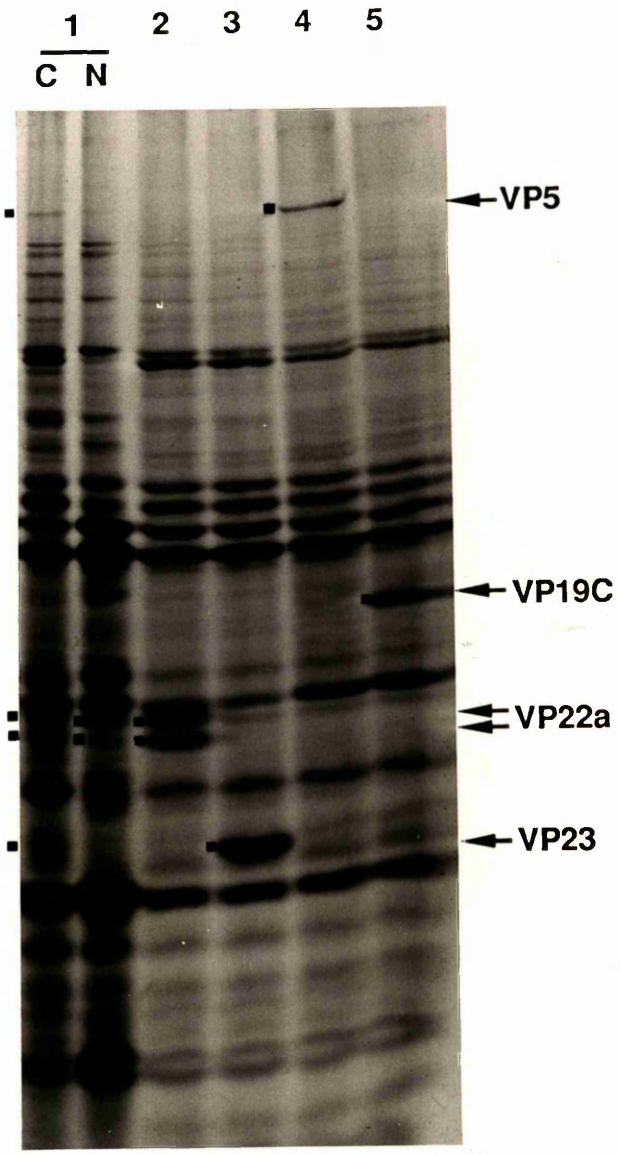

1
2
3
4
5
C
N
VP5
VP19C
VP22a
VP23

Figure 26. Fractionation of cells infected with all four vaccinia recombinants. Samples were prepared from CV-1 cells infected with 5 p.f.u. per cell of each of the following viruses: sample 1 - vMJ521, vMJ535, vMJ534 and vMJ542; sample 2 - vMJ542; sample 3 - vMJ521; sample 4 - vMJ535; sample 5 - vMJ534. Cells were labelled with ^{35}S-methionine from 3 to 18 h p.i., and then harvested. Sample 1 was separated into cytoplasmic (C) and nuclear (N) fractions, and aliquots were analysed on a 9% SDS-polyacrylamide slab gel. The cross-linker was bis-acrylamide. Sample 1 was analysed in lane 1, and 30 ul (from a total sample volume of 500 ul) of the cytoplasmic fraction was used (lane 'C'), and 12 ul (from a total sample volume of 25 ul) of nuclear fraction (lane 'N'). Samples 2 to 5 were analysed in lanes 2 to 5 respectively, and 20 ul (from a total sample volume of 200 ul) of whole-cell extract was used. Arrows indicate the positions of proteins which are the products of the HSV-1 capsid genes under investigation. These proteins are also marked at the left hand side of each lane with a dot (▪). VP19C is not visible in lanes 1C or 1N.

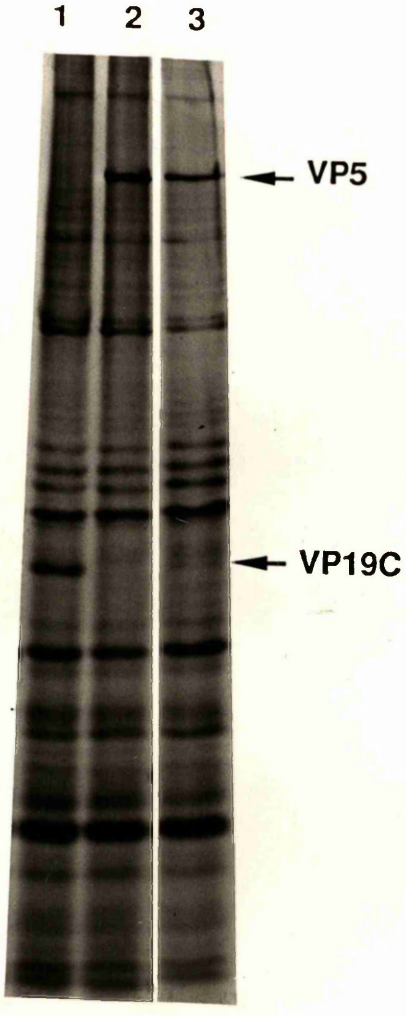

1
2
3
VP5
VP19C

Figure 27. Down-regulation of VP19C in the presence of VP5 in a recombinant vaccinia expression system. Samples were prepared from CV-1 cells infected with 5 p.f.u. per cell of each of the following viruses: lane 1 - vMJ534; lane 2 - vMJ535; lane 3 - vMJ534 and vMJ535. Cells were labelled with ^{35}S-methionine from 3 to 18 h p.i., and then harvested. Samples were analysed on a 9% SDS-polyacrylamide slab gel. The cross-linker was bis-acrylamide. Arrows indicate the positions of proteins which are the products of the HSV-1 capsid genes under investigation. Both VP19C (lane 1) and VP5 (lane 2) are expressed in abundant amounts from the respective vaccinia virus in a single infection. In cells infected with both viruses (lane 3), the level of VP5 is unaffected, but the level of VP19C is greatly reduced.

all three recombinants, did not affect distribution of VP5 or VP23. Figure 25 shows that the location of VP5 and VP23 in dual or triple coinfections remained almost exclusively cytoplasmic. However, in these experiments, VP19C was not visible in lanes where cells coinfected with vMJ534 and with any other recombinant were used (tracks 5, 6 and 7 in Figure 25). VP19C was, however, always well resolved on these gels in control tracks of whole-cell extracts of vMJ534-infected cells.

Figure 26 shows the result of a fractionation experiment performed on cells infected with all four recombinants. The distribution of VP22a appears to be unaltered, when compared to its distribution in cells infected only with vMJ542 (compare with Figure 24). Similarly, the distribution of VP23 appears to be unaltered, being almost exclusively cytoplasmic. However, although VP5 is still predominantly cytoplasmic, some VP5 is clearly present in the nuclear fraction. This presence of VP5 in the nucleus was not a reproducible feature of these experiments and may be due to a weakness in the fractionation procedure. Again, VP19C is not visible on this gel.

7.3 Downregulation of VP19C expressed by vMJ534 in dual infections with a second recombinant virus

In the experiments reported in section 7.2, VP19C could not be detected in cells coinfected with vMJ534 and any other of the recombinant viruses. In order to assess this more accurately, whole-cell extracts of cells infected with vMJ534, with vMJ535, and with both viruses, were analysed by electrophoresis. This experiment is shown in Figure 27. Lane 1 contains an extract of cells infected with vMJ534, and VP19C can be seen at the position marked. Lane 2 contains an extract of cells infected with vMJ535, and VP5 is clearly visible. Lane 3 contains cells infected with equal amounts of both viruses. VP5 is

A

B

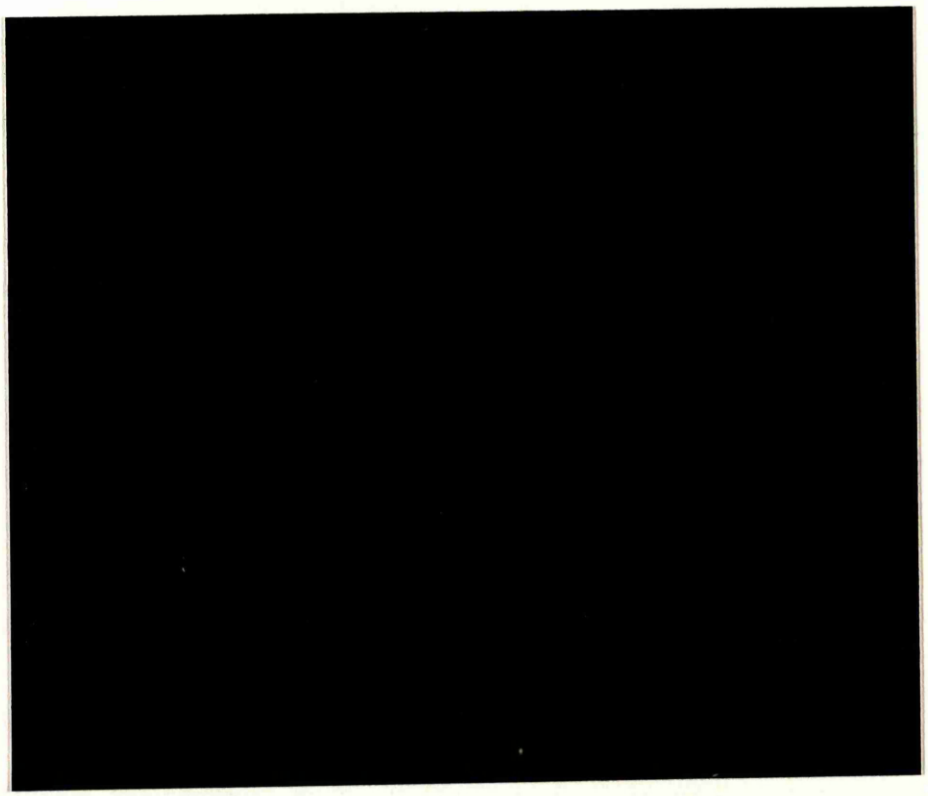


Figure 28. Immunofluorescent staining of HSV-1-infected cells using the anti-VP23 monoclonal antibody 1060. CV-1 cells were infected with 5 p.f.u. per cell of HSV-1 and were incubated at 37°C for 18 h. Cells were then stained with a 1/100 dilution of 1060 (A) or a 1/100 dilution of control ascites (B), followed by a 1/200 dilution of FITC-conjugated goat anti-mouse immunoglobulin G, to detect VP23.

A

B

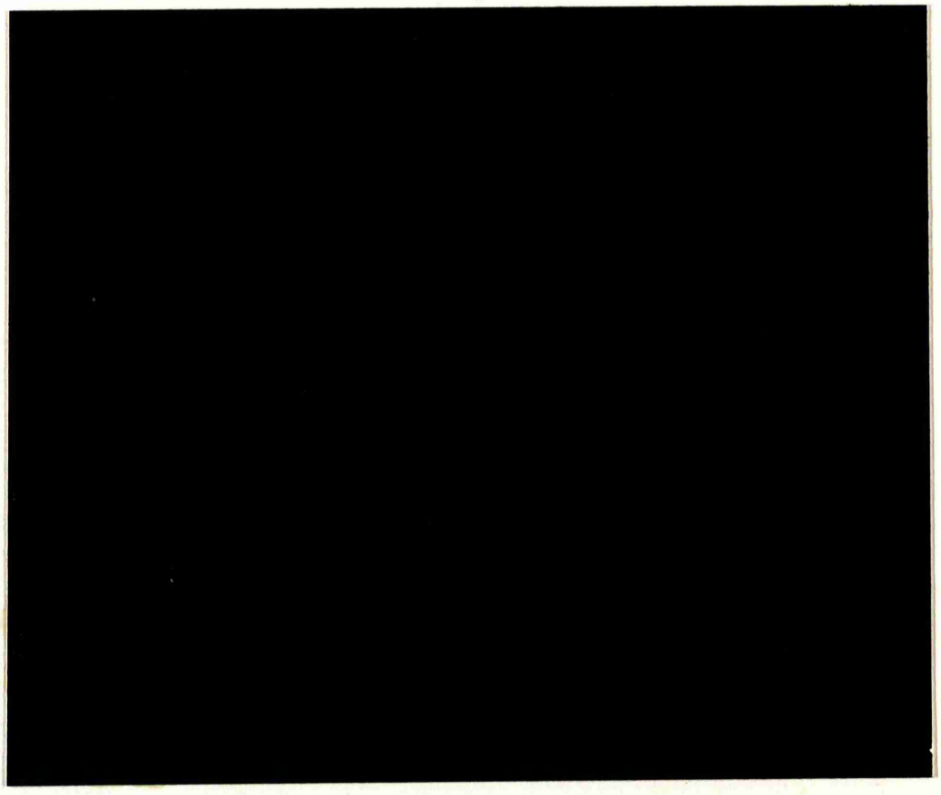


Figure 29. Immunofluorescent staining of vMJ521-infected cells using 1060. CV-1 cells were infected with 5 p.f.u. per cell of vMJ521 and were incubated at 37°C for 18 h. Cells were then stained with a 1/100 dilution of 1060 (A) or a 1/100 dilution of control ascites (B), followed by a 1/200 dilution of FITC-conjugated goat anti-mouse immunoglobulin G, to detect VP23.

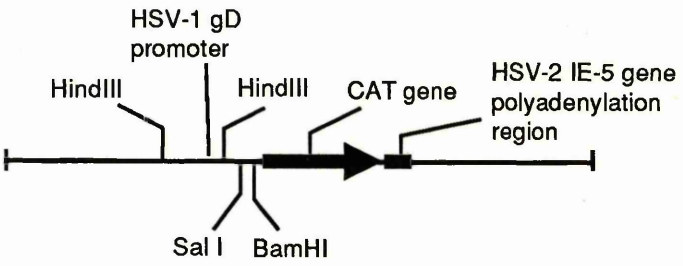
present in amounts similar to those found with vMJ535 alone, but VP19C is present only in greatly reduced amounts. The reason for this is not known.

7.4 The intracellular distribution of capsid proteins determined by immunofluorescence

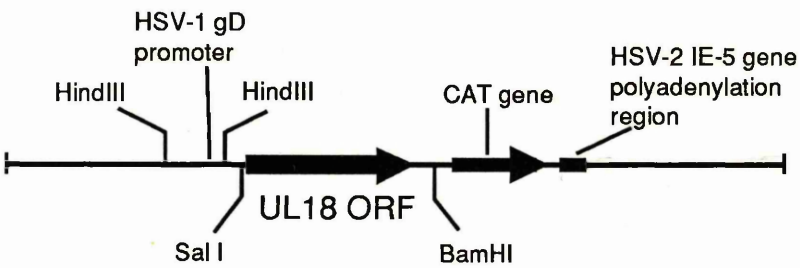
To further investigate the intracellular distribution of the capsid proteins, and in particular to confirm that the cytoplasmic distribution of the products of UL18 and UL19 was a true reflection of their intracellular distributions and not a result of the fractionation procedure, the subcellular localisation of individual capsid proteins was determined by immunofluorescence. The method used was one of indirect immunofluorescent staining, using either a mouse monoclonal or a rabbit polyclonal antibody as the primary antibody, and a goat FITC-conjugated anti-mouse or anti-rabbit secondary antibody.

7.4.1 Immunofluorescent localisation of VP23

This was performed using monoclonal antibody 1060 as primary antibody. Cohen et al. (1980) have previously shown by immunofluorescent techniques that VP23 is localised to the nucleus of HSV-1-infected cells. These results, and the results of the fractionation of HSV-1-infected cells presented above (section 7.1), led to the expectation that use of 1060 to stain HSV-1-infected cells would show a nuclear location of VP23. As expected, VP23 was found to be predominantly nuclear in HSV-1-infected cells (Figure 28). In marked contrast, in cells infected with vMJ521, VP23 was predominantly cytoplasmic in agreement with the cell fractionation experiments (Figure 29). vMJ521-infected cells do show a slight nuclear fluorescence, as evidenced by the presence of unstained nucleoli.



pgDCAT

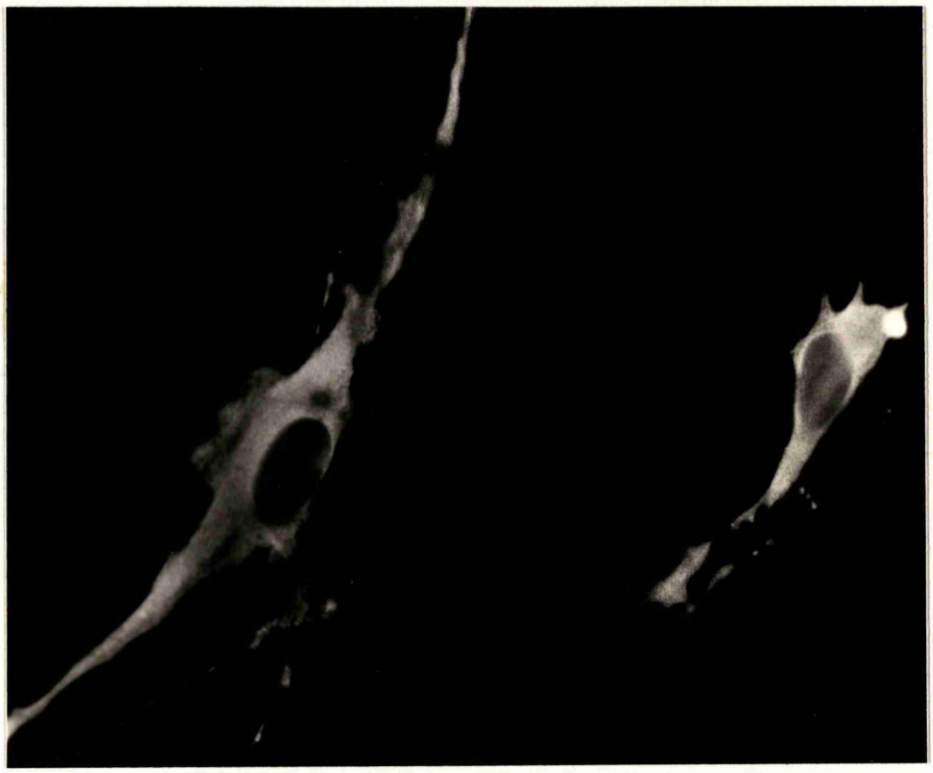


pBJ182

Figure 30. The structures of pgDCAT and pBJ182

The proportions of this diagram are approximately to scale and are based on the details of pgDCAT given by Everett (1986) and by Gaffney et al. (1985).

A



B

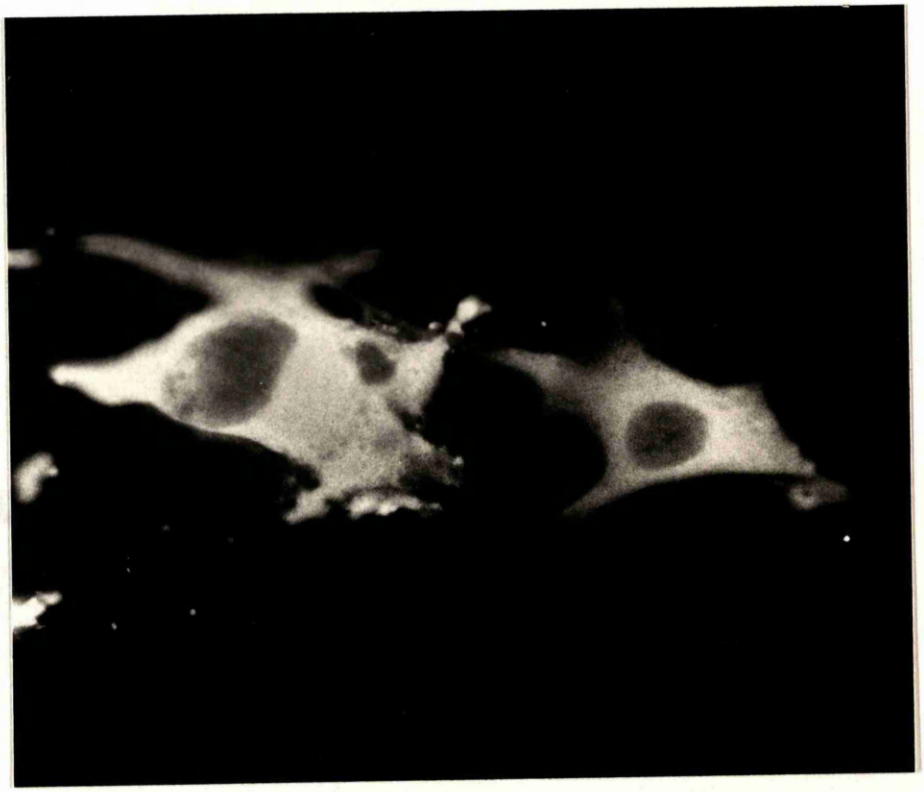
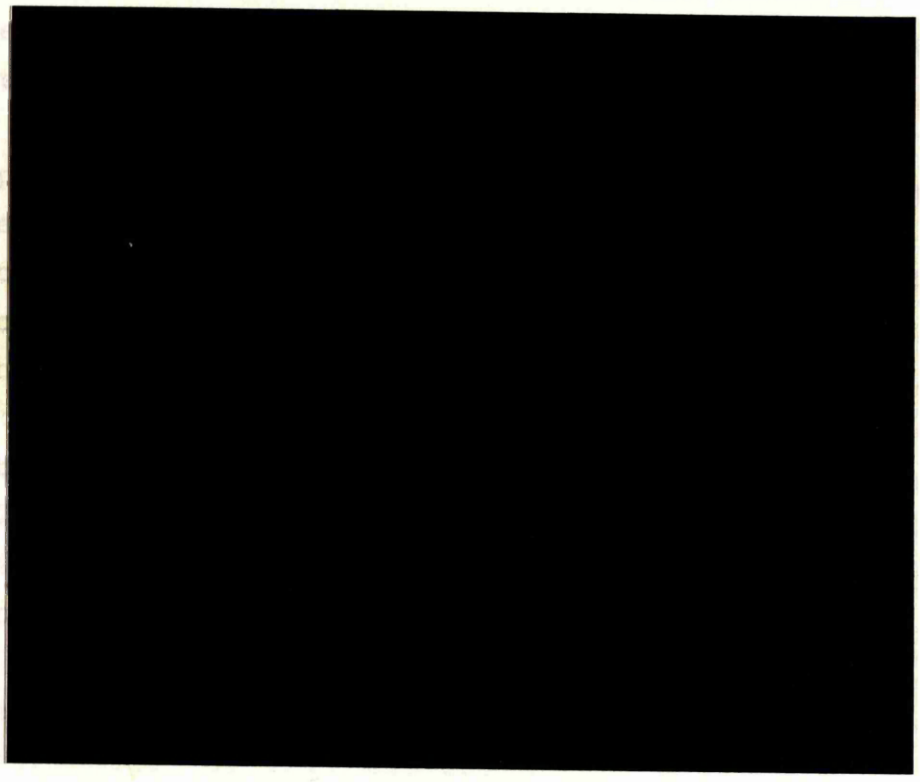


Figure 31. Immunofluorescence of cells transfected with pBJ182, showing cytoplasmic distribution of VP23. Confluent monolayers of BHK cells were transfected with 1 ug each of pBJ182, p175, and p111 DNA, and incubated at 38.5°C for 72 h. Cells were then stained with a 1/100 dilution of 1060 (A and B) or a 1/100 dilution of control ascites (C), followed by a 1/200 dilution of FITC-conjugated goat anti-mouse immunoglobulin G, to detect VP23.



C

7.4.2 Expression of VP23 from a plasmid vector

There are two possible reasons why VP23 adopts a cytoplasmic localisation in these experiments. The first is that VP23 lacks a nuclear targetting signal and is dependent on some other protein (presumably HSV-specified) to transport it to or to retain it in the nucleus. The second is that the normal transport mechanism is disrupted by the vaccinia host. It was thought unlikely that the failure of VP23 to accumulate in the nucleus was a consequence of the vaccinia vector interfering with the normal transport mechanisms since VP22a when expressed by vMJ542 (see below, sections 7.4.5, 7.4.6 and 7.4.7) and other herpesvirus proteins expressed from the same vector (personal communication, Dr A.J.Davison) were known to enter and to accumulate in the nucleus. However, to eliminate this possibility the distribution of VP23 expressed from a plasmid vector was examined. The plasmid pBJ182 was constructed by cloning the UL18 coding sequences contained on a 1370-base-pair SalI-BamHI fragment from pMJ521 (refer to Figure 9) into the SalI and BamHI sites of pgDCAT, such that UL18 was under the control of the HSV-1 gD promoter. The structures of pgDCAT and of pBJ182 are shown in Figure 30. The gD promoter is a strong early promoter exhibiting very high levels of activation in the presence of two HSV-1 immediate early transactivating proteins Vmw110 and Vmw175, which were expressed from the plasmids p111 and p175 respectively (Everett, 1986). 1 ug of each of pBJ182, p111 and p175 was cotransfected into BHK cells, which were then incubated for 72 hours at 38.5°C. When the distribution of VP23 in these cells was examined by fluorescent staining with 1060, a very small proportion of cells was found to be fluorescing. Presumably the efficiency of transfection was very low, and very few cells received sufficient amounts of all three plasmids necessary for expression of VP23. When fluorescence was detected, it was similar to that in vMJ521-infected cells, ie predominantly cytoplasmic (Figure 31).

A

B

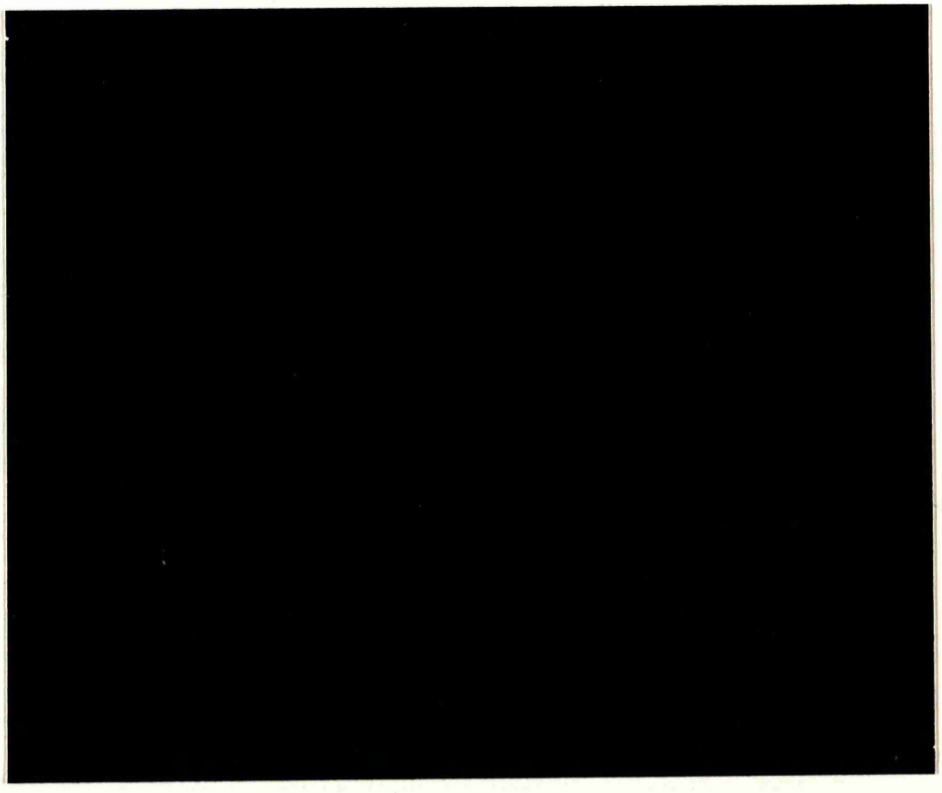


Figure 32. Immunofluorescence of cells infected with vMJ521, vMJ535 and vMJ534, showing predominantly cytoplasmic distribution of VP23. CV-1 cells were infected with 5 p.f.u. per cell each of vMJ521, vMJ535 and vMJ534 and were incubated at 37°C for 18 h. Cells were then stained with a 1/100 dilution of 1060 (A) or a 1/100 dilution of control ascites (B), followed by a 1/200 dilution of FITC-conjugated goat anti-mouse immunoglobulin G, to detect VP23.

A

B

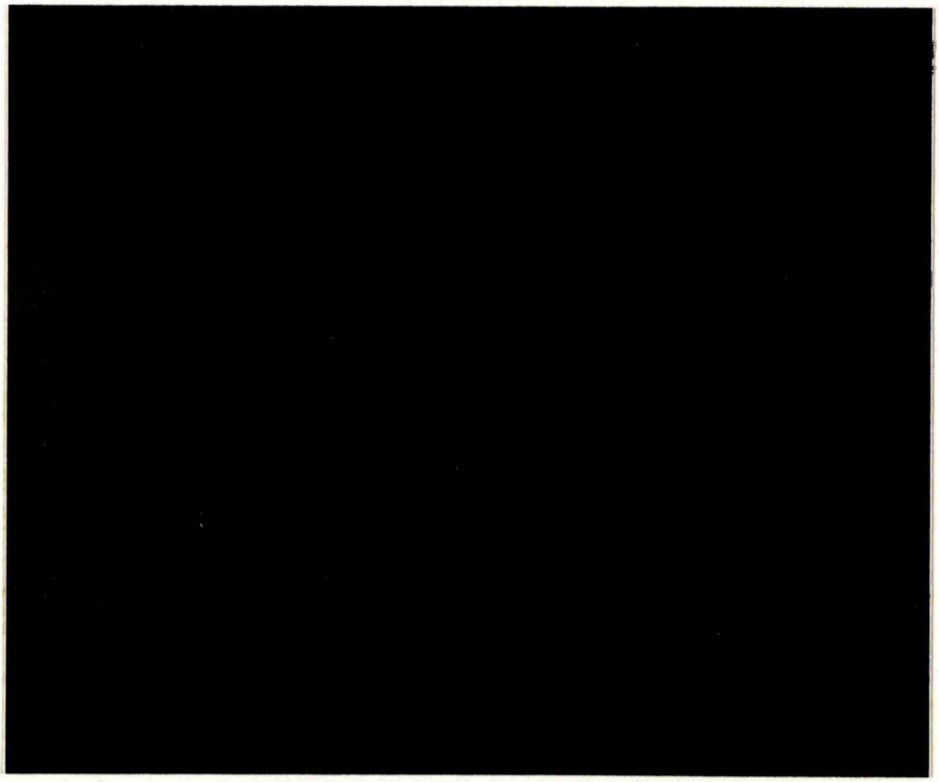


Figure 33. Immunofluorescence of vMJ535-infected cells. CV-1 cells were infected with 0.05 p.f.u. per cell of vMJ535 (A) or 5 p.f.u. per cell of WR vaccinia (B) and were incubated at 37°C for 18 h. Cells were then stained with a 1/200 dilution of 10555, followed by a 1/200 dilution of FITC-conjugated goat anti-rabbit immunoglobulin G.

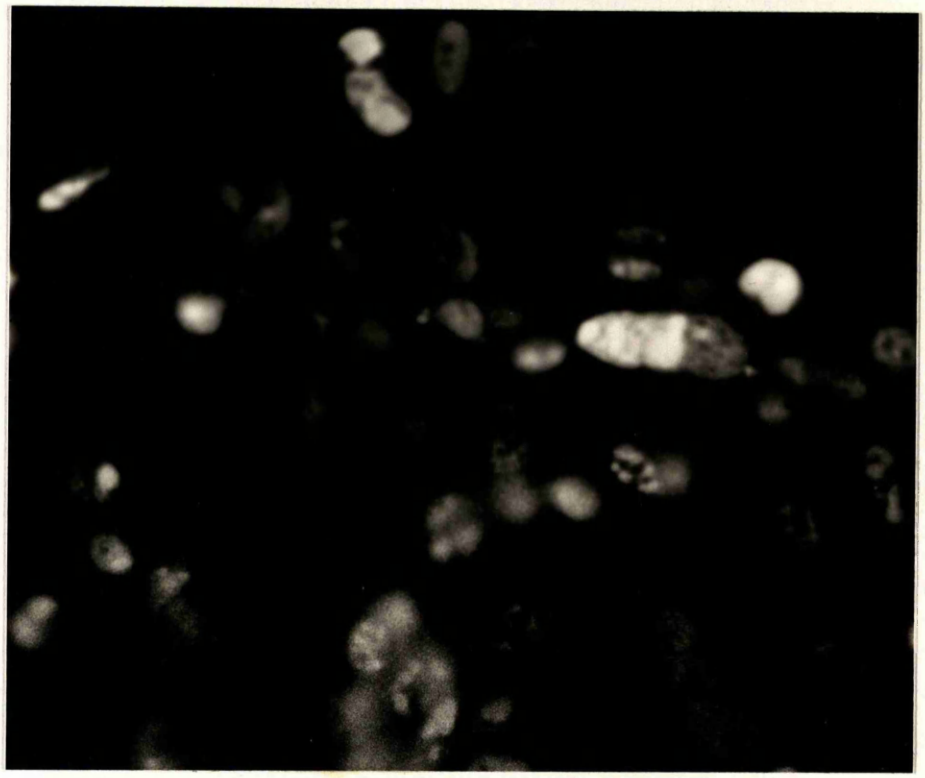
7.4.3 Immunofluorescent localisation of VP23 in coinfection experiments

The intracellular distribution of VP23 in cells infected with vMJ521 was further examined by coinfecting cells with vMJ521 and either or both of vMJ535 and vMJ534. The results showed that neither of the other two capsid proteins, VP5 nor VP19C, when expressed in the recombinant vaccinia system, had any effect on the distribution of VP23. Figure 32 shows that in CV-1 cells infected with vMJ521, vMJ535 and vMJ534, VP23 still has a predominantly cytoplasmic location, similar to that in Figure 29A.

7.4.4 Immunofluorescent localisation of VP5

The intracellular distribution of VP5 was also examined using immunofluorescent techniques. Powell & Watson (1975) and Cohen *et al*. (1980) have previously demonstrated that VP5 adopts a nuclear location in HSV-1-infected cells. The antibody used in the present experiments, 10555, was a rabbit anti-HSV-1 antibody, and as such, was not specifically raised against VP5. It was therefore unsuitable for use in HSV-1-infected cells. However, in studies of human HSV-1-immune sera, VP5 has been shown to be a dominant antigen (Eberle & Mou, 1983), and it was felt likely that 10555 would recognise VP5. In practice it was found that either 10555 or the anti-rabbit secondary antibody produced a level of background fluorescence which sometimes made the results difficult to interpret. The most effective way to control these experiments was by using a low m.o.i. which resulted in fields of view showing only a few infected cells against a background of uninfected cells. Figure 33A shows a CV-1 cell monolayer infected with vMJ535 at an m.o.i. of 0.05 p.f.u. per cell, and stained using 10555 as primary antibody. Two cells show levels of fluorescence higher than background. The fluorescence is stronger in the

A



B

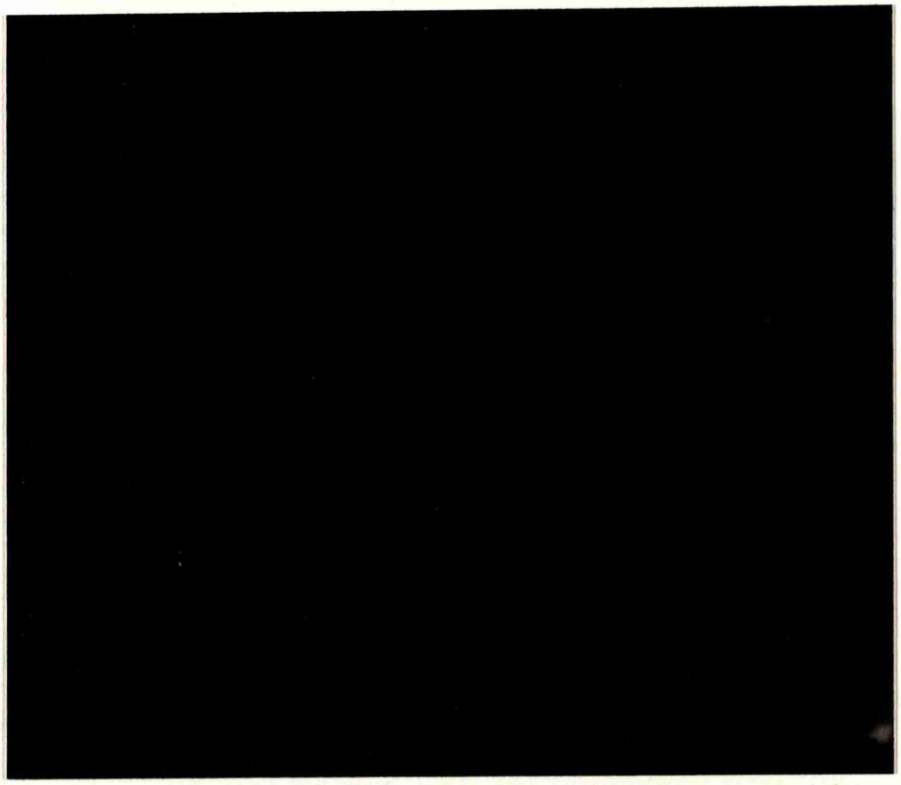
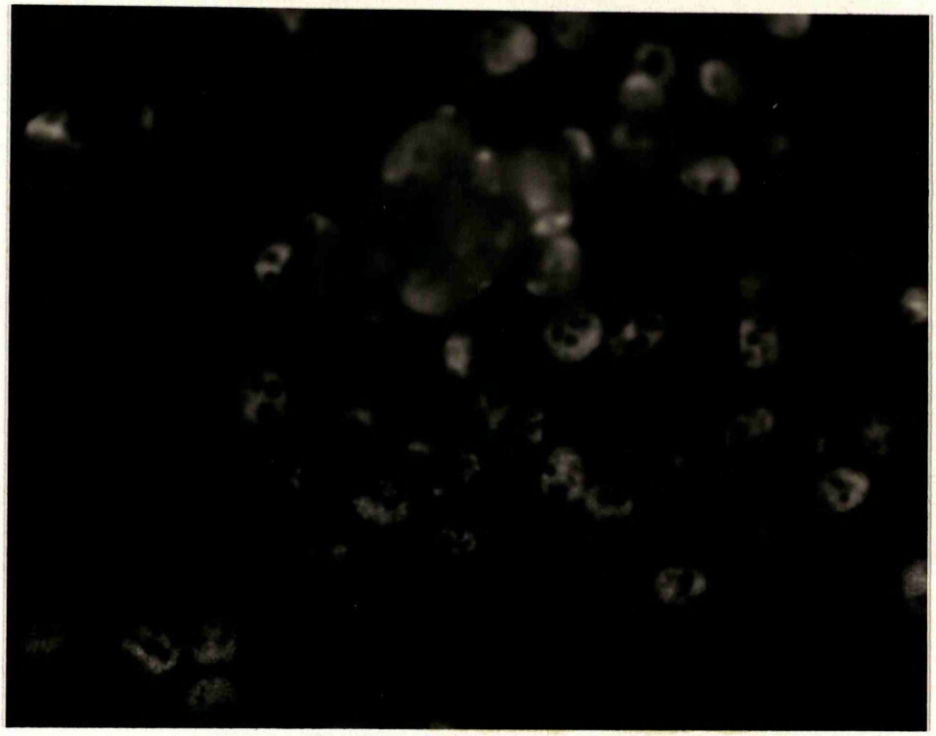


Figure 34. Immunofluorescence of HSV-1-infected cells using monoclonal antibody 5010, showing nuclear localisation of VP22a. CV-1 cells were infected with 5 p.f.u. per cell of HSV-1 and were incubated at 37°C for 18 h. Cells were then stained with a 1/50 dilution of 5010 (A) or a 1/50 dilution of control ascites (B), followed by a 1/200 dilution of FITC-conjugated goat anti-mouse immunoglobulin G, to detect VP22a.

A



B

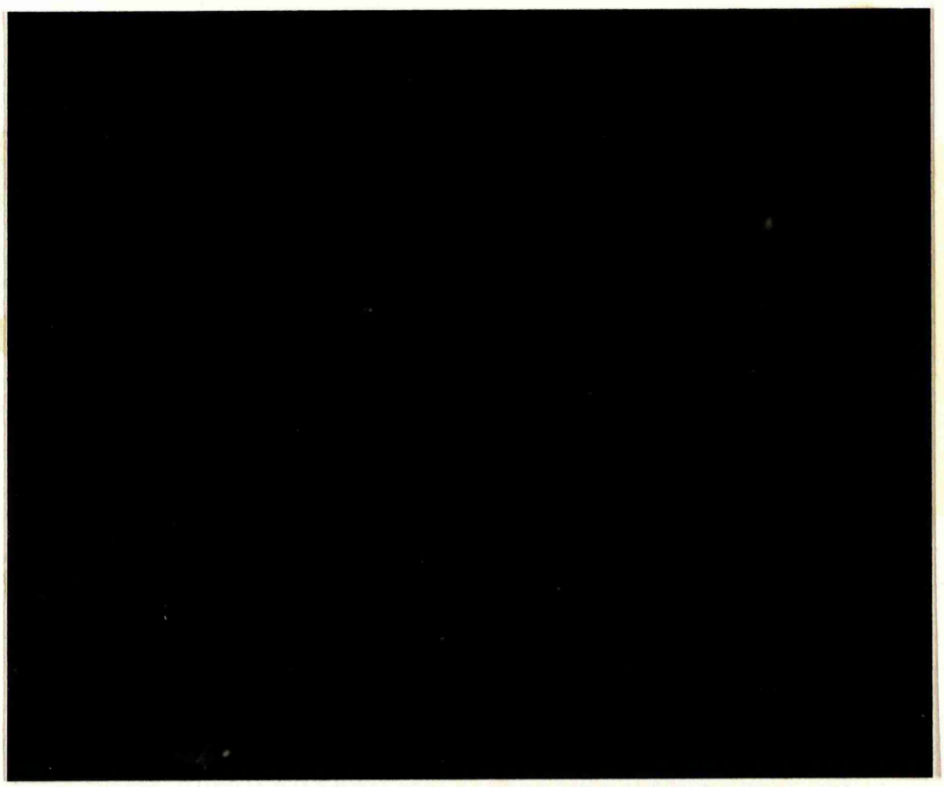


Figure 35. Immunofluorescence of vMJ542-infected cells, showing nuclear localisation of the products of UL26.5. CV-1 cells were infected with 5 p.f.u. per cell of vMJ542 and were incubated at 37°C for 18 h. Cells were then stained with a 1/50 dilution of 5010 (A) or a 1/50 dilution of control ascites (B), followed by a 1/200 dilution of FITC-conjugated goat anti-mouse immunoglobulin G, to detect VP22a.

cytoplasm, although there is apparent nuclear fluorescence also. This suggests a predominantly cytoplasmic location for VP5. Figure 33B shows CV-1 cells infected with WR vaccinia at an m.o.i. of 5 p.f.u. per cell, and stained with 10555. The relatively high background fluorescence obtained with this antibody is again illustrated.

Antibody 10555 was also used in similar experiments on cells infected with vMJ534. However, this antibody did not show a detectable response to VP19C in these experiments.

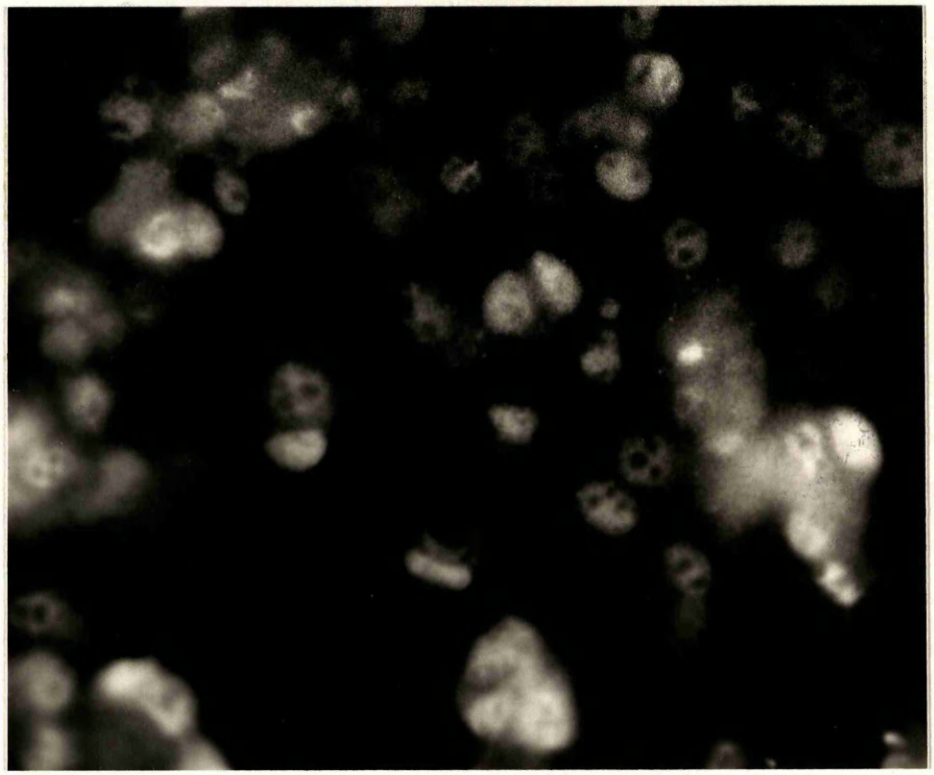
7.4.5 Immunofluorescent localisation of the products of UL26.5 expressed from vMJ542

The nuclear localisation of VP22a in HSV-1-infected cells has been demonstrated by immunofluorescent techniques by Heilman et al. (1979), Cohen et al. (1980) and Rixon et al. (1988). In the latter report, the anti-VP22a antibody employed was the monoclonal antibody 5010, which was also the antibody used in the experiments reported here. HSV-1-infected CV-1 cells showed strong nuclear fluorescence when stained with 5010 as primary antibody (Figure 34). In cells infected with vMJ542, the fluorescence was again strongly nuclear (Figure 35). This is in contrast to the results obtained for VP23 and for VP5. This result does, however, conflict with the result obtained for the distribution of the products of UL26.5 in the cell fractionation studies reported above in sections 7.1 and 7.2. This anomaly will be treated further in the Discussion.

7.4.6 The effect of the products of UL26.5 on the location of VP23 determined by immunofluorescence

The coinfection experiments reported in section 7.4.3

A



B

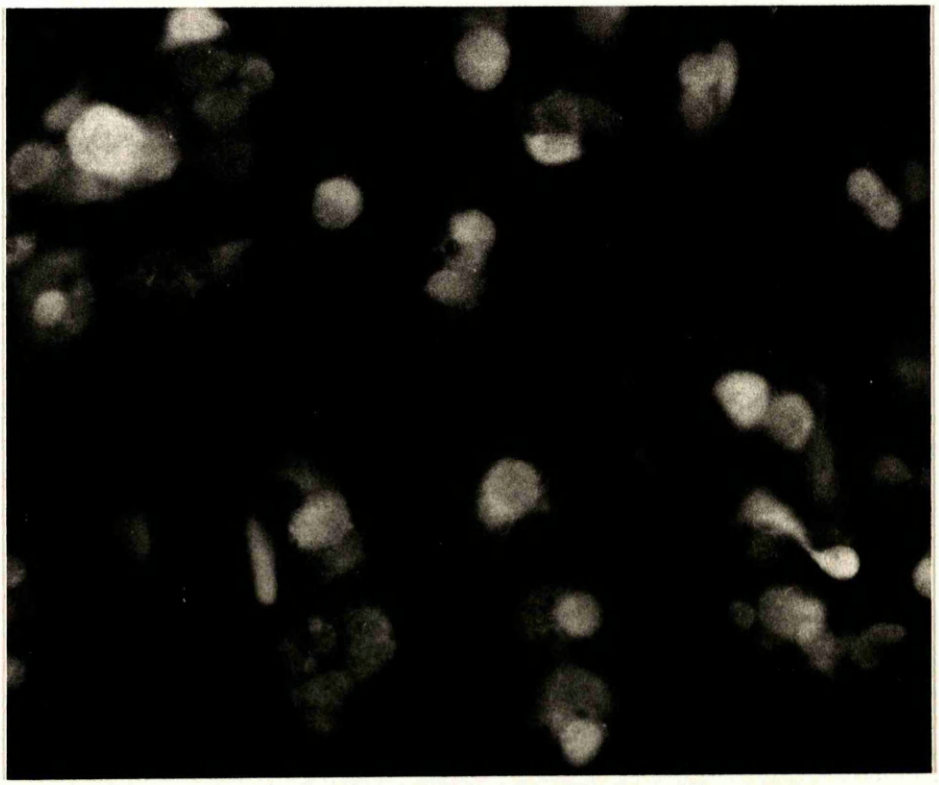


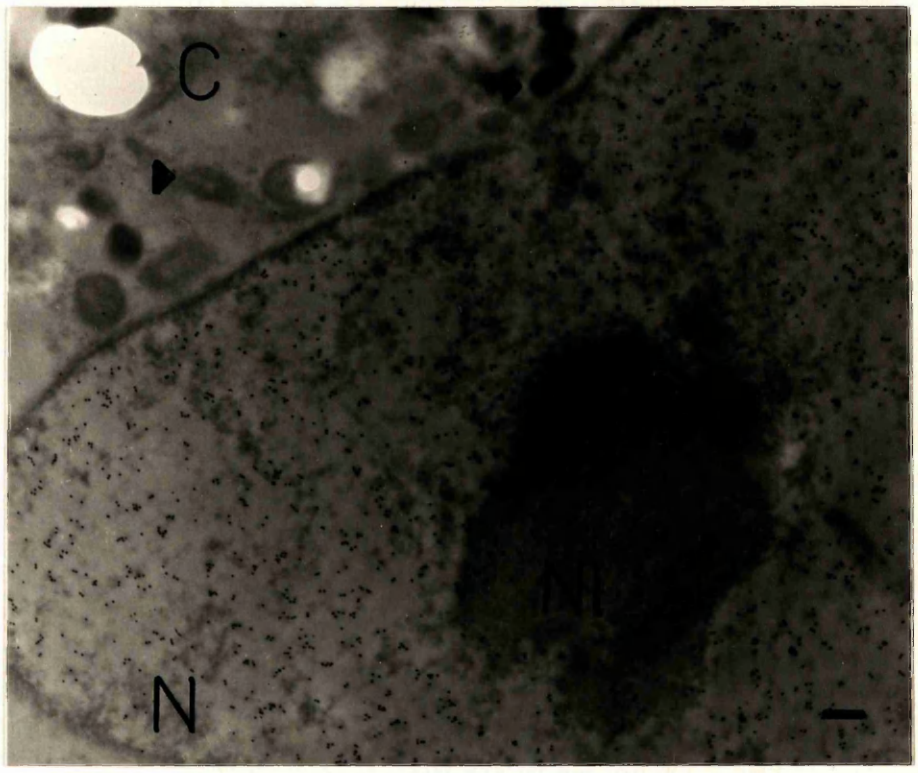
Figure 36. The effect of the presence of the products of UL26.5 on the intracellular distribution of VP23 in a recombinant vaccinia expression system. CV-1 cells were infected with 5 p.f.u. per cell each of vMJ521 and vMJ542 and were incubated at 37°C for 18 h. Cells were then stained with a 1/50 dilution of 5010 (A) or a 1/100 dilution of 1060 (B), followed by a 1/200 dilution of FITC-conjugated goat anti-mouse immunoglobulin G, to detect VP22a and VP23 respectively.

were extended by coinfecting cells with vMJ521 and vMJ542. Figure 36A shows that in cells infected with both viruses, the location of the products of UL26.5 is the same as in cells infected only with vMJ542 (see Figure 35). However, Figure 36B shows that when vMJ521 is coinfected with vMJ542, there is a change in the distribution of VP23. In infections with both viruses, a majority of cells exhibit nuclear fluorescence when 1060 is used as primary antibody. Most of the cells in Figure 36B show strong nuclear fluorescence as well as cytoplasmic fluorescence. This is in marked contrast to the distribution of VP23 shown in Figure 29. This result suggests that the products of UL26.5 not only have intrinsic ability to localise to the nucleus, but that they can also effect nuclear localisation of VP23. This result contrasts with the results obtained by fractionation of coinfected cells (section 7.2), where the products of UL26.5 did not appear to affect the location of VP23. In the experiments reported in this section a minority of cells were seen in which VP23 was retained in the cytoplasm. These may represent cells which were not infected with both viruses.

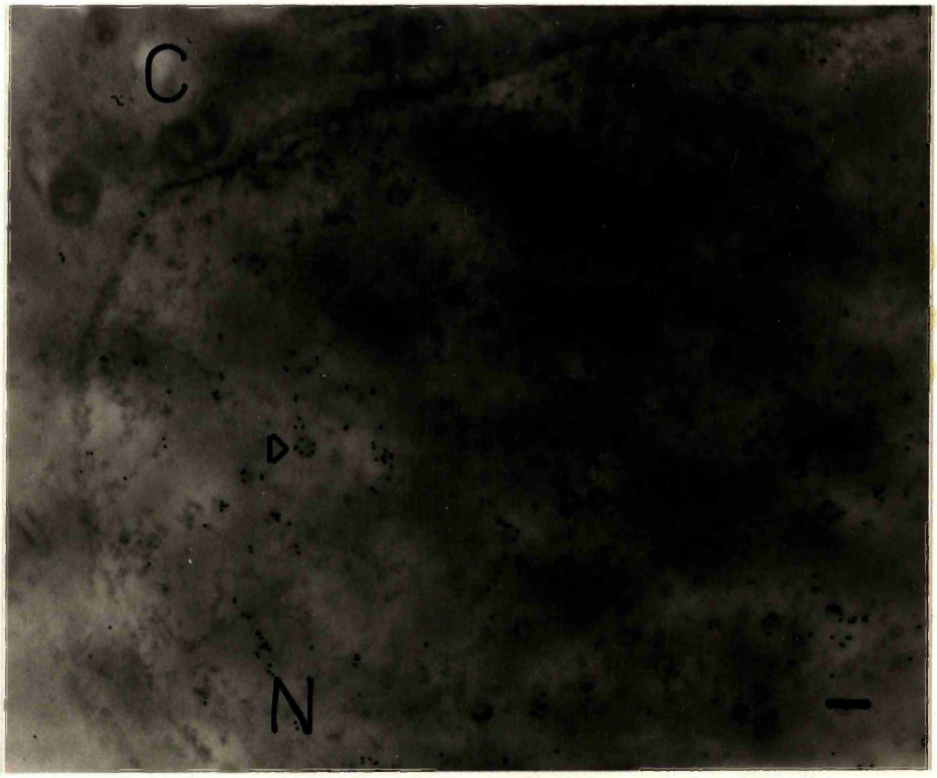
7.4.7 The intracellular location of the products of UL26.5 determined by immunoelectron microscopy

The anti-VP22a monoclonal antibody 5010 has been successfully used for identifying the sites of subcellular location of unprocessed and processed forms of VP22a in HSV-1-infected cells by electron microscopy using immunogold localisation procedures (Rixon et al., 1988). In order further to examine the intracellular distribution of the products of UL26.5 when expressed by the recombinant vaccinia virus vMJ542, CV-1 cells infected for 18 h with 5 p.f.u. per cell of vMJ542 were embedded in Lowicryl K4M resin. Sections were incubated with monoclonal antibody 5010 and this was visualised using a goat anti-mouse antibody tagged with 10-nm gold

A



B



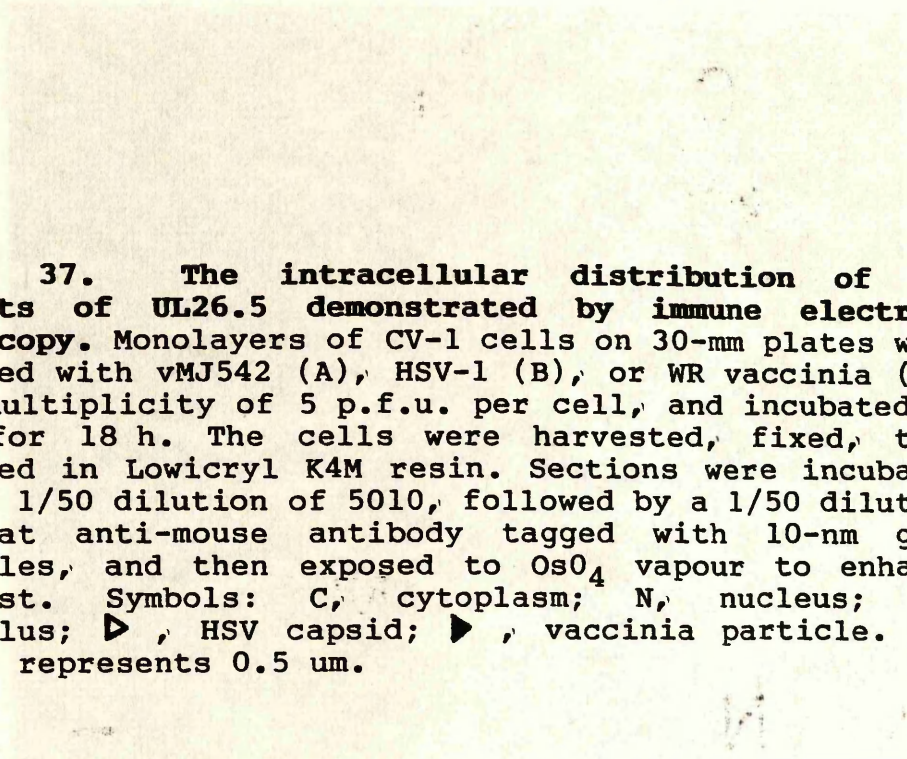
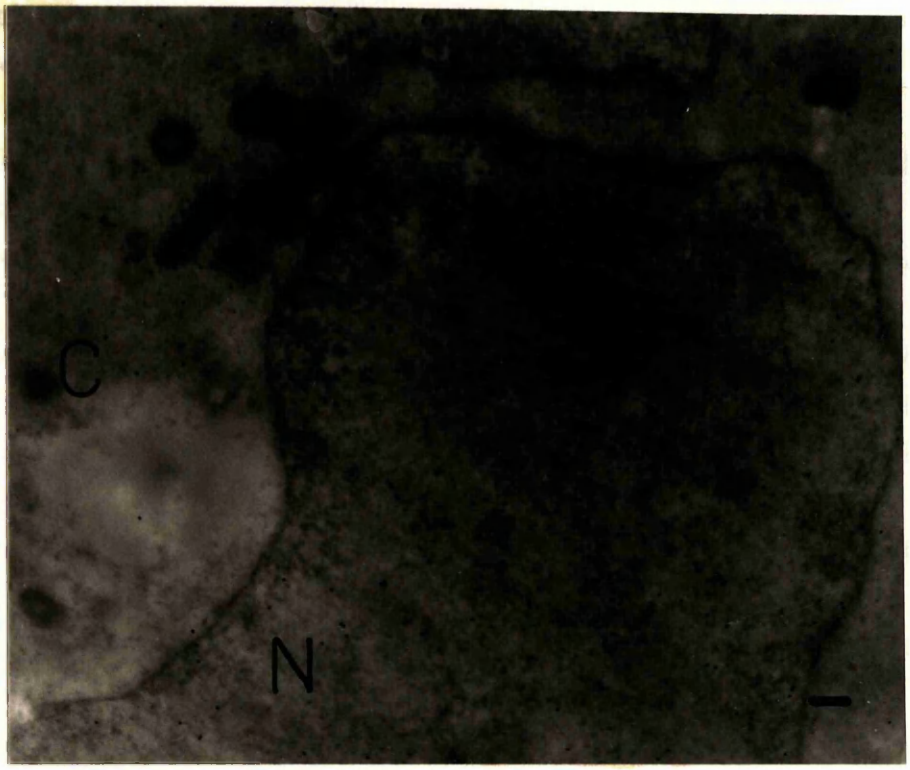


Figure 37. The intracellular distribution of the products of UL26.5 demonstrated by immune electron-microscopy. Monolayers of CV-1 cells on 30-mm plates were infected with vMJ542 (A), HSV-1 (B), or WR vaccinia (C), at a multiplicity of 5 p.f.u. per cell, and incubated at 37°C for 18 h. The cells were harvested, fixed, then embedded in Lowicryl K4M resin. Sections were incubated with a 1/50 dilution of 5010, followed by a 1/50 dilution of goat anti-mouse antibody tagged with 10-nm gold particles, and then exposed to OsO_4 vapour to enhance contrast. Symbols: C, cytoplasm; N, nucleus; Nl, nucleolus; ▷ , HSV capsid; ▶ , vaccinia particle. Bar marker represents 0.5 um.

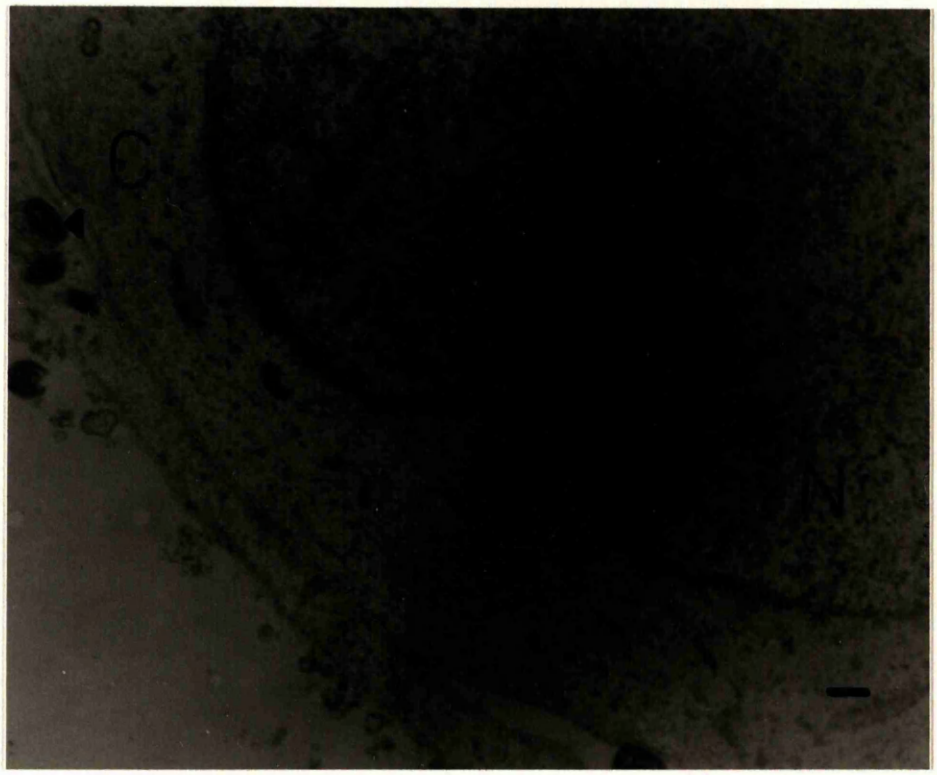

C
C
N

particles. The results shown in Figure 37 indicate that when expressed by vMJ542, the products of UL26.5 are located in the nucleus. vMJ542-infected cells did not show significant binding of gold particles in the cytoplasm. Figure 37A shows that the products of UL26.5 are specifically localised in the nucleus but are excluded from the nucleolus. This result conflicts with those presented in sections 7.1 and 7.2, but is in agreement with the result presented in section 7.4.5; these results will be treated further in the Discussion. HSV-1-infected cells (Figure 37B) also showed nuclear binding of gold particles, to intranuclear capsids, as was previously demonstrated by Rixon et al. (1988). In WR vaccinia-infected cells (Figure 37C) there was a low background level of gold particles over the whole section but no specific binding to any region of the cell was observed.

8 ATTEMPTS TO ASSEMBLE CAPSID SUB-STRUCTURES IN VIVO WITH CAPSID PROTEINS EXPRESSED FROM RECOMBINANT VIRUSES

In a variety of viruses, purified capsid proteins have been shown to self-assemble into capsids, capsid-like particles or subunits of capsids. Assembly of structural entities has also been achieved by expression of capsid genes using in vivo expression systems, including recombinant vaccinia viruses. Details of these results are given in the Discussion. Thus the results of other workers suggested that some form of particle self-assembly might be achieved using individual capsid protein genes expressed from recombinant vaccinia viruses. Of particular interest was vMJ535, because VP5 as the structural component of capsomers might be able to self-assemble to form capsomers, as is the case with the polyomavirus major capsid protein VP1 (Salunke et al., 1986). vMJ542 was also of interest, in view of the proposed role of VP22a as a scaffolding protein, and the finding of Newcomb & Brown (1991) that VP22a can exist in

A



B

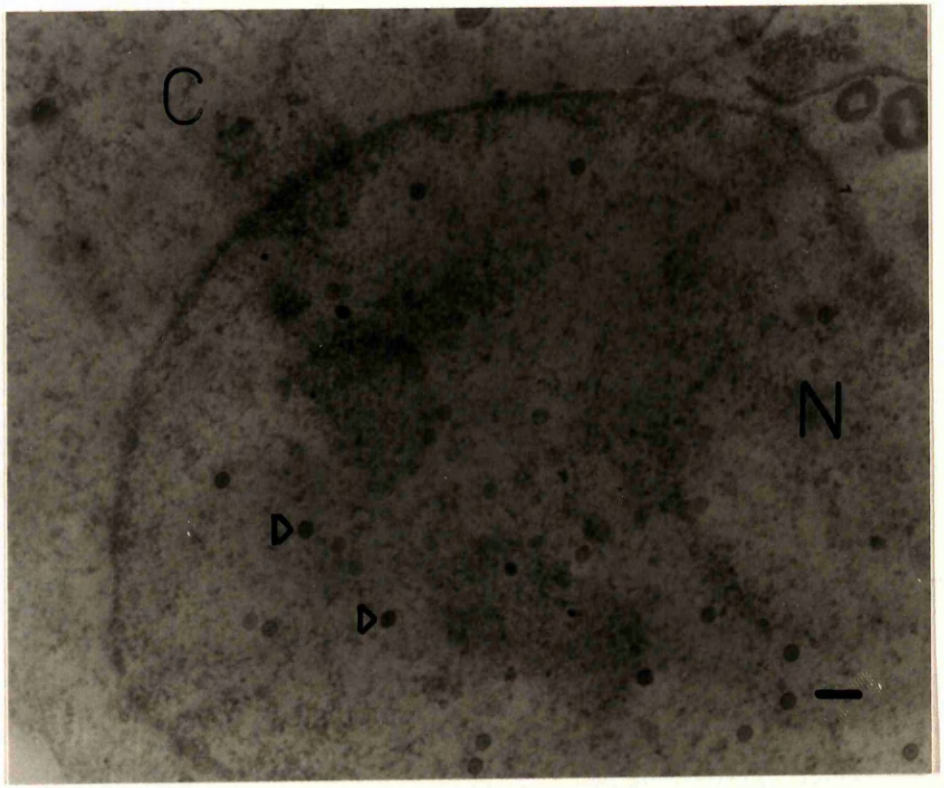
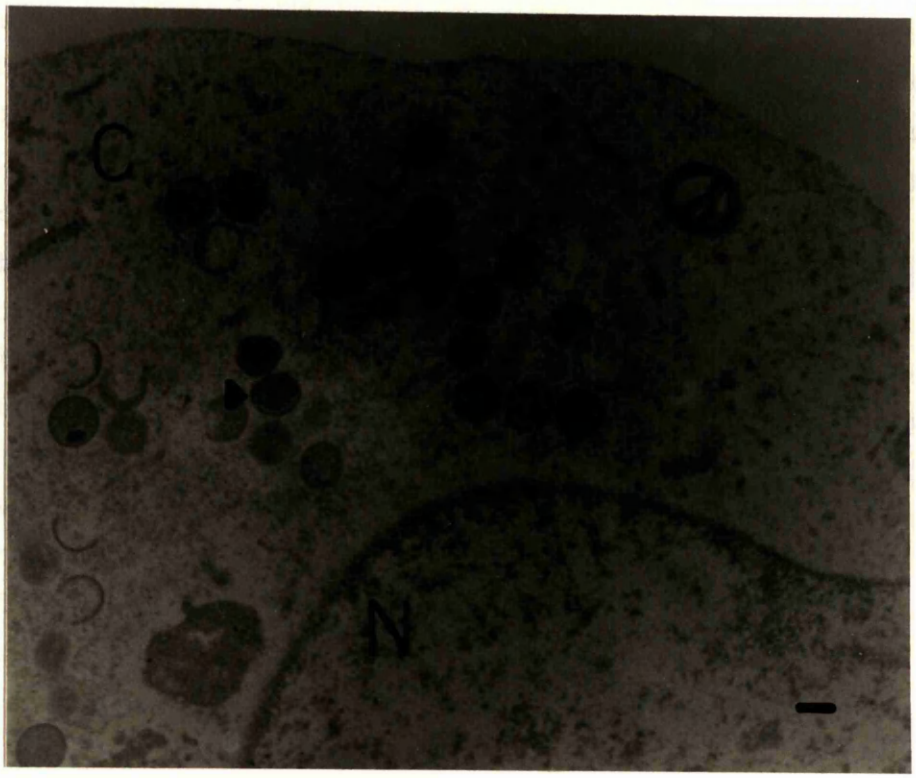


Figure 38. Electron microscopy of cells infected with vaccinia recombinants, showing no evidence of HSV-1 structural intermediates. Monolayers of CV-1 cells on 30-mm plates were infected with 5 p.f.u. per cell each of vMJ521, vMJ535, vMJ534 and vMJ542 (A), HSV-1 (B), or WR vaccinia (C), and incubated at 37°C for 18 h. The cells were harvested, fixed, then embedded in Epon 812 resin. Sections were stained with uranyl acetate and lead citrate. Symbols: C, cytoplasm; N, nucleus; ▷, HSV capsid; ▶, vaccinia factory/particle. Bar marker represents 0.5 um.



C

a free form as discrete condensations.

Monolayers of CV-1 cells infected with 5 p.f.u. per cell of HSV-1, WR vaccinia, vMJ521, vMJ535, vMJ534 or vMJ542 for 18 h were embedded in Epon 812 resin for examination by electron microscopy. Although formation of vaccinia particles occurred apparently normally in infections with recombinant viruses, no novel structural element could be seen in sections of these cells. As it was possible that more than one capsid protein is necessary for any kind of assembly to be formed, further experiments were attempted by coinfecting cells with all four recombinants. Again, no novel structural element was observed (Figure 38). The products of the four capsid genes UL18, UL19, UL38 and UL26.5 are not sufficient for assembly of capsid-like particles in this expression system.

9 TIME COURSE OF SYNTHESIS OF VP23

As part of the study of the properties of VP23, the availability of the newly characterised antibody 1060 made possible an investigation of the kinetics of synthesis of VP23 by HSV-1. The UL18 mRNA was reported to be regulated with early-late kinetics in lytic infections by Costa et al. (1985). In the present experiments the kinetics of synthesis of VP23 were examined in collaboration with Dr A.M.Cross. HSV-1 was allowed to adsorb onto BHK cells for 1 hour at 37°C and then replicate plates were labelled with ^{35}S methionine in succcessive 1 hour pulses. By immunoprecipitation of 1 h pulses Dr A.M.Cross showed that VP23 was already precipitable by 1060 in cells pulsed 2 to 3 hours post-infection. Longer exposures of the autoradiographs failed to show any VP23 in the 1-2 hour pulse. Amounts of VP23 steadily increased in later samples.

To examine this in more detail, a further experiment tested whether the DNA synthesis inhibitor

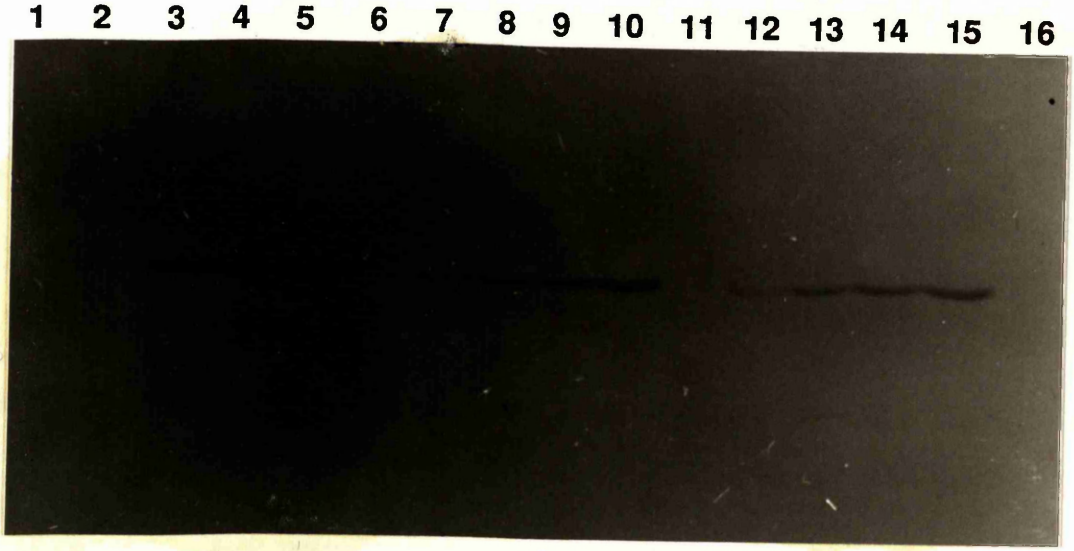

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 39. The time course of expression of VP23. Western blot of HSV-1-infected cell extracts. BHK monolayers on 50-mm plates were infected with 20 p.f.u. per cell of HSV-1 strain 17 (lanes 1 to 10), PAA^R-1 (lanes 11 to 15), or were mock-infected (lane 16). These cells were incubated at 37°C in the absence (lanes 1 to 5, and 16) or the presence (lanes 6 to 10, and 11 to 15) of PAA (300 ug/ml), and were harvested at 0 h (lanes 1,6,11), 3 h (lanes 2,7,12), 6 h (lanes 3,8,13), 9 h (lanes 4,9,14), and 12 h (lanes 5,10,15,16) post infection. Samples were separated on a 9% SDS-polyacrylamide slab gel and transferred to a nitrocellulose membrane filter by transverse electroblotting. The membrane was probed with 1060 to detect VP23. Bound 1060 was visualised using the Promega Protoblot horseradish peroxidase system.

phosphonoacetic acid (PAA) would affect the production of VP23. PAA was used at a concentration of 300 ug per ml, which reduces virus replication to a level of less than 5% of the no PAA control in cells infected with HSV-1 strain 17 (Johnson et al., 1986). The PAA-resistant mutant, PAA^r-1 (Hay & Subak-Sharpe, 1976), which induces wild-type levels of DNA synthesis in the presence of 300 ug per ml of PAA, was included in the experiment as a control. This control would have been important had VP23 been a true-late protein. A second control, demonstrating the efficient inhibition of DNA synthesis by PAA at the concentration used, is discussed below. The results of this experiment are shown in Figure 39. BHK monolayers on 50-mm plates were infected with HSV-1 strain 17 (lanes 1 to 10), PAA^r-1 (lanes 11 to 15), or were mock-infected (lane 16). These cells were incubated in the absence (lanes 1 to 5 and lane 16) or presence (lanes 6 to 10 and 11 to 15) of PAA, and were harvested at 0 hours (lanes 1,6,11), 3 h (lanes 2,7,12), 6 h (lanes 3,8,13), 9 h (lanes 4,9,14) or 12 h (lanes 5,19,15,16) post infection. Cells incubated in the presence of PAA had been maintained in PAA for 1 h prior to infection. Samples were separated on a 9% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane filter by transverse electroblotting. The membrane was probed with 1060 to detect VP23. Bound 1060 was visualised using the Promega Protoblot horseradish peroxidase system. Figure 39 shows that VP23 was first detectable in this system at 3 h p.i. (lane 2), and that levels of VP23 steadily increased in later samples (lanes 3 to 5). Similar amounts of VP23 were produced by strain 17-infected cells in the presence of PAA, and by PAA^r-1-infected cells in the presence of PAA, at corresponding times post infection. A second control experiment was carried out, using a second gel, identical to the one used for Figure 39. This gel was blotted, and probed with antibody 14327, which recognises the true-late products of gene US11 (MacLean et al., 1987). Bound antibody was visualised using ^{125}I-labelled Protein A. In this experiment, the

products of US11 were not detected in HSV-1-infected cells in the presence of PAA, but they were detected in HSV-1-infected cells in the absence of PAA and in PAA^r-1-infected cells in the presence of PAA. Thus the levels of PAA used in these experiments were successful in inhibiting synthesis of DNA (result not shown). VP23 is produced early in infection, steadily increases in later samples and is not dependent on viral DNA synthesis. This is in agreement with the finding of Costa et al. (1985).

10 THE DNA-BINDING PROPERTIES OF VP19C

Several of the capsid proteins of HSV have been reported to be DNA-binding. Bayliss et al. (1975) identified an HSV-1-infected cell protein of 43 kDa which bound DNA in affinity chromatography experiments. They identified this protein as the capsid protein VP21, because during PAGE it comigrated with the VP21 present in purified virions. In similar experiments, Powell & Purifoy (1976) and Purifoy & Powell (1976) identified 155-kDa infected-cell proteins in HSV types 1 and 2 which were DNA-binding. These were identified as the capsid protein VP5 because they comigrated with the VP5 present in purified virions. Binding was very inefficient, when compared to the binding activities of other infected-cell proteins. The study of Bayliss et al. (1975) did not identify VP5 as being DNA-binding. Blair & Honess (1983) reported a study of the DNA-binding structural proteins of herpesvirus saimiri which also included some observations on HSV-1. Proteins of interest were identified by binding of labelled DNA to nitrocellulose filters containing electrophoretically separated proteins of purified virions. They did not detect DNA-binding activity in VP5 or VP19C. They did detect an HSV-1 DNA-binding protein of 38 kDa, which they identified as the virion protein VP22. The study of Pinard et al. (1987) also identified the HSV-1 VP22 as a DNA-binding protein, using the same technique. They also identified the virion proteins VP13

and VP14 as being DNA-binding. Blair & Honess (1983), although they did not name the candidate proteins, also found strong DNA-binding activity in a protein or proteins of this size (approximately 82 kDa) in virions of HSV-1.

A 38-kDa HSV-2 protein was found by Burdett & Docherty (1987) and Burdett et al. (1990) to be DNA-binding in affinity chromatography experiments. This protein was claimed by these workers to be a component of the capsid. However, in an attempt to identify this protein, they specifically eliminated VP22a, and mapped the protein to a region on the HSV-2 genome spanning 0.605 to 0.720 map units. This region is not known to encode any capsid protein. In view of the lack of a candidate capsid component of this size, it would seem that the 38-kDa protein is not a capsid protein.

Another recent investigation of HSV DNA-binding structural proteins is that of Braun et al. (1984a). Again, proteins of interest were located on nitrocellulose filters, which this time contained electrophoretically separated proteins of purified B and C capsids. They did not detect any DNA-binding activity associated with VP5 or any protein of approximately 40 kDa. However, they did detect in HSV -1 and -2 an efficient DNA-binding protein which they identified as VP19C.

The availability of a recombinant vaccinia virus expressing VP19C enabled repetition of the work of Braun et al. (1984a) in an attempt to investigate the DNA-binding properties of this protein. vMJ534 expresses VP19C in abundant amounts and represents a more convenient source of VP19C than do purified capsids. The reported DNA-binding activity of VP19C is of special interest because its proposed location in the capsid floor suggests that it may have a role in anchoring DNA within the capsid.

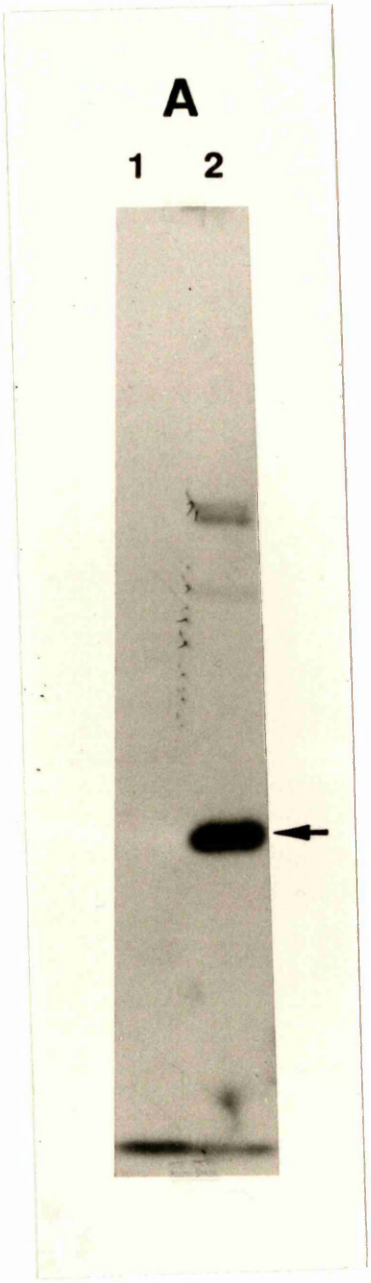

A
1
2

Figure 40A. Investigation of the DNA-binding properties of VP19C. CV-1 cells were infected with 5 p.f.u. per cell of vMJ534 and were labelled with ^{35}S-methionine from 3 to 24 h p.i. Whole-cell extract (lane 1), and unlabelled HSV-1 virions (lane 2), were electrophoretically separated on a 10.5% SDS-polyacrylamide slab gel. The cross-linker was DATD. The proteins were then transferred by transverse electrophoresis onto a nitrocellulose filter. The filter was then probed for DNA-binding activity using nick-translated ^{32}P-labelled pG35a DNA. Figure 40A shows an autoradiograph developed following a 2-hour exposure of the filter. There is no detectable DNA-binding activity in the vMJ534 lane (lane 1). In lane 2, there is a protein (arrowed) exhibiting strong DNA-binding activity. This protein is not VP19C, as can be seen by a comparison with Figure 40B.

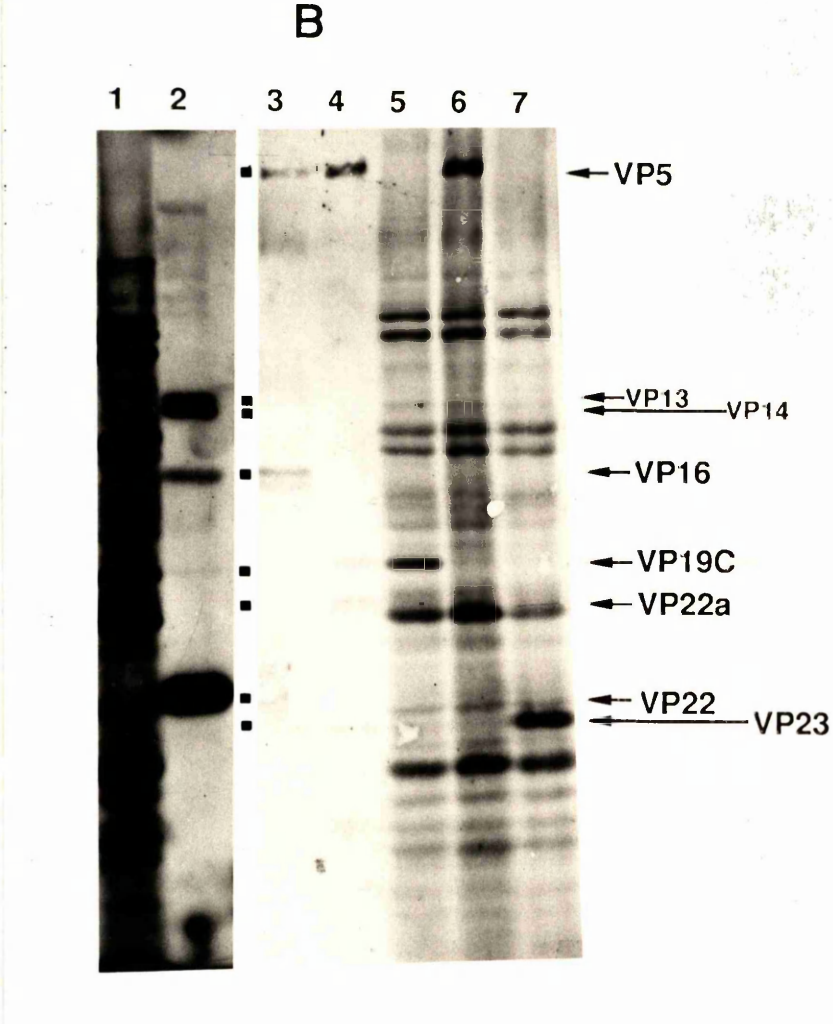

B
1
2
3
4
5
6
7
VP5
VP13
VP14
VP16
VP19C
VP22a
VP22
VP23

Figure 40B. Identification of a DNA-binding protein present in HSV-1 virions. Figure 40B shows an autoradiograph developed following a 2-day exposure of the same filter which was used in the production of Figure 40A, and shows the positions of ^{35}S-labelled proteins in lane 1. Lanes 3 to 7 is an autoradiograph of an SDS-polyacrylamide gel containing electrophoretically separated ^{35}S-labelled proteins which can be used to identify the proteins of interest in lanes 1 and 2. Lane 3 contains HSV-1 virions. Lane 4 contains HSV-1 capsids. Lanes 5 to 7 contain samples of CV-1 cells infected with 5 p.f.u. per cell of vMJ534 (lane 5), vMJ535 (lane 6) or vMJ521 (lane 7) and labelled with ^{35}S-methionine from 1 to 24 h p.i. Several vaccinia or cellular proteins are common to lanes 1, 5, 6 and 7, and have been used to align the two parts of Figure 40B. The positions of several HSV-1 structural proteins are indicated with a dot (▪) between lanes 2 and 3 and with arrows at the right hand side of the figure.

Figure 40A shows the result of an experiment in which a sample of CV-1 cells infected with 5 p.f.u. per cell of vMJ534 and labelled with ^{35}S-methionine from 1 to 24 h p.i. was electrophoresed (Figure 40A lane 1) alongside a preparation of HSV-1 virions (Figure 40A lane 2). The virions were not radiolabelled. The proteins were transferred by transverse electroblotting onto a nitrocellulose filter and incubated in the presence of nick-translated ^{32}P-labelled pG35a DNA. Figure 40A shows an autoradiograph developed following a 2-hour exposure of this filter. There is no detectable DNA-binding activity in the vMJ534 sample (40A lane 1). In the virion sample (40A lane 2) there is a protein present (arrowed) exhibiting strong DNA-binding activity. This protein is not VP19C, as can be seen by a comparison with Figure 40B.

Figure 40B lanes 1 and 2 show an autoradiograph developed following a 2-day exposure of the same filter which was used in the production of Figure 40A, and shows the positions of ^{35}S-labelled vMJ534-infected-cell proteins in lane 1. Figure 40B lanes 3 to 7 is an autoradiograph of an SDS-polyacrylamide gel containing electrophoretically separated ^{35}S-labelled proteins which can be used to identify the proteins of interest in lanes 1 and 2. Lane 3 contains HSV-1 virions. Lane 4 contains HSV-1 capsids. Lanes 5 to 7 contain samples of CV-1 cells infected with 5 p.f.u. per cell of vMJ534 (lane 5), vMJ535 (lane 6) or vMJ521 (lane 7) and labelled from 1 to 24 h p.i. Several vaccinia or cellular proteins are common to lanes 1, 5, 6 and 7, and have been used to align the two parts of Figure 40B. The positions of several HSV-1 structural proteins are indicated with dots between lanes 2 and 3 and with arrows at the right hand side of the figure. The capsid proteins VP19C, VP5 and VP23 can be seen in lanes 5, 6 and 7 respectively. These capsid proteins, and the capsid protein VP22a, can be seen in lane 4. VP5 and VP19C are also visible in lane 3. The position of VP19C in lane 1 is easily identifiable by

a comparison with lanes 3, 4 and 5. It is clear that the strong DNA-binding protein in lane 2 is not VP19C.

A minor band is present in Figure 40B lane 2 at the position of VP19C. A comparison with Figure 40A lane 2 reveals that several minor bands have appeared during the 2-day exposure of the filter. During the 2-day exposure, a second sheet of film was placed immediately behind the one shown as Figure 40B lanes 1 and 2. This sheet was activated only by emissions due to bound ^{32}P, and revealed several minor bands in lane 1 of similar intensity to those in Figure 40B lane 2, including a minor band at the position of VP19C. The low intensity of these bands, and the fact that several minor bands were detected, suggests that this does not represent significant DNA-binding activity by VP19C.

The identities of the four most intense bands in Figure 40B lane 2 are of interest and can be determined by a comparison with lanes 3 and 4 and by comparison with published HSV-1 virion protein profiles (Heine et al., 1974; Marsden et al., 1976; Dargan, 1986; McLean et al., 1990). The band immediately above VP19C is probably VP16. VP16 is visible in lane 3; the protein immediately below is probably gD. The doublet above VP16 in lane 2 is probably VP13 and VP14. The strong DNA-binding protein which migrates at a position just above the position of VP23 is probably VP22, which is the only virion protein of this size. Thus, although this experiment has failed to demonstrate significant DNA-binding activity in VP19C, it has confirmed the finding of Blair & Honess (1983) and of Pinard et al. (1987) that VP13, VP14 and VP22 are DNA-binding.

No DNA-binding activity due to VP5 is detectable in Figure 40B lane 2. vMJ535 was also used in similar experiments to assess the DNA-binding properties of VP5. Again, no DNA-binding activity was found to be associated with VP5 in this system.

11 THE DISULPHIDE LINKAGE OF VP5 AND VP19C

VP5 and VP19C were reported to be covalently linked by disulphide bonds in capsids of HSV-2 (Zweig et al., 1979a). In their experiments, B capsids were investigated using a two-dimensional PAGE system. Samples were run in the first dimension in a cylindrical gel under non-reducing conditions. The cylindrical gel was then treated with a reducing agent (dithiothreitol), in order to disaggregate proteins bound by disulphide bonds. The gel was then placed across the top of a slab gel and electrophoresis was conducted at right angles to the first dimension. In this system, a diagonal line of protein spots results from proteins which in both directions migrate according to their molecular weights. Proteins which were linked under non-reducing conditions migrate as higher molecular weight complexes in the first dimension. Under reducing conditions, these complexes are disaggregated, and in the second dimension these proteins show up as spots lying to the left of the diagonal.

Prior to the report of Zweig et al. (1979a), McCombs & Williams (1973) found that HSV-1 nucleocapsids underwent collapse if treated with the reducing agent 2-mercaptoethanol at 80°C. Capsids did not collapse at this temperature in the absence of the reducing agent. Electron microscopy, which revealed the collapse of capsids, showed that the intercapsomeric bonding had not been disrupted. They concluded that some disulphide linkages were involved in structural integrity. Given the proposed location of VP19C in the capsid floor, some form of interaction with capsomers would seem likely. This could be a crucial feature of structural integrity. Capsids of polyomavirus have been shown to be resistant to mild treatment with SDS, due to the presence of disulphide bonds (Walter & Deppert, 1974).

Repetition of the experiments of Zweig et al. (1979a) using cells co-infected with vMJ535 and vMJ534 was

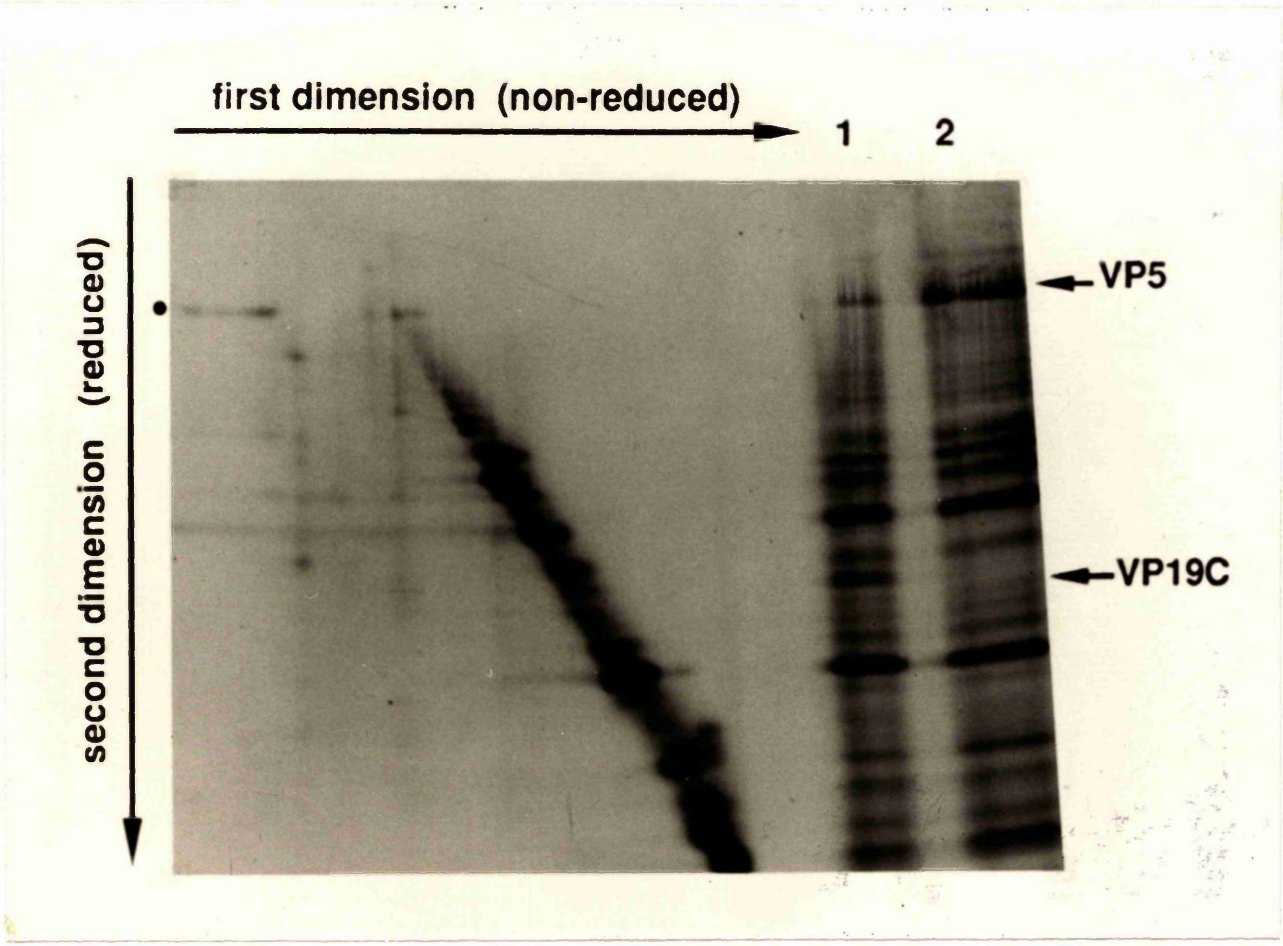

first dimension (non-reduced)
1
2
second dimension (reduced)
VP5
VP19C

Figure 41. Two-dimensional electrophoresis of VP5 and VP19C. CV-1 cells were infected with 5 p.f.u. per cell of vMJ534 (lane 1), vMJ535 (lane 2), or with 5 p.f.u. per cell of both viruses. Cells were labelled with ^{35}S-methionine from 1 to 24 h p.i., and were harvested. A sample of infected-cell extract from the dual infection was electrophoresed through a cylindrical gel (described in Materials and Methods) under non-reducing conditions. The cylindrical gel was then cemented along the top of a 9% SDS-polyacrylamide slab gel (the cross-linker was bis-acrylamide) and electrophoresis was conducted at right angles to that of the first direction, under reducing conditions. Control lanes 1 and 2 were also run in the second dimension. Lane 1 shows the position at which VP19C (arrowed) migrates in the second dimension. Lane 2 shows the position at which VP5 (arrowed) migrates in the second dimension. Lane 1 also contains some VP5, due to spill-over of sample during loading of the gel. A diagonal line can be seen which consists of proteins which migrate according to their molecular weights in both dimensions. To the left of this diagonal can be seen a smear of VP5 (marked at the left hand side with a dot).

undertaken in an attempt to determine whether disulphide linkages would form under these circumstances. One such experiment is represented in Figure 41. Control lanes show the positions of VP5 (lane 2) and VP19C (lane 1) in the second dimension. On the two-dimensional gel of coinfected cells, a number of protein species lie to the left of the diagonal, representing cellular or vaccinia polypeptides which were disulphide-linked under non-reducing conditions (in these experiments the reducing agent used was 2-mercaptoethanol). A smear migrating in the second dimension at the position of VP5 can be seen to the left of the diagonal, indicating that under non-reducing conditions it migrated with a molecular weight higher than 155 kDa. This is interesting because it shows that VP5 had formed disulphide bonds with some other protein. Such smearing of VP5 also occurred during the two-dimensional electrophoresis of purified capsids carried out by Zweig et al. (1979a), when VP5 was shown to form disulphide bonds with VP19C. In Figure 41, if VP19C had formed part of a higher molecular weight complex with VP5 in the first dimension, a spot migrating in the second dimension at the position of VP19C (indicated by the position of VP19C in lane 1) would be expected lying vertically below one point of the smear of VP5. No such spot could be detected in these experiments. Thus, these experiments did not confirm that the HSV-1 VP5 and VP19C can form disulphide-linked complexes. However, in view of the results presented in section 7.3, the existence of disulphide linkages between VP5 and VP19C can not be discounted. A further weakness is the lack of other factors. Zweig et al. (1979a) used preformed capsids. In the present experiments, free protein was used; formation of correct bonding arrangements may require another protein such as VP22a, or may require capsid assembly. Also, VP5 expressed by vMJ535 remains in the cytoplasm. Physiological conditions in the cytoplasm are not suitable for assembly of capsid proteins of polyomavirus (Montross et al., 1991). However, similar two-dimensional electrophoresis

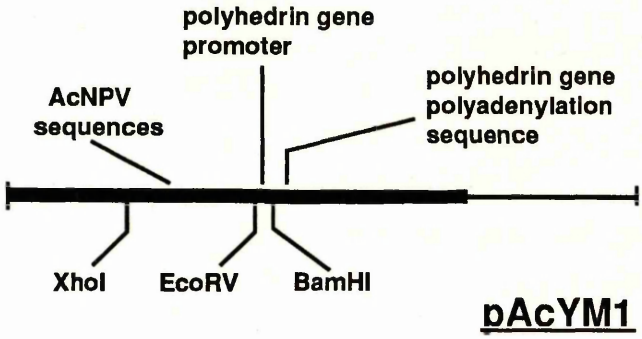
experiments using HSV-1 virions have failed to demonstrate disulphide linking between VP5 and VP19C (personal communication, Dr F.J.Rixon).

12 EXPRESSION OF UL19 IN A RECOMBINANT BACULOVIRUS

12.1 Cloning of UL19 into a baculovirus transfer vector

Many recent studies on the properties of a variety of proteins have been greatly facilitated by the use of the baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV) as an expression vector. These studies include several on a number of virus structural systems, and have enabled elucidation of aspects of structure and assembly in these viruses. Further details of these studies are given in the Discussion. Other studies have shown that a subset of HSV-1 proteins, when expressed by recombinant baculoviruses, are able to associate normally as in HSV-1 infections, retaining full biochemical activity (Dodson et al., 1989; Calder & Stow, 1990; Stow, 1992). Study of aspects of HSV-1 capsid structure and assembly using recombinant baculoviruses may prove to be a useful alternative to the vaccinia expression system used in this thesis. Recombinant baculoviruses may also prove useful for the production and purification of large amounts of capsid proteins. It would be of particular interest to be able to crystallise VP5 and analyse it by X-ray crystallography.

As an initial step in developing a recombinant baculovirus system, a virus was constructed which contained the UL19 gene. The transfer vector used for this purpose was pAcYM1 (Matsuura et al., 1987), which contains sequences from AcNPV which flank the polyhedrin gene. The polyhedrin gene has been deleted, and a BamHI restriction site allows insertion of foreign DNA between the polyhedrin gene promoter and polyadenylation sequences.



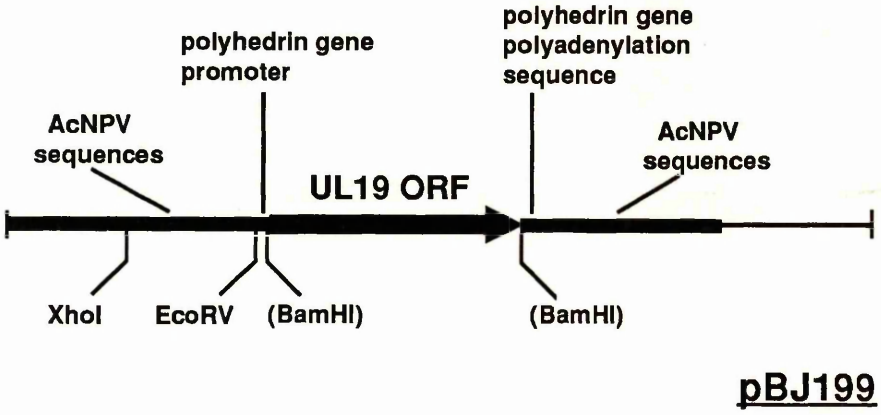


Figure 42. The structures of pAcYM1 and pBJ199

The proportions of this diagram are approximately to scale and are based on the details of pAcYM1 given by Matsuura et al. (1987).

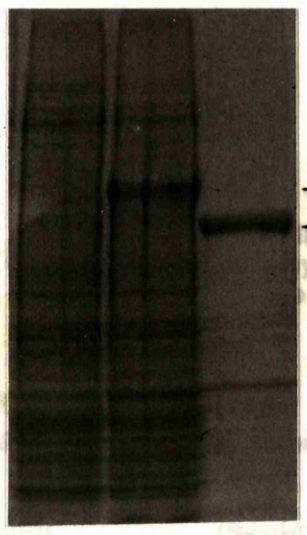

1
2
3
VP5
β–gal

Figure 43. Expression of VP5 by a recombinant baculovirus. Spodoptera frugiperda cells were harvested 48 h after infection with 10 p.f.u. per cell of AcNPV (lane 1), AcUL19 (lane 2), or AcRP23lacZ (lane 3). Samples of total cellular proteins were analysed on a 7.5% SDS-polyacrylamide slab gel. The cross-linker was bis-acrylamide. After electrophoresis the gel was stained with Coomassie brilliant blue, destained, dried, and photographed. The positions of VP5 (155 kDa) and β-galactosidase (135 kDa) are indicated.

The UL19 coding sequences on a 4184-base-pair BglII fragment from pBJ196 were ligated into the BamHI site of pAcYM1. A clone, pBJ199, was selected having the UL19 ORF under the control of the baculovirus polyhedrin gene promoter. A diagram of the structures of pAcYM1 and pBJ199 is given in Figure 42.

12.2 Construction of a recombinant baculovirus

A recombinant baculovirus containing UL19 was constructed by Dr R.M.Elliott essentially as described by Elliott & McGregor (1989). pBJ199 DNA was cotransfected with AcNPV DNA into Spodoptera frugiperda cells by calcium phosphate precipitation. After incubation at 28°C for 48 h, serial dilutions of the supernatant fluid were used to infect S.frugiperda cells in 96-well microtitre plates which were then incubated at 28°C for 48 h. Supernatants from these cultures were used to produce plaques on S.frugiperda monolayers, and polyhedrin-minus plaques were identified by microscopic examination. Several such plaques were picked, and purified by two further rounds of plaque purification. One plaque was grown up to produce a high-titre stock, and this virus was designated AcUL19.

12.3 Expression of VP5 by AcUL19

S.frugiperda cells were infected with 10 p.f.u. per cell of wild-type AcNPV, AcUL19 and AcRP23lacZ, and incubated at 28°C for 48 h. AcRP23lacZ contains the lacZ gene (Possee & Howard, 1987). Samples containing total cellular proteins were analysed by SDS-PAGE. Figure 43 shows the separated proteins visualised by Coomassie brilliant blue staining. Lane 1 contains the sample prepared from AcNPV, lane 2 that prepared from AcUL19, and lane 3 that from AcRP23lacZ. The products of UL19 and of lacZ are readily apparent in lane 2 (VP5, 155 kDa) and

lane 3 (β-galactosidase, 135 kDa) and are arrowed. This indicates efficient expression of VP5 by AcUL19. In some samples VP5 was estimated to form over 10% of total cell protein (personal communication, Dr F.J.Rixon).

12.4 Electron microscopy of AcUL19-infected cells

In view of the large amounts of VP5 made by AcUL19, electron microscopy of AcUL19-infected cells was undertaken in order further to assess the expression of VP5. S.fruqiperda cells infected with 10 p.f.u. per cell of AcUL19 were incubated at 28°C for 48 h. The cells were harvested, fixed, then embedded in Epon 812 resin prior to electron microscopic examination of thin sections. However, as was the case with vMJ535-infected cells (section 8), no novel structural element or inclusion body could be detected in AcUL19-infected cells.

DISCUSSION

1 CAPSID PROTEIN PROFILES

General agreement exists as to the protein components of HSV-1 capsids (Gibson & Roizman, 1972; Zweig et al., 1979a; Cohen et al., 1980; Rixon et al., 1990). These proteins are: VP5, VP19C, VP21, VP22a, VP23, VP24 and VP26 (refer to Table 1). However, not all of these proteins are present in all capsid types. The genes which encode six of the seven known capsid proteins have now been identified: VP5 - UL19; VP19C - UL38; VP22a - UL26.5; VP23 - UL18; VP24 - UL26; VP26 - UL35. The seventh capsid protein, VP21, is also believed to be a product of UL26. However, direct demonstration of the exact regions of UL26 which encode VP21 and VP24 is lacking, and this problem will be discussed in a later section.

The protein profiles shown in Figure 7 afford an interesting demonstration of the HSV-1 capsid proteins and their distributions through a sucrose velocity gradient. The capsid proteins described by previous workers are all present. Three additional proteins were also detected in A capsids in Figure 7. The protein of approximate molecular weight of 110 kDa, running at a position between VP5 and VP19C, is previously undescribed in HSV-1 capsids, and some evidence suggests that it is a breakdown product of VP5 (personal communication, Dr F.J.Rixon). It is interesting to note that the capsid protein profiles of two other herpesviruses contain proteins which are possible counterparts of this protein. Stevely (1975) reported a protein profile for capsids of PRV which included a major capsid protein of 150 kDa and a protein running immediately below the MCP at a position of 120 kDa. Similarly, Dolyniuk et al. (1976) reported a protein profile for capsids of EBV which included a MCP of 160 kDa and a protein running immediately below this at a position of 144 kDa, which was not present in EBV virions. It is not clear why this novel protein in the present study is strongly associated with A capsids. The

two other novel proteins highlighted in Figure 7 are also strongly associated with A capsids. It is possible that they also are breakdown products of VP5.

The herpesvirus capsid is a highly defined structure which presumably means that architectural constraints select against gross variations in the conformations of constituent proteins (Heine et al., 1974; Dargan, 1986). It seems unlikely that an essential structural component in one type of herpesvirus would be dispensible in another. Capsid protein profiles published for a variety of herpesviruses indicate that this is probably the case, with the notable exceptions of human and simian CMV, which apparently lack a VP19C homologue, and CCV, which has only three capsid proteins. Sequencing studies have recently raised the possibility that CCV may not be a herpesvirus, and it will be interesting to see if further structural analyses of the capsid support this suggestion.

Many investigations of herpesvirus capsid composition have reported more than seven proteins. Thus, in the study of Grose et al. (1983), the capsids of VZV were found to contain approximately 15 proteins, but electron microscopy of the capsid samples showed that they contained virions in a ratio of unenveloped:enveloped particles of 10:1. The study of Kemp et al. (1974) on EHV-1 also isolated capsids on a gradient containing whole-cell extracts. Electron microscopy did not detect the presence of contaminating virions in the capsid samples, but the possibilty exists that these samples did contain capsids originating from the cytoplasm which had been enveloped but which had lost the envelope during the disruptive preparative procedures. Such capsids usually retain some tegument material, which would not easily be detected by electron microscopy, but which would account for the experimental finding of 11 capsid proteins. A related problem is that some workers have prepared capsids by chemical stripping of the envelope and

tegument from virions. Since the stripping process does not normally achieve removal of all tegument material, the resultant capsids might still possess several tegument species. Thus in the study of Shiraki et al. (1989) on HHV-6, virions were stripped of envelope and tegument by treatment with NP40, and the resultant capsids were found to contain approximately 10 proteins. Using a similar procedure, Dolyniuk et al. (1976) found a 275-kDa protein in the capsids of EBV. It is probable that this protein is in fact a homologue of the 273-kDa HSV-1 tegument protein VP1, which is known to be closely associated with capsids (Gibson & Roizman, 1972). The possibility also exists of there being contaminating cellular proteins in any preparation of capsids. Even when apparently 'clean' preparations of intranuclear capsids are used, anomalies can result. Sanchez-Pinel et al. (1989) reported a monoclonal antibody which specifically reacted with a 75-kDa component of EBV capsids. The study of Dolyniuk et al. (1976) did not detect a capsid protein of this size in EBV. It is possible that the capsids used by Sanchez-Pinel et al. (1989) to prepare the monoclonal antibody, and in subsequent investigations of that antibody, were contaminated with a non-capsid protein. Thus in these studies the large numbers of 'capsid' proteins observed probably reflect deficiencies in the purification protocols resulting in incomplete separation of intranuclear (unenveloped) from cytoplasmic (enveloped) particles. Some workers have not used isolated nuclei, but instead have used whole-cell extracts, and the gradient systems employed have not been able to achieve good resolution of capsids from virions.

Thus it would appear that all the constituent proteins of the HSV-1 nucleocapsid are known, and that the genes which encode them have been identified. That a relatively small number of genes is directly involved in capsid assembly is suggested by the fact that despite the large number of mutants available for study, those in only two

complementation groups (mapping in UL19 and UL38) are known to have a direct effect on capsid assembly. This knowledge enables more detailed study of capsid structure and assembly than has hitherto been possible.

2 CLONING OF GENES

This thesis reports the cloning of four capsid protein genes of HSV-1: UL18, UL19, UL26 and UL38. Three of these genes, UL18, UL19 and UL38, were successfully expressed in a recombinant vaccinia virus system. The capsid protein gene UL26.5, cloned by other workers (Preston et al., 1992), was also expressed in a recombinant vaccinia virus.

UL35 was not cloned because it had not been identified as a capsid gene at the time of this work. It has recently been cloned successfully using the polymerase chain reaction (personal communication, Dr F.J.Rixon).

The cloning of UL35 brings the number of HSV-1 capsid protein genes which have been cloned to six: UL18, UL19, UL26, UL26.5, UL35 and UL38. This being the case, the genes encoding all seven HSV-1 capsid proteins have now been cloned. With the cloning of all the capsid protein genes, there is now much potential for further work to investigate HSV-1 capsid structure and assembly.

3 CHOICE OF EXPRESSION SYSTEM

High-level transient expression of heterologous DNA has been achieved in mammalian cells by a variety of methods, which fall into two general classes. Direct administration of DNA usually requires less effort than is involved in construction of a recombinant viral vector, but the efficiency of DNA transfer by conventional methods is poor. The experiment in which

VP23 was expressed from UL18 under the control of the HSV-1 gD promoter is an example of this problem. The experiment was useful in that it confirmed that the failure of VP23 to accumulate in the nucleus of vMJ521-infected cells was not a consequence of the vaccinia vector interfering with the normal transport mechanisms. However, introduction of pBJ182 into BHK cells by calcium phosphate coprecipitation was clearly an inefficient process, in that only a very small proportion (approximately 1%) of cells exhibited any fluorescence, and this severely limits the potential for further experiments using this technique. It has been reported that efficiency of transfection can be significantly increased by using unilamellar phospholipid vesicles (liposomes) to mediate DNA transfection, and in particular the synthetic cationic lipid DOTMA may prove useful in extending these experiments (Keown et al., 1990; Karger & Komro, 1990). If high levels of transfection could be achieved by this means, it might prove possible to use plasmids expressing capsid genes to examine capsid assembly.

By contrast, the ability of viruses to infect cells efficiently means that effectively 100% targeting of cells can be achieved using a recombinant virus. HSV-1 is itself suitable for use as an expression vector in that its large genome size allows for accomodation of additional DNA sequences without adversely affecting virus replication. Foreign DNA is usually introduced by homologous recombination. An HSV-1 vector has been engineered which is based on the ts mutant tsk, which expresses only immediate early genes at the NPT. This vector allows the insertion of heterologous DNA sequences under the control of an HSV IE promoter into a unique XbaI site in an intergenic position in the short unique genome region (Rixon & McLauchlan, 1990). The HSV-1 capsid genes UL26 and UL26.5 have been expressed using this vector (Preston et al., 1992), which would also have potential for expression of other capsid proteins.

The project reported in this thesis began with the intention of expressing capsid proteins in recombinant vaccinia viruses. Vaccinia virus is now widely used as an expression vector (reviewed by Mackett et al., 1985; Mackett & Smith, 1986; Piccini et al., 1987). In common with HSV the large genome (approximately 185 kb) of vaccinia has many non-essential sites suitable for insertion of DNA sequences. Other proteins which have been expressed in recombinant vaccinia viruses have shown correct synthesis, processing and transport (eg Ansardi et al., 1991; Hong & Engler, 1991).

The insertion plasmids (Davison & Moss, 1990) used in this project for introduction of capsid genes into vaccinia aim to improve the ease of isolation of recombinants by two means. The first is that the foreign DNA is inserted into and disrupts the vaccinia TK gene. Thus TK^- recombinants can be selected by the incorporation into the culture medium of BUdR, which inhibits TK^+ virus. Incorporation of the β-galactosidase gene into the insertion plasmid further enhances selection of recombinants, which are easily recognisable by the resultant blue plaque colour, not possessed by plaques arising from spontaneous TK^- mutants. However, considerable difficulty was encountered during the course of this project in attempts to isolate recombinant vaccinia viruses. The chief problem was the low efficiency of recombination, resulting in formation of very few blue plaques. A second problem was the poor selection by BUdR. Poor inhibition of growth of TK^+ virus meant that plates were infected at relatively high m.o.i. resulting in microscopic plaques following recombination reactions using pMJ535 (UL19) and pMJ542 (UL26.5). The recombinant viruses only yielded plaques visible to the naked eye following several passages in BUdR. Recombinant viruses could not be obtained using pMJ540 and pMJ541 (full-length and truncated versions respectively of UL26). The UL26 protein which would be expressed by such viruses is a protease, and it may be that it is toxic to

vaccinia virus, thus preventing replication and plaque formation. However, the UL26 protease has since been successfully cloned into baculovirus (personal communication, Dr V.G.Preston).

High-level expression of heterologous DNA has also been achieved successfully using the baculovirus Autographa californica nuclear polyhedrosis virus as a vector (Doerfler, 1986; Emery, 1991). The viral genome of approximately 126 kbp can, like that of vaccinia virus accommodate relatively large segments of additional foreign DNA. Also, as is the case with the vaccinia expression system, the insect cells used in the propagation of baculoviruses appear capable of accomplishing most post-translational modifications that are required to render a foreign gene product biologically active (eg Urakawa et al., 1989). A number of herpesvirus proteins have been expressed from baculovirus vectors. Of particular interest is the recent work showing that a subset of HSV-1 proteins, when expressed by recombinant baculoviruses, are able to associate normally as in HSV-1 infections, retaining full biochemical activity (Dodson et al., 1989; Calder & Stow, 1990; Stow, 1992). It would be of great interest to be able to express HSV-1 capsid proteins in such a system in order to study aspects of capsid structure and assembly. The large amounts of foreign protein which can be synthesised by placing the gene of interest under the control of the polyhedrin gene promoter make this a potentially useful system for purifying proteins for structural studies. This thesis reports the expression of VP5 by a recombinant baculovirus.

4 EXPRESSED PROTEINS

This thesis reports the construction of four recombinant vaccinia viruses each of which contains an HSV-1 capsid protein gene. These viruses, and the HSV-1 genes they

contain, are vMJ521 (UL18), vMJ535 (UL19), vMJ542 (UL26.5) and vMJ534 (UL38). In three cases, the capsid protein expressed by the recombinant vaccinia virus was shown to migrate at the position of the equivalent protein in a profile of purified HSV-1 capsids. Thus vMJ535 produced a protein migrating at the position of VP5, vMJ534 one which migrated at the position of VP19C, and vMJ521 one migrating at the position of VP23 (Figure 18). The apparent similarity of size between the native HSV-1 proteins and those expressed by the recombinant viruses supports the view that there is little or no post-translational modification of VP5, VP19C or VP23.

The products of UL26.5 do undergo significant post-translational processing (Liu & Roizman, 1991b; Preston et al., 1992), and the size of the processed product present in capsids would not necessarily be the same as that of the protein expressed by the recombinant. In the case of vMJ542, containing UL26.5, two proteins of approximate size 40 kDa were expressed (Figure 19), which were characterised using a monoclonal antibody (5010) known to react specifically with processed and unprocessed forms of VP22a. Immunoprecipitations performed using this antibody showed that the proteins expressed by vMJ542 are the unprocessed forms of VP22a (Figure 20). This was predictable, in that the processing event is a cleavage accomplished by the product of UL26, which is not present during infections with vMJ542. UL26 was cloned during this project, but attempts to introduce this gene into vaccinia virus were unsuccessful. Such a recombinant could be used in a coinfection experiment with vMJ542, to determine whether the protease product of UL26 is able to accomplish the cleavage of the products of UL26.5 in a vaccinia system, as it is able to do in a transient expression assay (Liu & Roizman, 1991b) and when expressed under IE conditions in an HSV-1 vector (Preston et al., 1992). It is not known why vMJ542 produced two novel protein bands. The UL26.5 sequences used to construct vMJ542, when expressed in an HSV vector

under IE control, also produced two protein bands (Preston et al., 1992).

5 ANTIBODIES

Several aspects of this project were hampered by a lack of suitable antibodies against VP5 and VP19C. The antibody 10555 was a rabbit anti-HSV antibody which was able in an immunofluorescence assay to recognise VP5 when expressed by vMJ535. However, it did not recognise VP19C when expressed by vMJ534. Presumably VP19C is not such a prominent antigen as is VP5. Two rabbit antibodies raised against HSV-1 virions and kindly supplied by Dr J.C.M.MacNab were also used in these IF experiments. However, neither recognised VP5 or VP19C. Attempts have been made in this department to produce monoclonal antibodies against capsid proteins following immunising mice with purified capsids (personal communication, Dr A.M.Cross), but these have so far been unsuccessful. An attempt was made during the course of this project to raise an antibody against VP19C by immunising rabbits with a synthetic branching peptide. The sequence of the branches was designed to be identical to that of the N-terminal 15 amino acids of VP19C. Sera from two rabbits thus immunised were both found to have very high titres to the synthetic peptide, when tested by ELISA. However, neither showed any detectable response to VP19C when tested in immunofluorescence or Western blot assays. Antibodies against VP19C were successfully prepared by Yei et al. (1990) by two methods. Three synthetic peptides, of lengths of 15, 15 and 13 amino acids respectively were prepared and each was conjugated with a carrier protein. The sequences of the synthetic peptides were those of three different internal regions of the VP19C of HSV-2. The conjugates were used to immunise rabbits. A fourth antibody was prepared by immunising rabbits with a 59-kDa fusion protein containing 197 amino acids of an internal region of the HSV-2 VP19C. All four

antibodies showed cross-reactivity with the type 1 protein.

6 SUBCELLULAR LOCALISATION OF CAPSID PROTEINS

6.1 Cell Fractionation

Whilst some newly synthesised herpesvirus proteins remain in the cytoplasm, the structural proteins are amongst those which migrate to the nucleus of infected cells (Spear & Roizman, 1968; Mark & Kaplan, 1971), the site of nucleocapsid assembly (Morgan et al., 1954). In experiments similar to those reported in this thesis, Fenwick et al. (1978) demonstrated the transferral of VP5 from cytoplasm to nucleus during the first 6 h of infection with HSV-1. This process was found to be inhibited by the arginine analogue canavanine and by the proline analogue azetidine, although the mechanism of this inhibition was not known. That nuclear localisation of VP5 in HSV-infected cells is an efficient process was demonstrated by Bibor-Hardy et al. (1985a), who showed that 80% was tightly bound to the nuclear matrix as early as 4 h after infection. In a study of the kinetics of the intracellular transport of the HCMV MCP, Yamauchi et al. (1985) found that 90% of the newly synthesised protein localised to the nucleus during the 3 h period from 72 to 75 h post infection.

The results of experiments reported in this thesis show that VP5, VP19C and VP23 are able to locate to the nucleus in HSV-1 infections (Figure 22). In recombinant vaccinia infections, VP5 and VP23 are almost exclusively cytoplasmic, indicating lack of an endogenous nuclear localisation signal (NLS). VP19C and VP22a did partially localise to the nucleus in recombinant vaccinia infections (Figures 22, 23 and 24). VP22a appeared to have the ability to affect the location of VP5 (Figure 26), although this effect was limited and was not

reproducible. This effect may have been due to a possible weakness of the fractionation procedure, in that nuclear-bound proteins could leach into the cytoplasmic fraction during fractionation. A proportion of capsid proteins did fractionate with the cytoplasm in HSV-infected cells - this may represent leakage but probably also represents the true state of affairs. Capsid proteins are synthesised in the cytoplasm so must be expected to be present there. Capsid proteins present in the cytoplasm late in infection may also represent the presence of progeny virions.

6.2 Immunofluorescence

Immunofluorescent staining techniques have been used to demonstrate that VP5 adopts a nuclear location in HSV-1-infected cells (Powell & Watson, 1975; Cohen *et al.*, 1980). The intracellular distributions of other herpesvirus MCPs have also been investigated using this technique, and have also shown strong nuclear localisation: VZV (Vafai *et al.*, 1990), PRV (Yamada *et al.*, 1991), HCMV (Rudolph *et al.*, 1990a), HHV-6 (Littler *et al.*, 1990) and EBV (Vroman *et al.*, 1985). The HSV-1 capsid proteins VP19C, VP23 and VP26 were also shown to adopt a nuclear location in IF experiments by Cohen *et al.* (1980). Using an antibody raised against VP21/VP22a, Cohen *et al.* (1980) also demonstrated a nuclear location for VP21/VP22a in HSV-infected cells. Heilman *et al.* (1979) and Rixon *et al.* (1988) have also used IF to demonstrate a nuclear location of VP22a. The VP22a homologue in HCMV has been shown by IF to be distributed roughly evenly between the cytoplasm and the nucleus of infected cells (Landini *et al.*, 1991b). Experiments reported in this thesis confirm the nuclear locations of VP22a and VP23 in HSV-1-infected cells by IF using the monoclonal antibodies 5010 and 1060 respectively.

The cytoplasmic locations of VP23 when expressed by a vaccinia vector (Figure 29) and by a plasmid vector (Figure 31), and of VP5 when expressed by a vaccinia vector (Figure 33), are of interest as a demonstration that these proteins do not have intrinsic ability to localise to the nucleus of infected cells. These results are in accordance with the result obtained by cell fractionation.

IF experiments using 5010 demonstrated that, when expressed by a vaccinia vector, VP22a adopts a nuclear location (Figure 35) in a similar fashion to the situation in HSV-infected cells (Figure 34). VP22a was also shown to be specifically located to the nucleus of vMJ542-infected cells in immunoelectron microscopy experiments (Figure 37). This distribution differs from that obtained by cell fractionation, and as described above, probably reflects weaknesses in the fractionation approach. It is worth noting that, using cell fractionation, Braun et al. (1984b) found that some unprocessed forms of VP22a were specifically restricted to the cytoplasm. However, Rixon et al. (1988) using IF and immune EM showed that the unprocessed forms of VP22a made by ts1201 are specifically located in the nucleus. This suggests that cell fractionation can generate misleading conclusions. The unprocessed forms of VP22a made by ts1201 probably correspond to those made by vMJ542.

The ability of VP22a to affect the intracellular location of VP23, as demonstrated by IF (Figure 36), conflicts with the fractionation result. This again is difficult to explain. The potential inherent in the fractionation procedure for degradation of proteins and for leaching of nuclear-bound proteins into the cytoplasmic fraction suggests that this is the least reliable method of the two. Further work is necessary to analyse in more detail the effect of VP22a on the intracellular transport of VP23.

An interesting example of a capsid protein which affects the intracellular location of another capsid protein is the 42-kDa polyomavirus MCP VP1. This protein contains an endogenous NLS (Moreland & Garcea, 1991) which is sufficient for nuclear localisation (Stamatos et al., 1987; Montross et al., 1991). The polyomavirus capsid contains two other proteins, VP2 (35 kDa) and VP3 (23 kDa), and it has been shown by cell fractionation studies that VP1 directs the nuclear localisation of VP3, whereas VP2 is able to locate to the nucleus independently (Stamatos et al., 1987).

7 KINETICS OF SYNTHESIS OF CAPSID PROTEINS

Results presented in this thesis are in agreement with the designation of VP23 as an early-late protein. VP5 and VP22a have also been defined as early-late proteins, as would be expected for capsid proteins given the temporal appearance of capsids. The status of VP19C is less clear; UL38 has been described as a true late gene. However, some capsid assembly does occur under conditions of inhibition of synthesis of DNA, indicating that sufficient VP19C must be available since this protein is essential for capsid formation. The different properties of VP19C and the other capsid proteins is intriguing in view of the finding that the levels of VP19C found in cells coinfected with vMJ534 and vMJ535 are greatly reduced compared to the levels present in cells infected only with vMJ534. If expression of VP5 in some way interferes with expression of VP19C, it may be necessary for these two proteins to be synthesised at different stages of the HSV lytic cycle. It has recently been shown that VP26 is also regulated as a true-late protein (McNabb & Courtney, 1992). However, it has not been established that VP26 is essential for capsid assembly. No mutant in UL35 has been isolated. Stable capsids can exist without VP26 (Newcomb & Brown, 1991), which might indicate that VP26 is not essential for capsid assembly.

8 DNA-BINDING PROPERTIES OF CAPSID PROTEINS

Experiments carried out as part of this thesis have failed to demonstrate the DNA-binding ability of VP19C reported by Braun *et al*. (1984a). VP19C expressed by a recombinant vaccinia virus did not bind DNA in a Southwestern assay, nor did VP19C present in virions bind DNA in this assay even though other known virion DNA-binding proteins (Blair & Honess, 1983; Pinard *et al*., 1987) showed DNA-binding activity.

It is interesting that another research group has been unable to demonstrate DNA-binding activity in intact VP19C. They do however have some evidence to suggest that in some virus stocks a DNA-binding protein is released by cleavage of approximately 100 amino acids from the N-terminus of VP19C (personal communication, Dr P.Sheldrick). They are continuing investigation of this phenomenon using vMJ534.

The polyomavirus MCP VP1 is a high-affinity, non-sequence-specific DNA-binding protein. DNA inhibits the self-assembly of VP1 into capsid-like structures *in vitro* (Salunke *et al*., 1986). The binding activity is localised at the amino terminus (Moreland *et al*., 1991), and a recent study of SV40 virion structure shows that the N-terminal 15 amino acids of VP1 are oriented towards the interior of the virion (Liddington *et al*., 1991). The proposed location of VP19C in the capsid floor (Newcomb & Brown, 1989) would be an ideal site for interaction with DNA. If VP19C does have significant DNA-binding activity, it may be that it, rather than the now-discounted core 'plug', is the means of anchorage of DNA within the capsid.

9 ARTIFICIAL CAPSID ASSEMBLY

During the course of the work presented in this thesis,

assembly of capsids or structural intermediates of a number of viruses has been achieved by expression of viral structural proteins in heterologous vectors. For example, a recombinant vaccinia virus which expresses the gag and pol genes of HIV-1 and which produces HIV-like particles has been constructed by two research groups (Karacostas et al., 1989; Shioda & Shibuta, 1990). Other workers have assembled HIV-like particles during dual infections using two recombinant vaccinia viruses which express the gag and env genes (Haffar et al., 1990; Vzorov et al., 1991). Ansardi et al. (1991) constructed two recombinant vaccinia viruses, one expressing the poliovirus P1 capsid precursor polyprotein, and the other expressing the poliovirus protease 3CD. Dual infections using these viruses resulted in the correct cleavage of the P1 protein to yield the three capsid proteins VP0, VP3 and VP1, which assembled correctly to form empty, immature poliovirus virions. A further interesting example is that of the vaccinia virus constructed by Hong & Engler (1991) which expresses the adenovirus fibre protein. The protein expressed was correctly assembled into trimers, glycosylated, and transported to the nucleus. A NLS was identified in this protein.

Baculovirus vectors have also been used in studies of virus assembly. The complete coding region of a poliovirus, when expressed in a recombinant baculovirus, resulted in synthesis of empty, immature poliovirus particles comprising VP0, VP3 and VP1 (Urakawa et al., 1989). The major core protein of bovine rotavirus, VP2, assembles into core-like particles when expressed by a recombinant baculovirus. In dual infections with another baculovirus expressing the component of the bovine rotavirus inner shell (VP6), single-shelled capsids were formed which contained cores of VP2 (Labbe et al., 1991). Sabara et al. (1991) assembled double-shelled rotavirus-like particles by expressing bovine rotavirus proteins in two recombinant baculoviruses. One recombinant expressed VP6, and the other expressed the major component of the

outer shell, VP7. A baculovirus vector expressing the two major core proteins VP3 and VP7 of the reovirus bluetongue virus (BTV) assembled core-like particles (French & Roy, 1990). A second baculovirus recombinant expressing the two outer capsid proteins VP2 and VP5 of BTV, when used in dual infections with the core protein recombinant, led to the synthesis of double-shelled BTV particles (French et al., 1990).

In the above-cited examples, assembly was an intrinsic feature of the capsid proteins involved. Thus it was hoped that the same would be true for HSV, and that by simultaneous expression of HSV-1 capsid proteins using recombinant vaccinia viruses, assembly of capsids or capsid-like structures might occur. However, electron microscopy failed to detect any capsids, suggesting that the four capsid proteins VP5, VP19C, VP22a and VP23 are not sufficient for the assembly of capsids in the vaccinia system. It is not known whether VP24 and/or VP26 are essential for assembly of structural units, or whether another non-capsid protein or proteins is necessary to achieve assembly. Capsid assembly may not have occurred because expression from a recombinant vaccinia virus might have altered transport or localisation of capsid proteins preventing them from coming together in the appropriate circumstances. This was thought a possible reason for the cytoplasmic locations of VP5 and VP23 and was the reason for the experiment expressing VP23 from a plasmid, which ruled out this possibility. It is also possible that considerations of stoichiometry or features of temporal regulation of expression of capsid proteins might be important. These factors are not regulated in the vaccinia system. The observation that VP5 suppresses levels of VP19C in vaccinia suggests that they might need to be expressed at different times as in wild-type HSV infections. However, the most likely reason for the lack of capsid assembly in vaccinia is simply that not all the HSV capsid proteins were present in these experiments,

and that some of these are essential for assembly. Minor capsid proteins can be essential for capsid stability. An example of such a protein is found in the 15-kDa adenovirus protein known as polypeptide IX (van Oostrum & Burnett, 1985). Particles of adenovirus, when disassembled, consistently yield groups-of-nine (GON) hexons, which are the major shell component (Burnett, 1985; van Oostrum et al., 1987). Polypeptide IX acts as a capsid cement, bonding hexons into the highly stable GON assemblies (Furcinitti et al., 1989; Stewart et al., 1991). Thus the role of polypeptide IX appears to be similar to that proposed for VP23 (Schrag et al., 1989). The role of VP26 is not known. It may be that VP26 plays an essential role in capsid stability during assembly. The fact that VP26 can be removed from capsids without disruption (Newcomb & Brown, 1991) does not necessarily militate against this suggestion, since VP22a which is essential during capsid assembly is not essential for stability of mature capsids.

A further experiment carried out as part of the studies reported in this thesis was an attempt made to complement ts2 at the NPT in a coinfection with vMJ534. However, electron microscopy did not detect formation of HSV capsids under these circumstances. This is interesting in that the only known mutation in ts2 is in the essential capsid gene UL38. Apparently, VP19C, the product of UL38, does not direct the assembly of capsids by ts2 when supplied by a recombinant vaccinia virus. Infection with vaccinia interferes with HSV capsid assembly. However, control experiments using sequentially staggered coinfections of wild-type vaccinia and wild-type HSV-1 showed that HSV capsids can be formed under these circumstances.

Now that all the recognised HSV-1 capsid protein genes have been identified and cloned, they can be included in future experiments. It may be possible to achieve the project aim of assembly of capsids in a heterologous

system. As A and B capsids are the only known immature capsid forms, it would be interesting to be able to assemble other structural units, which might yield valuable information on the pathway of assembly and maturation. The work of Newcomb & Brown (1991) showing that capsids lacking VP26 are stable suggests that it may be possible to construct capsids without VP26. Artificial assembly of capsids may prove a useful method for mass production of capsids for cryoelectron microscopy. If capsids can be assembled with this system, it might also be possible to construct chimeric capsids using capsid proteins of other herpesviruses. This has been done with rotavirus (Labbe et al., 1991), with BTV (Loudon et al., 1991) and with poliovirus (Rombaut et al., 1991). Experiments with assembly of capsids could be extended by the use of truncated forms of capsid proteins. This and other techniques such as the use of site-directed mutagenesis can be useful in identification of NLSs and other interactive sites, and regions of structural significance.

Although all known capsid genes are now available for study it is still possible that there may be other, non-capsid, proteins essential for assembly. Cellular proteins necessary for capsid assembly would be supplied in the vaccinia system, but other HSV-encoded proteins which might have a transient role in structure or assembly would not. Capsid assembly may not be possible in the vaccinia (or baculovirus) system using only the recognised capsid proteins.

10 SCAFFOLDING PROTEINS and CHAPERONES

The potential role of VP22a as a scaffolding protein during HSV-1 capsid assembly is of great interest. The precedents for such a function of VP22a are the scaffolding proteins of the double-stranded DNA bacteriophages (reviewed in Casjens & Hendrix, 1988). A

good example of this is in the assembly of heads of the Salmonella typhimurium lambdoid phage P22 (reviewed by Georgopoulos et al., 1983). A double-shelled prohead is assembled consisting of a shell of about 420 55-kDa coat protein molecules around an inner shell of about 250 molecules of the 42-kDa scaffolding protein. DNA is encapsidated and as a concomitant the scaffolding protein molecules are removed from the prohead, to take part in further rounds of the process (King & Casjens, 1974). Mutants in either protein gene prevent this process. Using proteins purified from proheads, the assembly process can be repeated and studied in vitro. When both proteins are present together, self-assembly of normal proheads occurs. However, the scaffolding protein does not form any structural units when present alone, and in the absence of scaffolding protein the coat protein only forms aberrant shell structures when present in concentrations much higher than are necessary to form normal proheads in conjunction with a scaffold. These results suggest that assembly of P22 proheads in vivo occurs by a process of copolymerisation (Fuller & King, 1980). VP22a can be removed from HSV-1 capsids using GuHCl leaving a stable outer capsid shell (Newcomb & Brown, 1991), and the P22 scaffolding protein can be removed from proheads in a similar way (Fuller & King, 1981). Thus although the P22 coat protein is capable of a degree of self-assembly, the scaffold is required for correct assembly; but once assembled the prohead shell of coat protein remains stable when the scaffold is removed.

Another interesting example of capsid assembly using a scaffold is found in the assembly of heads of the Es-cherichia coli bacteriophage T4 (reviewed by Black & Showe, 1983). In T4 infections a scaffolding core is formed which comprises one major and seven minor proteins (Traub et al., 1984). Proheads are formed by the addition of two major coat proteins. Use of mutants defective in the coat proteins has shown that the scaffold core can form in vivo without the coat proteins (Traub et al.,

✳(However, in view of the discovery of the UL26 protease it can no longer be certain that there is a single core protein in HSV: Davison et al., 1992).

1984; Traub & Maeder, 1984), and that these cores can act as intermediates in the T4 head assembly pathway (Kuhn et al., 1987). Assembly of T4 is therefore not a copolymerisation event. A further significant difference from the P22 assembly pathway is that after prohead assembly is complete, the scaffolding core structure is proteolytically removed. In this process the core proteins are either totally or partially degraded, and the degradation products of some core proteins remain inside the mature head (Onorato & Showe, 1975; Onorato et al., 1978).

Thus many similarities can be seen between these bacteriophages and the assembly of HSV capsids. Like P22, HSV capsids have a single known core protein, VP22a, which is present during assembly but which is afterwards removed. The fact that intermediate structures in the herpesvirus assembly pathway are not known, and the lack of assembly of cloned capsid gene products reported in this thesis suggest that HSV capsid assembly occurs by a process of copolymerisation as in P22. VP22a is lost from capsids at or near the time that viral DNA is packaged (Sherman & Bachenheimer, 1988; Rixon et al., 1988), and DNA packaging appears to be a concomitant of exit of scaffold in the dsDNA bacteriophages also (Earnshaw & Casjens, 1980). Both VP22a and the P22 scaffolding protein can be removed with GuHCl to leave basically intact capsid shells. The mechanism by which VP22a exits the HSV capsid is not clear. However, it is known that VP22a does undergo proteolytic modification (Liu & Roizman, 1991b; Preston et al., 1992), but the full roles of the UL26 protease and of VP24 have yet to be elucidated. These will be discussed in a later section. The precise roles of scaffolding proteins in virus assembly have not been fully elucidated, but Georgopoulos et al. (1983) summarise three possibilities. These are that the role of the scaffolding proteins may be (1) to help guide the coat protein into a proper assembly, (2) to exclude host macromolecules from the interior of the

coat protein shell, or (3) to allow coassembly of the coat protein with other, minor capsid proteins. None of these possibilities has been rigourously excluded but neither are they, in general, mutually exclusive.

A broader class of proteins which are involved in the self-assembly of protein structures are the molecular chaperones. These have been defined by Ellis & van der Vies (1991) as proteins which are transiently associated with one or more proteins to prevent improper interactions between potentially complementary surfaces. Chaperones can be involved in preventing incorrect folding during synthesis or transport of a protein. Even where chaperones are involved in the assembly of protein structures they do not convey steric information. They merely act to prevent an incorrect folding or interaction, allowing correct folding and interaction to predominate.

Certain stages in the assembly pathways of some viruses are known to depend upon proteins which clearly act as molecular chaperones but which are not scaffolding proteins. An example of a molecular chaperone involved in virus assembly is found in the papovavirus SV40. The SV40 MCP VP1 has an endogenous nuclear localisation signal (Wychowski et al., 1986). However, nuclear localisation of VP1 is enhanced by a 61-amino-acid, highly basic, SV40-specified polypeptide known as the agnoprotein (Carswell & Alwine, 1986; Resnick & Shenk, 1986). The agnoprotein may facilitate formation of infectious particles by inhibiting polymerisation of VP1 molecules until the time they interact with viral minichromosomes (Barkan et al., 1987). This is a recognised function of one type of molecular chaperone (Ellis & van der Vies, 1991). The ability of VP22a to effect nuclear localisation of VP23 as shown by IF experiments might be due to a similar phenomenon. Thus VP23 may adopt an incorrect conformation or form aggregations which prevent its translocation through nuclear pores. Association with

VP22a could maintain VP23 molecules in a form which can be translocated into the nucleus.

The adenovirus capsomer, the hexon, is composed of three, identical 110-kDa molecules (Burnett et al., 1985; Roberts et al., 1986). Assembly of this trimer is mediated by an essential 100-kDa virus-encoded non-virion protein. The 100-kDa protein complexes with nascent hexon monomer, and trimerisation is concomitant with separation of the complex (Cepko & Sharp, 1982; Cepko & Sharp, 1983). These authors suggest that the 100-kDa protein may serve actively to fold the hexon monomers into the trimer, or to transport monomers to a site where trimerisation can occur. The function of one class of chaperones is to prevent incorrect folding during transport. Another class of chaperones prevents incorrect folding of the aminoterminal region of a protein while the carboxyterminal region is still being synthesised (Ellis & van der Vies, 1991), and the association of the 100-kDa protein with hexon monomers while still attached to polyribosomes suggests that it may be acting in this way. In any case, it is possible that proteins of similar function to the 100-kDa protein might be essential for HSV capsid assembly. If such a protein does exist, it remains unidentified, but this would explain why HSV capsid assembly was not achieved in the experiments described here.

11 ASSEMBLY OF THE MAJOR CAPSID PROTEIN, VP5

Although assembly of viral capsids typically involves the ordered interaction of a number of components, individual structural proteins are capable of self-assembly into recognisable structures. The polyomavirus MCP VP1 has been purified after expression of the recombinant gene in an Eshcherichia coli vector. It was found that the purified VP1 had not undergone the extensive post-translational processing (acetylation and

phosphorylation) seen in virus-infected eukaryotic cells (Leavitt et al., 1985). Image analysis of low-dose electron micrographs revealed that the purified VP1 existed as pentamers which resembled the capsomers derived from dissociated capsids. At high ionic strength, these pentamers associated into capsid-like assemblies. Thus, neither the minor capsid proteins VP2 or VP3 nor post-translational processing of VP1 is essential for assembly of VP1. The stability of these assemblies was found to be increased by the addition of calcium to the medium (Salunke et al., 1986). A recombinant baculovirus expressing VP1 alone was found to assemble capsid-like particles in the nuclei of infected cells. However, treatment of infected cells with the calcium ionophore ionomycin led to formation of these particles in the cytoplasm also (Montross et al., 1991). These authors suggested that nuclear assembly might result from increased available calcium in this subcellular compartment. Increased calcium ion concentration has also been shown to improve the assembly of rotavirus-like particles in a recombinant baculovirus (Sabara et al., 1991).

Further interesting work on the assembly of the polyomavirus MCP VP1 was reported by Salunke et al. (1989). In these experiments it was found that by varying the ionic strength, pH and calcium concentration of preparations of purified VP1, a variety of structures would assemble from the pentameric VP1 subunits. Thus under certain conditions elongated tubules of VP1 could be formed. By varying the conditions, 12-capsomer icosahedral assemblies and 24-capsomer octahedral assemblies could be made. Cryoelectron microscopic analysis of these assemblies demonstrated that pentamers of VP1 had assembled by means of several significantly different inter-pentamer bonding arrangements. The normal polyomavirus capsid is an icosahedron consisting of 72 identical capsomers which are all pentamers of VP1. In this structure the 12 vertex pentamers adopt a

pentavalent bonding arrangement while the remaining 60 pentamers are hexavalent (Rayment et al., 1982). Such changes in bonding specificity exceed the possibilities of the elastic distortion assumed by Caspar & Klug (1962), and in fact involve distinct changes in quaternary structure of oligomers (Rossman, 1984).

A site-directed deletion at the carboxy terminus of the gene encoding VP1 resulted in the production of a mutant protein which was 57 amino-acid residues shorter than the wild-type (Garcea et al., 1987). Although this protein assembled in vitro into pentamers as normal, it did not assemble into capsid-like assemblies or any other higher-order structure. Thus the carboxy-terminal region of VP1 was shown to be involved in the specific bonding responsible for the non-equivalent association of capsomers.

Another example of polymorphism in virus structural assemblies is to be found in the bovine rotavirus major structural protein VP6. Hexameric capsomers of VP6, when purified from rotavirus particles, can be induced to assemble into a variety of structures according to conditions of ionic strength and pH. These forms include tubules and sheets as well as single-shelled capsids (Ready & Sabara, 1987). Thus even where chaperones or scaffolding proteins are not necessary, normal capsid assembly is not an inevitable outcome of the self-assembly of capsid proteins.

A feature of the lytic cycle of bacteriophage T4 is the appearance late in infection of polyheads. These open-ended tubular structures consist of a shell of the main coat protein gp23 built around a core of scaffolding proteins. Mutants in the main core protein genes do not produce proheads but do produce polyheads which lack a core. Thus gp23 is able to assemble in vivo without a scaffold (Steven et al., 1976). These coreless polyheads can be purified and used in studies of head formation.

Purified polyheads can be dissociated into hexameric capsomers of gp23; these capsomers are then able to re-assemble *in vitro* into polyheads without the need for a scaffold (Caldentey & Kellenberger, 1986). This re-assembly is dependent on conditions of ionic strength, temperature and pH. T4 capsomers can be further dissociated into lower-order oligomers, and ultimately to monomers (Caldentey & Kellenberger, 1986).

It was also observed that assembly of purified gp23 occurred above a critical concentration, in a manner comparable to crystal formation (Caldentey & Kellenberger, 1986). A similar phenomenon has been demonstrated in the assembly of poliovirus. The poliovirus 14S structural subunit consists of five copies each of the structural proteins VP0, VP1 and VP3. These 14S subunits are precursors in the poliovirus assembly pathway of 75S procapsids (Hellen & Wimmer 1992a). Purified 14S subunits have been shown to assemble *in vitro* only if their concentration exceeds a 1.6 nM threshold. This is also true for the 14S subunits in infected cell extracts. Thus assembly of poliovirus particles appears to be driven by the supply of 14S subunits (Rombaut *et al.*, 1991).

11.1 Why doesn't VP5 self-assemble?

Although it has not yet been possible to assess whether cloned VP5 assembles into oligomers including hexamers, it has been shown that higher-order assemblies resembling capsids, tubules or sheets do not occur in either vaccinia- or baculovirus-infected cells. The reason for this is not clear. HSV capsid assembly may take place by a process of copolymerisation. If this is the case, the four capsid proteins present in the experiments reported in this thesis are clearly not sufficient. It is possible that the minor capsid proteins VP24 and/or VP26 are essential for the copolymerisation process.

VP5 may not even assemble into oligomers. Like the adenovirus hexon monomer, it may require a chaperone to assist in assembly of capsomers (Cepko & Sharp, 1982). If this is the case, such a chaperone remains to be identified and was not present/functional in the experimental systems used here.

Assembly of T4 and poliovirus subunits has been shown to be dependent on a concentration threshold. The high levels of VP5 expressed by vMJ535 and by AcUL19 would probably exceed such a threshold if it is a limiting condition in the assembly of VP5. Both vMJ535 and AcUL19 express greater amounts of VP5 than are expressed by wild-type HSV-1. However, HSV-infected cells may achieve higher localised concentrations of VP5. For instance, assembly of VP5 in HSV occurs in the nucleus and not in the cytoplasm. VP5 was not present in the nucleus of vMJ535-infected cells. Conditions for VP5 assembly are not correct in the cytoplasm of HSV-infected cells, and presumably the same is true in the nuclei of AcUL19-infected cells. If certain physiological conditions are critical, it may be that the S.frugiperda cells used for the cultivation of baculoviruses do not provide the necessary conditions. It is interesting to note that the capsid-negative mutant ts2, which has a lesion in UL38, does not assemble any capsid-like structures, despite synthesising normal levels of VP5 (Pertuiset et al., 1989).

12 ROLE OF THE UL26 PROTEASE

The demonstration by Liu & Roizman (1991a) that a second ORF, UL26.5, is contained within the UL26 ORF was of great interest, and is the first of several recent developments in the understanding of the UL26 ORF, the proteins encoded within this region and their relevance to capsid assembly and structure. The significant features of this region are that the UL26.5 ORF is

contained entirely within the UL26 ORF, that the two ORFs are in frame and 3'-coterminal, and that the promoter regulatory domain of UL26.5 is entirely within the coding sequences of UL26. Although this is the first such example in HSV, a known parallel to this nested arrangement is found in the overlapping pre-S1, pre-S2 and S genes of hepatitis B virus. The pre-S2 gene is contained entirely within the pre-S1 gene, and the S gene is contained entirely within the pre-S2 gene. All three genes are 3'-coterminal. All three gene products are components of HBV particles (Heerman et al., 1984; Neurath et al., 1985). The paper of Liu & Roizman (1991a) also showed that UL26.5, rather than UL26, encodes VP22a. They also demonstrated for the first time the product of UL26, which is predicted to be a 635-amino-acid protein, and is of M_r 75 kDa.

Further research on the ORFs UL26 and UL26.5 demonstrated that the product of UL26 is a protease, and that this protease effects the removal of approximately 20 amino acids from the carboxy terminus of VP22a, and that it also autocatalyses the removal of its own corresponding carboxy terminus (Liu & Roizman, 1991b; Preston et al., 1992). This is the first demonstration of a protease encoded by HSV-1.

Homologues of UL26/UL26.5 have been identified in several other herpesviruses, as discussed in the Introduction. Further elucidation of these systems would also be of interest. The best characterised example is that of simian CMV, which has a nested set of four in-frame 3'-coterminal genes, and a protease activity localised in the amino-terminal 249 amino acids of the largest protein (Welch et al., 1991a; Welch et al., 1991b).

The region of the UL26 protein which contains the protease activity has been delineated by two sets of data. The mutant ts1201, defective in this protease activity (Preston et al., 1983), has a lesion at position

50897 on the HSV-1 genome (refer to Figure 14) (Al-Kobaisi, 1989). This lesion is 89 base-pairs downstream of the amino terminus of UL26, and corresponds to amino acid number 30 of the UL26 protein. The report of Liu & Roizman (1992) analyses the 635-amino-acid UL26 protein by deletion and insertion analysis. Whilst the first nine amino acids could be deleted without loss of the protease activity, a stop codon inserted after amino acid 286 inactivated protease activity. A stop codon inserted after amino acid 306 did not. Thus the protease activity is localised to the region of amino acids 10 to 306 of the 635-amino-acid UL26 protein. The protease is shown to be a serine protease (Liu & Roizman, 1992).

The recent demonstration by Davison et al. (1992) that the capsid protein VP24 is encoded by the amino-terminal region of UL26 is a further interesting development. Their results suggest that VP24 is produced by cleavage of the amino-terminal 247 amino acids from the 635-amino-acid UL26 gene product. It has long been thought that VP21 is encoded by a region lying within the UL26 ORF. This is on the basis that VP21 is immunologically cross-reactive with VP22a (Zweig et al., 1979b; Braun et al., 1984a), although direct demonstration of the exact region of UL26 which encodes VP21 is lacking. Davison et al. (1992) speculate that VP21 may result as a consequence of the production of VP24 by cleavage from the UL26 gene product, i.e. that VP24 represents the amino-terminal 247 and VP21 the carboxy-terminal 388 amino acids respectively of the 635-amino-acid UL26 protein. This suggestion is a major change from the view held prior to the discovery of UL26.5, which was that VP21 was a precursor form of VP22a (Braun et al., 1984a).

It is not clear what controls the release of VP24 from the UL26 protein. Following the suggestion of Welch et al. (1991b), Davison et al. (1992) suggest that the UL26 protein itself is responsible. However, although the UL26 protein has been shown to autocatalytically cleave its

own 20 carboxy-terminal amino acids (Liu & Roizman, 1991b; Preston et al., 1992; Liu & Roizman, 1992), it has not yet been shown to cleave itself to release VP24 and VP21.

It would also be interesting to know when these cleavages occur. The UL26 protein is not a recognised component of capsids, but VP24 and VP21 are. Therefore release of VP24 and VP21 from the UL26 protein must occur before capsid isolation. Since assembly can take place without the carboxy-terminal processing of VP22a (Preston et al., 1983), it seems likely that this proteolytic event occurs subsequent to assembly. This suggests that the protease activity is present in capsids. Davison et al. (1992) suggest that the 247-amino-acid VP24 molecule would retain the protease activity after cleavage from the UL26 protein, and thus would be able to effect the carboxy-terminal cleavage of VP22a within capsids. However, the work of Liu & Roizman (1992) suggests that this is not the case, as they show that a stop codon placed after amino acid 286 of the UL26 protein is sufficient to eliminate protease activity. Clearly there is a need to elucidate more fully the roles of VP24, VP21 and VP22a. This thesis reports the expression of UL26.5 in a vaccinia vector, and the cloning of UL26, providing the basis for further investigation of the products of these genes.

Proteolytic processing is a commmon phenomenon in the maturation of virus particles. Hellen & Wimmer (1992b) divide such processing into two classes: formative and morphogenetic. Formative proteolytic processing occurs when structural proteins are synthesised as a large polyprotein and require to be cleaved to release individual proteins. A good example of this are the picornaviruses, where the capsid proteins VP0, VP1 and VP3 are synthesised as a polyprotein (reviewed by Rueckert, 1990). Morphogenetic proteolytic processing involves a conformational change in a precursor capsid

form, and is usually associated with aquisition of infectivity (Hellen & Wimmer, 1992b). Picornaviruses also exhibit morphogenetic processing, at a later stage in the assembly process. A 125S provirion has been identified which contains 60 molecules each of VP0, VP1 and VP3. Conversion to mature 150S virions occurs following cleavage of the VP0 molecules to release VP2 and VP4. The cleavage site appears to be inaccessible to exogenous viral or cellular proteases and it has been suggested that cleavage is effected by some part of the virion structure itself. Cleavage is probably concomitant with encapsidation of RNA (Hellen & Wimmer, 1992a).

Bacteriophage T4 is an example of a large DNA virus which exhibits morphogenetic proteolytic processing, and which has some parallels with HSV. The complex prohead of T4 contains over 20 proteins. Conversion of the prohead to a mature head involves cleavage of most of these proteins, several conformational changes, and packaging of DNA (reviewed by Black & Showe, 1983). The protease responsible for the cleavages is contained within the prohead, and is an 18.5-kDa molecule produced by autocleavage of a 27.5-kDa precursor. The protease is then inactivated by extensive self-digestion, although 1 to 4 intact copies of the protease remain in the mature head (Showe et al., 1976a; Showe et al., 1976b). The fact that VP24 is a component of mature HSV capsids has led to the suggestion that it may play an important structural role as well as having protease activity (Davison et al., 1992).

It is not clear how to classify the proteolytic events involved in the assembly of HSV capsids (Hellen & Wimmer, 1992b). It is not known what, if any, structural roles are played by VP24 and VP21. The cleavage producing these two proteins may even be an example of a formative processing event. Although no changes in morphology or structure are known to occur in the capsid shell, there is a reduction in the size of the core associated with

the carboxy-terminal cleavage event (Preston et al., 1983). Furthermore, the carboxy-terminal processing of VP22a is also known to be essential for packaging of DNA (Preston et al., 1983). The alteration of core size and the association with aquisition of DNA suggest that this proteolytic event could be classed as morphogenetic. It is clear that the products of the genes UL26 and UL26.5 play a central role in capsid morphogenesis and it is perhaps not surprising that the aim of capsid assembly was not achieved in the absence of a UL26-expressing vaccinia recombinant.

Viral proteases are increasingly being studied as potential targets for antiviral compounds, and Liu & Roizman (1991b) observed that the UL26 protease is a potential antiviral target. The approaches taken to this problem can be divided into two classes: direct inhibition of enzyme activity, and alteration of substrate conformation (Korant, 1989). With this in mind, vMJ542 should prove useful for studies on VP22a, the substrate of the UL26 protease.

Elucidation of the roles of the proteolytic processing events in HSV capsid assembly should prove to be a fruitful area of further enquiry.

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