MUTATIONAL ANALYSIS OF THE HERPES SIMPLEX VIRUS TYPE I UL8 PROTEIN

By

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SUMMARY

Seven genes encoded by herpes simplex virus type 1 (HSV-1) are essential and sufficient for viral origin-dependent DNA synthesis in transfected tissue culture cells. The products of three of these - UL5, UL8 and UL52 - form a heterotrimeric complex exhibiting DNA helicase and primase activities. Studies using temperature-sensitive HSV-1 recombinants have shown that co-expression of all three subunits is essential for efficient nuclear localisation of any one of them. Since a sub-assembly consisting of UL5 and UL52 is enzymatically active but remains in the cytoplasm, it was suggested that UL8 may play an important role in facilitating nuclear uptake of the trimeric complex. Biochemical assays utilising recombinant baculovirus-expressed DNA replication proteins subsequently demonstrated that UL8 stimulates the synthesis of primers by UL5/UL52 and/or their utilisation in DNA synthesis, although the mechanisms by which this occurs remain unclear. Since homologues of UL8 have not been identified outside the herpesvirus family, little information about the functions of UL8 has been gained from its amino acid sequence.

The aims of the work presented in this thesis were therefore to perform a mutational analysis of the UL8 protein, in an attempt to define important regions in UL8 and to relate these to the known functions of the protein. A random set of N- and C-terminally truncated versions of the 750 amino acid UL8 protein were created and expressed from plasmids in transfected tissue culture cells under the control of a strong constitutive promoter (the human cytomegalovirus major immediate-early promoter). An additional mutant containing an internal deletion spanning amino acids 78-339 of UL8 was also generated.

To investigate the effect of the mutated UL8 proteins on the intracellular location of UL5 and UL52, without the requirement for constructing temperature-sensitive HSV-1 recombinants, a plasmid-based assay for intracellular localisation of the helicase-primase complex was developed. This assay, coupled with the availability of monoclonal antibodies to UL8, permitted the previous findings using recombinant viruses to be confirmed and extended. The results demonstrated that co-expression of the three

components of the helicase-primase complex in the absence of all other viral proteins is sufficient for their efficient nuclear localisation, and that UL8 can apparently enter the nucleus in the absence of UL5 and UL52.

The mutated UL8 proteins were screened for ability to facilitate nuclear localisation of UL5 and UL52 in the plasmid-based assay, and for ability to replace wild-type UL8 in transient transfection assays of HSV-1 origin-dependent DNA synthesis. In addition, selected mutants were used to generate recombinant baculoviruses, resulting in expression of sufficient quantities of the mutated UL8 proteins to permit examination of their ability to co-immunoprecipitate with UL5 and/or UL52 from extracts of infected insect cells.

The results demonstrated that a deletion of 5 amino acids from the N-terminus of UL8 can be tolerated without affecting its ability to support HSV-1 origin-dependent DNA synthesis or facilitate nuclear localisation of the helicase-primase complex, whereas larger deletions of 23 to 165 amino acids abolish both of these activities. When expressed by a recombinant baculovirus, the protein in which the N-terminal 165 amino acids had been deleted,coprecipitated with both UL5 and UL52 from extracts of appropriately infected insect cells.

Removal of the 4 most C-terminal amino acids from UL8 did not affect its ability to facilitate efficient nuclear localisation of the helicase-primase complex, but the protein supported less than wild-type levels of HSV-1 origin-dependent DNA synthesis in the transfection assay. Larger C-terminal deletions of 33 to 497 amino acids, and deletion of residues 78-339 of UL8, abolished both transient replicative ability and efficient nuclear localisation of the helicase-primase complex in transfected cells. When expressed by recombinant baculoviruses, mutated UL8 proteins with deletions spanning amino acids 78-339, 471-750 and 718-750 were each capable of coprecipitating both UL5 and UL52 from extracts of infected insect cells.

The ability of the mutated UL8 proteins to support origin-dependent DNA synthesis therefore correlates well with their ability to facilitate efficient nuclear localisation of UL5 and UL52. Moreover, most of the mutants can, or are predicted to be able to, interact with UL5 and UL52. The possibility therefore arises that the UL8

mutants fail to support DNA synthesis in the transient assay solely due to their failure to facilitate efficient nuclear localisation of the helicase-primase complex. It is possible that complexes incorporating mutant UL8 in place of the wild-type protein may be sufficiently altered in conformation as to fail to present the appropriate signals for nuclear uptake and retention.

Although known functions of UL8 can not as yet be associated with particular stretches of amino acids, it is clear from these studies that residues close to each terminus (amino acids 6-23 and 718-746) of UL8 are indispensable for UL8 function. These studies provide the basis for a number of future experiments aimed at identifying functional domains within UL8.

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Eleanor Claire Barnard October 1996

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ABBREVIATIONS

AcNPV	*Autographa californica* Nuclear Polyhedrosis Virus
Amp^r	ampicillin resistant/resistance
Amp^s	ampicillin sensitive/sensitivity
BHK (cells)	baby hamster kidney (cells)
bp	base pair(s)
BSA	bovine serum albumin
°C	degrees Celsius
CAV	cell associated virus
Ci	Curie
CIP	calf intestinal phosphatase
CLB	cell lysis buffer
cm	centimetre(s)
cpe	cytopathic effect
cpm	counts per minute
CRV	cell released virus
CT (DNA)	calf thymus (DNA)
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dNTP	2'-deoxynucleoside-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
DDAB	dimethyl dioctadecyl-ammonium bromide
DEAE	diethylamino-ethyl
DMS	dimethyl sulphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DOPE	dioleoyl L-alpha-phosphatidyl ethanolamine
ds	double stranded
DTT	dithiothreitol
EBV	Epstein-Barr virus
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra-acetic acid (disodium salt)
EGTA	ethylene glycol-bis(ß-amino-ethylether) N,N,N',N'-tetra acetic acid
EHV-1	equine herpesvirus 1
EHV-2	equine herpesvirus 2
EtBr	ethidium bromide

Fc	crystallisable fragment (of immunoglobulin)
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
H (mix)	hybridisation (mix)
HBS	hepes buffered saline (for lipofection)
HCMV	human cytomegalovirus
HeBS	hepes buffered saline (for $CaPO_4$ precipitation)
hepes	N-[2-hydroxyethyl]piperazine-N'-[2-ethane sulphonic acid]
HHV-6	human herpesvirus 6
HHV-7	human herpesvirus 7
hpi	hour(s) post-infection
hr	hour(s)
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
HVS	herpesvirus saimiri
IE	immediate-early
IgG	immunoglobulin G
K	thousand
kb(p)	kilobase pair(s)
kDa	kiloDalton(s)
LB	L-broth
M	molar
mA	milliamp(s)
mg	milligram(s)
min	minute(s)
mJ	milliJoule(s)
ml	millilitre(s)
mM	millimolar
mm	millimetre(s)
m.o.i.	multiplicity of infection
mRNA	messenger RNA
m.w.	molecular weight
NBCS	newborn calf serum
ng	nanogram(s)
nm	nanometre(s)
NT buffer	nick translation buffer
OD	optical density
ORF	open reading frame

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
pfu	plaque-forming units
pg	picogram(s)
p.i.	post-infection
PMSF	phenyl methyl sulphonyl fluoride
PRV	pseudorabies virus
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SE (buffer)	soluble extract (buffer)
sec	second(s)
S.f.	*Spodoptera frugiperda*
ss	single-stranded
SV40	Simian virus 40
TBS	Tris buffered saline
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
TRITC	tetramethyl rhodamine isothiocyanate
Triton X-100	octyl phenoxy polyethoxyethanol
tRNA	transfer ribonucleic acid
ts	temperature sensitive
TS (buffer)	Tris saline (buffer)
TSB	transformation and storage buffer
UV	ultra violet
V	Volt(s)
v or vol.(s)	volume(s)
VZV	varicella-zoster virus
wt	wild type
w/v	weight per volume (ratio)
X-gal	5-bromo-4-chloro-3-indolyl ß-D-galactopyranoside
µCi	microCurie(s)
µg	microgram(s)
µl	microlitre(s)

Nucleotide Bases

A	adenine	C	cytosine
G	guanine	T	thymine
U	uracil	N	any base
R	purine	Y	pyrimidine

Amino acids

alanine	A	Ala	leucine	L	Leu
arginine	R	Arg	lysine	K	Lys
asparagine	N	Asn	methionine	M	Met
aspartate	D	Asp	phenylalanine	F	Phe
cysteine	C	Cys	proline	P	Pro
glutamate	E	Glu	serine	S	Ser
glutamine	Q	Gln	threonine	T	Thr
glycine	G	Gly	tryptophan	W	Trp
histidine	H	His	tyrosine	Y	Tyr
isoleucine	I	Ile	valine	V	Val

CHAPTER 1 INTRODUCTION

Section 1.1 THE HERPESVIRUSES: DISTINCTIVE CHARACTERISTICS AND STRUCTURE

1.1.1 The *Herpesviridae*

Members of the family *Herpesviridae* share common morphological features. Their viral particles are 120-200 nm in diameter and are composed of four elements - the core, capsid, tegument and envelope. The core contains the large (120-220 kbp) double-stranded (ds) DNA genome and is enclosed within the icosahedrally shaped capsid (100-110 nm in diameter). Between the capsid and the envelope lies an amorphous proteinaceous layer known as the tegument. The envelope itself consists of a lipid bilayer derived from the host cell membranes, from which protrude spikes of virally encoded glycoprotein (Francki *et al.*, 1991).

Historically, this family has been sub-divided into *alphaherpesvirinae* (*e.g.* HSV-1, HSV-2, VZV, EHV-1 and PRV), *betaherpesvirinae* (*e.g.* HCMV) and *gammaherpes-virinae* (*e.g.* EBV, EHV-2 and HVS) subfamilies according to biological properties such as host range in tissue culture, length of reproductive cycle, cytopathology and characteristics of latent infection (Roizman *et al.*, 1992). Many of the alphaherpesviruses are neurotrophic, with a wide *in vitro* host range and a relatively short reproductive cycle. Latent infection in ganglia is frequent. The betaherpesviruses exhibit a more restricted host range and a relatively long reproductive cycle. In productive infections cells frequently become enlarged (cytomegalia) whilst latent infections may be established in a variety of tissues including kidney and secretory glands. Gammaherpesviruses are lymphotropic, with a narrow host range and a variable reproductive cycle. Latent infection is frequently established in lymphoid tissue.

With a few exceptions, this classification corresponds with the genetic relationships determined by comparisons of amino acid sequences and gene organisation (reviewed in McGeoch *et al.*, 1993). The identification of approximately forty 'core' genes, common to all the subfamilies, suggests a common evolutionary

origin for all members of the *Herpesviridae* family an estimated 200 million years ago (McGeoch *et al.*, 1995). An exception to this may be the recently sequenced channel catfish virus (CCV), originally classified as an alphaherpesvirus, which appears to be so distantly related to the mammalian and avian herpesviruses that any evolutionary connection must greatly predate 200 million years before the present (Davison, 1992; McGeoch *et al.*, 1995). A herpesvirus infection of oysters has also been identified, extending the natural host range of the herpesviruses to include invertebrates (A.J. Davison, personal communication)

Of the herpesviruses described to date, seven are known to infect humans: HSV-1, HSV-2, VZV, HCMV, EBV, HHV-6 and HHV-7. An eighth human herpesvirus associated with Kaposi's sarcoma (KSHV) has now been classified as a gammaherpesvirus, most closely related to HVS (Moore *et al.*, 1996).

Primary infection of humans by HSV-1 usually occurs in epithelial cells of the lips and mouth, causing cutaneous lesions (cold sores), although the eyes and genitals are also sites of infection. Historically HSV-2 has been associated more with genital tract infections than HSV-1, but genital HSV-1 and oral HSV-2 have become more common in recent years.

Most members of the herpesvirus family have an ability to establish and maintain a latent state in their natural host. Following primary infection, HSV can establish long-term latency in the neuronal cells of the sensory ganglia. Reactivation of the virus, resulting in cutaneous lesions at the site of primary infection, can occur sporadically throughout life and is associated with trigger factors such as trauma, fatigue and exposure to UV light.

Although HSV is a human virus,it grows in cells of a wide range of species in tissue culture. It also replicates in mice which are widely used as models for latency and pathogenesis.

1.1.2 The HSV-1 Genome

a) Structure

The HSV-1 genome is a linear, double-stranded molecule of DNA, 152 kbp in size. It has a high G+C% content (68%) and a relatively complex arrangement of two covalently linked components: the long (L: 126 kbp) and short (S: 26 kbp) segments (**Figure 1a**). The L segment contains a 108 kbp unique sequence element (U_L) flanked by 9.2 kbp inverted repeat sequences (terminal and internal repeats of the long segment, TR_L and IR_L). The S segment is also composed of a unique sequence U_S (13.0 kbp) flanked by 6.6 kbp inverted repetitive sequences TR_S and IR_S (Sheldrick & Berthelot, 1975; Wadsworth *et al.*, 1975). A direct repeat of approximately 400 bp - known as the "a" sequence - is present at the genomic termini (within TR_L and TR_S) and in inverted orientation at the L-S junction (Wadsworth *et al.*, 1976; Davison & Wilkie, 1981; Mocarski & Roizman; 1981). Multiple copies of the "a" sequence can occur at the L terminus and joint region, but only one copy is found at the S terminus. The regions of TR_L and TR_S excluding the "a" sequence are designated the "b" and "c" sequences respectively.

Both genome termini have a single residue 3' overhang, which may facilitate circularisation of the DNA following infection (Mocarski & Roizman, 1982b). This process does not require viral gene expression or DNA replication (Poffenberger & Roizman, 1985).

The two segments of the genome can invert relative to one another, generating four isomeric forms (**Figure 1b**): prototype (P), inversion of the long segment (I_L), inversion of the short segment (I_S) and inversion of both segments relative to the prototype (I_{LS}) (Hayward *et al.*, 1975; Delius & Clements, 1976). Preparations of viral DNA from plaque-purified HSV-1 contain an equimolar mix of the four isomers and it is thought that all four isomers can participate equally in both lytic and latent infections (Hayward *et al.*, 1975; Davison & Wilkie, 1983; Efstathiou *et al.*, 1986). The isomers are generated by intramolecular recombination, apparently mediated by the "a" sequence (Mocarski *et al.*, 1980; Smiley *et al.*, 1981). High frequency recombination between "a" sequences requires *trans*-acting factors supplied by HSV-1 and is closely

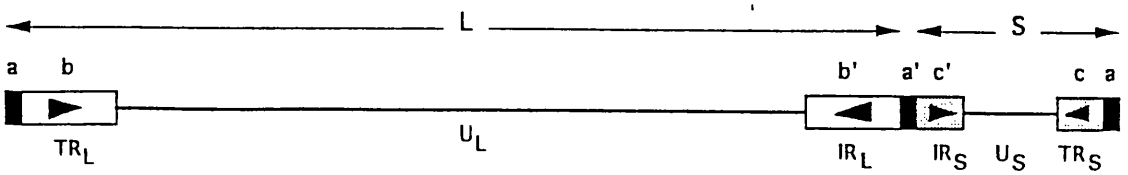
Figure 1. Structures of the genomes of human herpesviruses.

(a) The HSV-1 genome consists of two covalently linked segments L and S. Each segment contains unique sequences U_L and U_S (solid lines) flanked by terminal and internal repeat elements TR_L and IR_L, TR_S and IR_S respectively (open and shaded boxes, with relative orientations indicated by arrows). A direct repeat, termed the "a" sequence (solid box), is present at the genomic termini and in inverted orientation at the L-S junction.

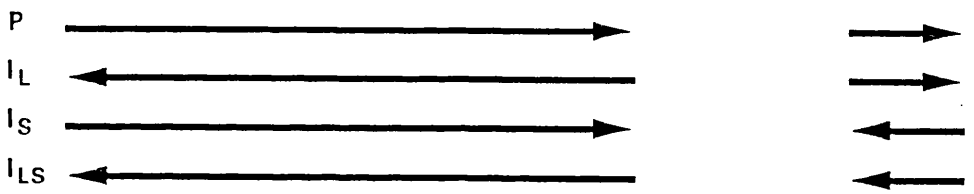
(b) Preparations of HSV-1 DNA contain an equimolar mix of four isomeric forms, generated by inversion of the L and S components (orientation of segments indicated by arrows): P (prototype), I_L (inversion of L), I_S (inversion of S) and I_{LS} (inversion of both L and S).

(c) Structures of the genomes of the other human herpesviruses are shown, with unique sequences as solid lines and repeat elements as boxes (relative orientations indicated by arrows). The HSV-2 genome is identical in structure to that of HSV-1and is therefore not shown. The inverted repeats flanking U_L in VZV are not marked, since they are only 88 bp in length. Direct terminal repeats (TR) flank the EBV genome and the major internal repeat (MIR) separates U_S from U_L. The EBV U_L contains the almost identical direct repeat elements D_L and D_R. The genome of HCMV is similar in layout to that HSV-1. The genome of HHV-6 consists of a single unique sequence flanked by direct repeats. The HHV-7 genome (not shown) is similar in layout to that of HHV-6. Adapted from McGeoch, 1989.

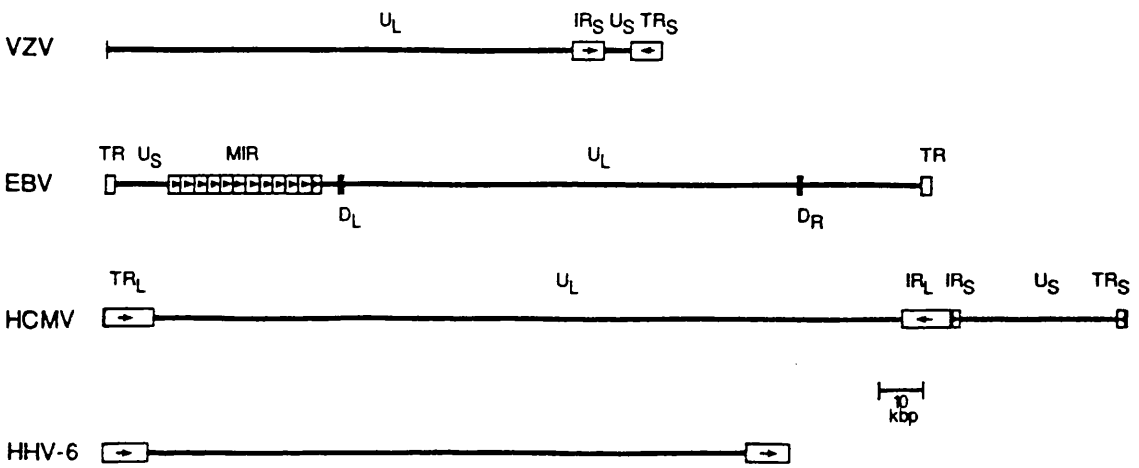
(a)


L
S
a b
b' a' c'
c a
TR$_L$
U$_L$
IR$_L$
IR$_S$
U$_S$
TR$_S$

(b)


P
I$_L$
I$_S$
I$_{LS}$

(c)


VZV
U$_L$
IR$_S$ U$_S$ TR$_S$
EBV
TR U$_S$
MIR
U$_L$
TR
D$_L$
D$_R$
HCMV
TR$_L$
U$_L$
IR$_L$ IR$_S$
U$_S$
TR$_S$
10 kbp
HHV-6

associated with HSV-1 DNA replication (Mocarski & Roizman, 1982a; Weber *et al.*, 1988; Dutch *et al.*, 1992). The isolation of viable HSV-1 mutants with genomes frozen in each of the four isomeric forms suggests that inversion is not essential for viral replication (Poffenberger *et al.*, 1983; Jenkins & Roizman, 1986).

The structures of the other human herpesvirus genomes are outlined in **Figure 1c**. The VZV genome is 125 kbp in size and resembles HSV in gross structure except that the inverted repeats flanking U_L are much shorter (only 88 bp) and there is no terminal redundancy in the VZV genome. As with HSV, each terminus has a single residue overhang, and the L and S segments can invert to generate different isomeric forms, although for VZV, the two isomers with the L segment in one orientation (corresponding to HSV-1 I_L) are approximately 20-fold more abundant than the other two. The 186 kbp genome of EBV differs markedly in the organisation of repeat elements. The termini are formed by a varying number of directly repeated copies of a 540 bp sequence. Another set of large directly repeated elements, termed the major internal repeat (MIR), also varies in copy number. The MIR separates the unique sequences U_L and U_S. The approximately 1 kbp regions D_L and D_R are almost identical to each other in sequence and are adjacent to tandem repeat families which are related but not identical. The HCMV genome, although larger than HSV at 230 kbp, is structurally similar in its layout of repeats and unique sequences and contains an eqiuvalent of the "a" sequence. It also occurs in four isomeric forms. The HHV-6 genome is much simpler in gross structure, consisting of a single unique sequence of 143 kbp flanked by 8 kbp direct repeats. Recent sequence data has confirmed that HHV-7, like HHV-6, consists of a unique sequence (133 kbp) flanked by direct repeats of 6 kbp (Ruvolo *et al.*, 1996).

b) Genetic Content

The complete sequence of the HSV-1 strain $17syn^+$ genome has been determined (McGeoch *et al.*, 1988a). Over 90% of the viral genome has protein coding potential. A total of 72 open reading frames (ORFs) coding for 70 distinct polypeptides were initially identified: fifty-six ORFs (UL1 - UL56) within U_L, twelve (US1 - US12)

within U_S and two immediate-early genes (IE-1 and IE-3) located in the R_L and R_S repeat sequences, which are therefore represented twice in the genome (**Figure 2**). HSV-1 contains few spliced genes: UL15 - whose intron contains the UL16 and UL17 ORFs, and three genes of the immediate-early class (section 1.3.3). [Although genes are often written in italics (*e.g. UL15*) to distinguish them from their products (UL15), italics will not be used in this thesis. Rather the word 'gene' or 'protein' will be included when ambiguity might result, for example to distinguish betwen the UL15 gene and the UL15 protein product.]

Several other protein coding ORFs have subsequently been reported. A second ORF within R_L, encoding the protein ICP34.5, was first described in HSV-1 strain F (Chou & Roizman, 1986; 1990) and later confirmed in HSV-1 strain 17 (Dolan *et al.*, 1992). An additional transcriptional unit has also been mapped within the UL26 gene. Its product, UL26.5 corresponds to the C-terminal 329 amino acids of the UL26 protein (Liu & Roizman, 1991). Likewise an additional gene between the UL49 and UL50 ORFs, designated UL49.5 (49A in **Figure 2**), has been reported (Barker & Roizman, 1992; Barnett *et al.*, 1992). Two transcripts, UL8.5 and UL9.5, which are 3' coterminal with the UL8 and UL9 transcripts, have been described. The UL8.5 ORF is in frame with and overlaps the C-terminal half of the UL9 gene. The product is a 53 kDa protein, OBPC, which is synthesised during viral infection and is recognised by antibody directed against the C-terminal region of the UL9 gene product, the origin binding protein (Baradaran *et al.*, 1994). A gene designated US1.5 has been reported to specify a mRNA which is 3' coterminal with that of the US1 gene which encodes Vmw68. The protein product of US1.5 corresponds to amino acids 147 to 420 of the Vmw68 protein (Carter & Roizman, 1996). Most recently an ORF mapping antisense to the UL43 gene has been described. The UL43.5 protein colocalises with capsid proteins in discrete nuclear structures, and has been postulated to play an accessory role in capsid assembly in cultured cells (Ward *et al.*, 1996).

Approximately two-thirds of the genes thus far identified have had functions assigned to their products, including roles in transcriptional regulation, DNA replication and virion structure and assembly. The complete sequencing of five other human

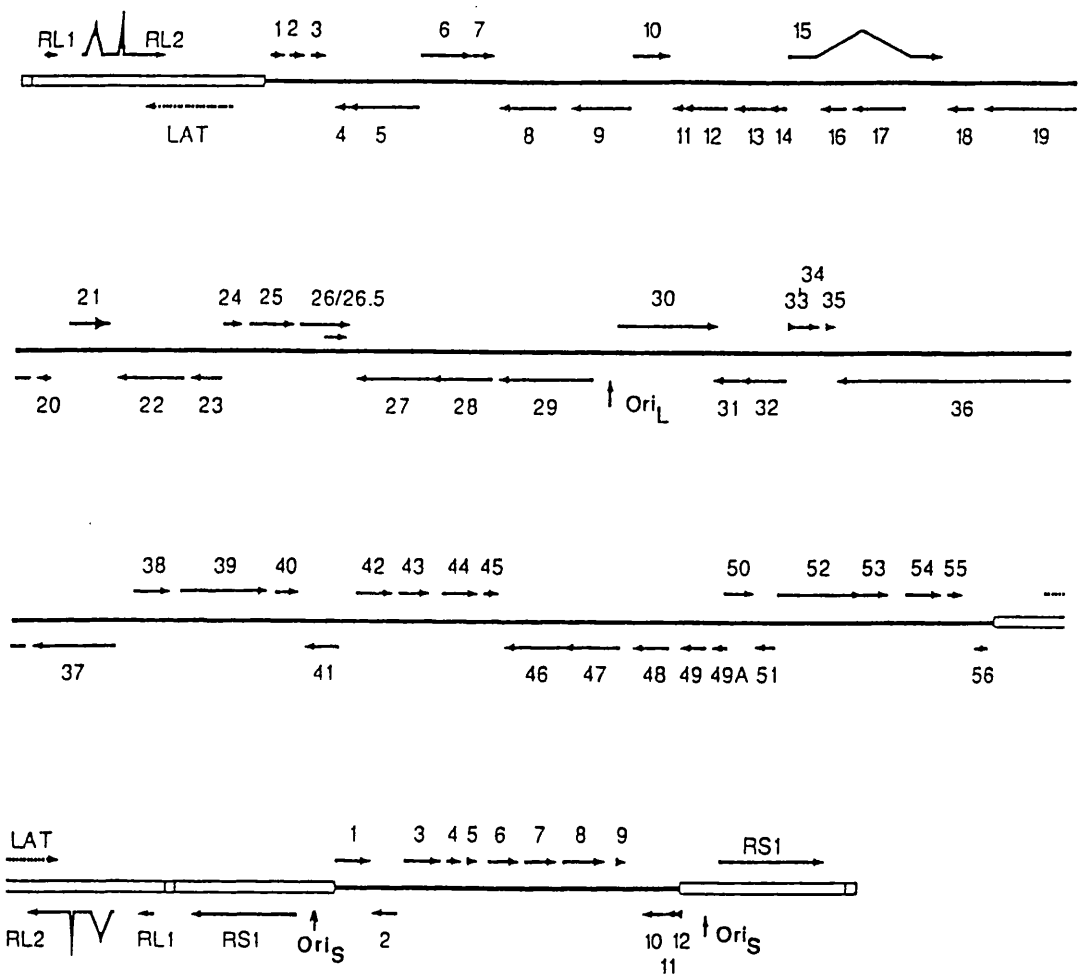


Figure 2. Genetic content of the HSV-1 genome.

The viral genome is represented as four successive lines with unique sequences shown as solid lines and major repeat elements as open boxes. The location and orientation of open reading frames are indicated by solid lines and LAT transcripts as arrowed, dotted lines. Genes UL1-UL56 are shown as 1-56 in the top three lines, US1-US12 as 1-12 in the bottom line, along with RL1 (encoding ICP 34.5), RL2 (IE-1 encoding Vmw110), and RS1 (IE-3 encoding Vmw175). The origins of DNA replication (oris and oriL) are also indicated. Refer to the text (page 5) for descriptions of additional ORFs not shown on this diagram. This diagram was provided by Dr D.J. McGeoch.

herpesviruses - VZV (Davison & Scott, 1986), HCMV (Chee *et al.*, 1990), HHV-6 (Gompels *et al.*, 1995), HHV-7 (Nicholas, 1996) and EBV (Baer *et al.*, 1984) - and the similar gross structures and colinearity of the HSV-1 and HSV-2 genomes (Davison & Wilkie, 1983), have enabled the identification of, and assignment of function to, many homologous genes within these related viruses.

1.1.3 The HSV-1 Virion

More than thirty HSV-1 genes are known to encode virion structural proteins. The majority of these are expressed in the late phase of the viral life-cycle, and can be classified as envelope glycoproteins, tegument proteins or capsid proteins (reviewed by Haarr & Skulstad, 1994).

a) Glycoproteins

Eleven virus-encoded glycoproteins have been identified to date: gB (encoded by UL27), gC (UL44), gD (US6), gE (US8), gG (US4), gH (UL22), gI (US7), gJ (US5), gK (UL53), gL (UL1) and gM (UL10). None of these glycoproteins is needed for virion assembly *per se*, but gB, gC, gD, gH and gL are involved in the initial stages of infection (section 1.3.1) and gC, gE and gI may modulate the immune response to infection - gC as a complement receptor and the gE/gI complex as an Fc receptor (Friedman *et al.*, 1984; Johnson *et al.*, 1988). gD also mediates resistance of infected cells to superinfection (Campadelli-Fiume *et al.*, 1988; Johnson & Spear, 1989), and gE/gI may facilitate neuron-to-neuron spread of the virus (Dingwell *et al.*, 1995).

Although the above glycoproteins constitute the major virus-specific components of the envelope (reviewed by Marsden, 1987 and Campadelli-Fiume, 1994), other HSV-1 potential membrane proteins such as those encoded by UL34, UL20 and UL43 have been putatively assigned to the envelope.

b) Tegument proteins

The tegument is an apparently amorphous structure between the capsid and the envelope. It includes several structural phosphoproteins (encoded by genes UL46,

UL47, UL49 and US9) and one myristylated protein (UL11) of unknown function. In addition to their structural roles, several of the tegument proteins have regulatory functions. These include a transactivator of IE gene expression, Vmw65 (αTIF: encoded by UL48; Batterson & Roizman, 1983; Campbell *et al.*, 1984) and the 58K protein (product of the UL41 gene) that acts to shut off host cell protein synthesis early after infection (Fenwick & Walker, 1978; Kwong *et al.*, 1988; Read *et al.*, 1993). Another tegument phosphoprotein, the product of gene UL36, has been reported to be a component of a complex capable of binding specifically to the "a" sequence (Chou & Roizman, 1989; McNabb & Courtney, 1992). The tegument protein encoded by gene US11 is abundant late in infection, associates with ribosomal RNA and may be involved in premature transcription termination (Roller *et al.*, 1996).

c) Capsid proteins

Seven proteins are involved in HSV-1 capsid formation (for a review see Rixon, 1993). Three different types of capsids have been isolated from HSV-infected cells (Gibson & Roizman, 1972). Empty, or type A, capsids lacking viral DNA consist of five structural proteins: VP5, VP19C, VP23, VP24 and VP26 (Gibson & Roizman, 1972; Cohen *et al.*, 1980). Intermediate or type B capsids, which also lack DNA but have an internal protein structure, include the additional proteins VP21 and VP22a. The latter protein is involved in maturation of the virion during encapsidation of DNA, and is subsequently lost such that it is absent from full or type C capsids containing viral DNA (Rixon *et al.*, 1988).

VP5, the product of gene UL19, is referred to as the major capsid protein. The capsid consists of 162 capsomeres of which 150 are hexons and 12 are pentons. Six molecules of VP5 constitute a hexon, and five form a penton (Vernon *et al.*, 1981; Newcomb & Brown, 1989, 1991). These hexameric and pentameric capsomeres are connected by Y-shaped structures termed triplexes (Newcomb *et al.*, 1993), which are formed by VP19C and VP23 (encoded by genes UL38 and UL18, respectively). VP19C is apparently linked to VP5 via disulphide bonds (Zweig *et al.*, 1979). The product of

the UL35 gene, VP26, is located at the tips of the hexons (Booy *et al.*, 1994; Trus *et al.*, 1995).

VP24 projects into the interior of the capsid and may interact with the viral DNA. It has protease activity essential for the maturation of virions and is a cleavage product of an 80K precursor protein encoded by gene UL26 (Davison *et al.*, 1992; Weinheimer *et al.*, 1993). This 80K protein cleaves 25 amino acids from its own C-terminus and from that of the UL26.5 gene product, generating the VP24/VP21 precursor and the protein VP22a, respectively. Further self-cleavage of the precursor yields the serine protease VP24, and the protein VP21 (Deckman *et al.*, 1992; Preston *et al.*, 1992; Dilanni *et al.*, 1993; Liu & Roizman, 1993; Person *et al.*, 1993). The function of the latter protein is unknown. VP22a acts as a scaffolding protein, aiding assembly of the capsid.

Section 1.2 HSV-1 LATENCY

Latency represents the maintenance of the virus in a non-pathogenic, non-replicating state in neuronal cells. The precise state of the viral DNA in latently infected cells is not clear. It does not appear to be linear, since terminal fragments have not been detected, nor is it integrated into the host DNA, but is thought to exist extrachromosomally as a circular episome (Rock & Fraser, 1983; Efstathiou *et al.*, 1986; Mellerick & Fraser, 1987). Despite many *in vivo* studies of latency in mice, rabbits and guinea pigs and tissue culture models utilising explanted ganglia and cell lines, the molecular mechanisms underlying establishment of and reactivation from latency remain poorly understood (for an overview of latency, see Steiner & Kennedy, 1995).

During latency the viral genome is transcriptionally silent with the exception of the latency-associated transcripts, or LATs (for a review, see Rock, 1993). LATs are largely non-polyadenylated transcripts which accumulate in the nuclei of latently infected cells (Deatly *et al.*, 1987; Rock *et al.*, 1987; Spivack & Fraser, 1987; Stevens *et al.*, 1987). The major LAT species is a 2 kb RNA which is synthesised in both lytic

and latent infections and may be a highly stable intron spliced from an 8.3 kb polyadenylated species, mLAT, transcribed from within the repeat regions flanking U_L (Spivack & Fraser, 1987; Wagner *et al.*, 1988a,b; Dobson *et al.*, 1989; Devi-Rao *et al.*, 1991). Smaller LATs of 1.4-1.5 kb are spliced variants of the 2 kb LAT (Wagner *et al.*, 1988b; Spivack *et al.*, 1991).

Two latency-active promoters (LAPs) have been identified, one upstream of mLAT (LAP1, Dobson *et al.*, 1989) and the other extending into mLAT, but 5' to the 2 kb LAT (LAP2, Goins *et al.*, 1994). LAP1, primarily responsible for LAT expression during latency (Chen *et al.*, 1995), consists of sequences associated with RNA polymerase II promoters and neuron-specific transactivators (Batchelor & O'Hare, 1992; Zwaagstra *et al.*, 1990), whilst LAP2 has the properties of a housekeeping gene promoter and is primarily responsible for LAT expression during lytic infection (Chen *et al.*, 1995).

Despite the progress in characterising LATs, their function remains unclear. The presence of two potential ORFs within the LATs have led to suggestions of a product expressed in latently infected cells (Perry & McGeoch, 1988; Wechsler *et al*, 1988), although no LAT-encoded polypeptides have been identified (Wagner *et al*, 1988a;b). Since the LAT transcripts overlap the Vmw110 ORF (IE gene 1) but are transcribed from the opposite strand, it has been suggested that they may cause anti-sense inhibition of Vmw110 synthesis, a viral transactivator with important roles in lytic infection (section 1.3.3a) and also in reactivation from latency (Russell *et al.*, 1987; Harris *et al.*, 1989; Clements & Stow, 1989). Expression of LATs might then play a role allowing establishment and maintenance of latency. Mutants deficient in LAT expression are, however, capable of establishing latent infections in rabbits and mice but show an impaired ability to reactivate *in vivo* and from explanted ganglia (Javier *et al.*, 1988; Steiner *et al.*, 1989; Leib *et al.*, 1989; Ho & Mocarski, 1989; Hill *et al.*, 1990; Bloom *et al.*, 1994). This indicates that the LATs may not be necessary for latency establishment, but may play a role in mediating the frequency of reactivation.

Section 1.3 HSV-1 LYTIC INFECTION

1.3.1 Initiation Of Infection

The initial interaction of HSV-1 with a susceptible cell involves several viral glycoproteins and probably more than one receptor. The pathway can be divided into three processes: viral attachment, membrane fusion and nucleocapsid penetration, and virion disassembly.

Non-specific adsorption of HSV-1 to the cell surface is followed by specific irreversible attachment which may be mediated either by gC or via a gC-independent pathway. This was shown using polarised Madin Darby canine kidney (MDCK) cells, which carry different surface proteins at their apical and basolateral domains. Attachment of HSV-1 to the apical domains required gC, whereas attachment to the basal domain was gC-independent (Sears *et al.*, 1991). The involvement of gC in attachment was demonstrated by the observation that HSV-1 mutants lacking gC adsorb more slowly to cells than *wt* virus (Herold *et al.*, 1991; Langeland *et al.*, 1990), and that antibodies to gC also reduce attachment. It has since been reported that gC mediates binding of virus to heparan sulphate moieties on the surface of erythrocytes (Trybala *et al.*, 1993). Heparan sulphate proteoglycans are known to be important for attachment of HSV-1 to host cells (WuDunn & Spear, 1989; Shieh *et al.*, 1992). The mechanism of gC-independent attachment, and the viral proteins involved, remain elusive, but the involvement of heparan sulphate and gB has been reported (Herold *et al.*, 1994).

Penetration of the HSV-1 nucleocapsid into cells occurs by fusion of the virion envelope with the plasma membrane, and studies utilising neutralising antibodies and mutants incapable of penetration show that this is mediated by the four essential glycoproteins gB, gD and gH/gL (Cai *et al.*, 1988; Highlander *et al.*, 1987; Ligas & Johnson, 1988; Fuller *et al.*, 1989; Forrester *et al.*, 1992). There is as yet no molecular model to describe the fusion event, but entry is known to occur at the plasma membrane and not from within endocytic vesicles (Wittels & Spear, 1991).

Following penetration, the nucleocapsid is transported to the nucleus where it is uncoated and the DNA released into the nucleus via nuclear pores. Studies with an

HSV-1 mutant carrying a *ts* lesion in the UL36 gene identified a block in the release of viral DNA and an accumulation of capsids at the nuclear pores (McNabb & Courtney, 1992). It is unclear whether the tegument protein encoded by UL36 participates directly in the nuclear entry process: it may be that the mutant UL36 protein prevents the normal activity of cellular uncoating proteins.

1.3.2 Effect Of Infection On Host Cell Macromolecular Synthesis

Infection of cells with HSV-1 results in a rapid decrease in host cell macromolecular synthesis arising from the disaggregation of polyribosomes and the degradation of cellular mRNAs (reviewed by Fenwick, 1984; Schek & Bachenheimer, 1985; Kwong & Frenkel, 1987). This "host cell shut-off" activity can be divided into two phases.

The "early shut-off" phase is mediated by components of the virion and hence does not require viral gene expression (Fenwick *et al.*, 1979; Hill *et al.*, 1985). Studies of HSV-1 mutants mapped the virion host cell shut-off (*vhs*) activity to the UL41 gene (Kwong *et al.*, 1988; Fenwick & Everett, 1990). Infection with *vhs*-defective mutants not only results in host mRNAs having significantly longer half-lives than in *wt* infections, but also produces more stable viral mRNAs (Read & Frenkel, 1983; Strom & Frenkel, 1987; Oroskar & Read, 1987; 1989). The *vhs* polypeptide therefore provides a non-specific mRNA degradation function, regulating the abundance of both viral and cellular mRNAs (Kwong & Frenkel, 1987; Oroskar & Read, 1987). It has been proposed that the *vhs* polypeptide may possess RNase activity, or may interact with cellular factors to activate a cellular RNase or to modify the host translational machinery, causing de-stabilisation of mRNAs (Kwong & Frenkel, 1989).

The "delayed shut-off" phase requires the expression of viral genes and involves further reductions in the levels of host protein synthesis (Nishioka & Silverstein, 1978; Fenwick & Clark, 1982). The viral proteins responsible for this have not been formally identified, although the IE gene product Vmw63 is known to contribute to a decrease in host mRNA levels by regulating small nuclear ribonucleoprotein distribution and inhibiting host cell splicing (Phelan *et al.*, 1993; Hardwicke & Sandri-Goldin, 1994;

Hardy & Sandri-Goldin, 1994). Vmw63 also increases the 3' processing and stability of certain viral mRNAs (McLauchlan *et al*, 1992; Brown *et al.*, 1995).

Although synthesis of the majority of host cell polypeptides is reduced during HSV-1 infection, the synthesis of some, including cellular stress and heat-shock proteins, is in fact stimulated (LaThangue *et al.*, 1984; Patel *et al.*, 1986; Kemp *et al.*, 1986).

1.3.3 Regulation Of Gene Expression

The expression of viral genes during HSV-1 lytic infection can be divided into three principle temporal classes, termed immediate-early (IE), early and late (Honess & Roizman, 1974; Clements *et al.*, 1977; Jones & Roizman, 1979). The IE genes can be transcribed in the absence of *de novo* protein synthesis, but prior IE gene expression is required to activate transcription from later classes of genes. Early gene expression takes place prior to the onset of viral DNA replication, and IE and early gene products are in turn essential for the synthesis of viral DNA and the expression of true late proteins (reviewed by Wagner, 1985).

All HSV-1 genes are transcribed by the host RNA polymerase II enzyme (Costanzo *et al.*, 1977), and most contain recognisable TATA-boxes, as well as "CCAATT" box homologies and cellular transcription factor (*e.g.* Sp1) binding sites at positions analogous to those seen with other eukaryotic pol II promoters.

a) immediate-early genes

Upon entry of HSV-1 into the host cell, the tegument protein VP16 (αTIF or Vmw65) migrates to the nucleus, where it plays a critical role in triggering transcription of the five viral immediate early (IE) genes (Post *et al.*, 1981; Campbell *et al.*, 1984; Spector *et al.*, 1991). VP16 does not bind directly to DNA but forms a multicomponent complex with the cellular DNA-binding protein Oct-1 (Gerster & Roeder, 1988; O'Hare & Goding, 1988; O'Hare *et al.*, 1988; Kristie *et al.*, 1989; Stern *et al.*, 1989), and at least one other cellular protein, designated HCF, C1 or CFF (Gerster & Roeder, 1988; Katan *et al.*, 1990; Wilson *et al.*, 1993). This complex assembles on IE-specific

sequence elements related to ATGCTAATGARAT (where R is a purine) occurring upstream of the viral IE genes, thereby bringing the acidic C-terminal activating domain of VP16 (Sadowski *et al.*, 1988; Triezenberg *et al.*, 1988; Greaves & O'Hare, 1989) into close proximity with cellular factors required for initiation of transcription, such as TFIIB, the TATA-binding protein TBP, the TBP-associated factor $TAF_{II}40$, TFIIH and PC4 (Preston *et al.*, 1988; Kristie & Sharp, 1990; Ge & Roeder, 1994; Goodrich *et al.*, 1993; Lin *et al.*, 1991; Stringer *et al.*, 1990; Xiao *et al.*, 1994). The products of the HSV-1 UL46 and UL47 genes have been reported to modulate the activity of VP16 (McKnight *et al.*, 1987; Zhang *et al.*, 1991).

Four of the five IE gene products (Vmw63, Vmw68, Vmw110 and Vmw175) are phosphorylated nucleoproteins which regulate the expression of HSV early and late genes, although only two (Vmw63 and Vmw175) are absolutely essential for growth of HSV in cell culture (Preston, 1979a;b; Dixon & Schaffer, 1980; Watson & Clements, 1980; Post & Roizman, 1981; Sacks *et al.*, 1985; Stow & Stow, 1986). The fifth IE gene product, Vmw12 (ICP47 or IE-5, encoded by US12) is cytoplasmic, unphosphorylated, and dispensable for viral growth in tissue culture (Longnecker & Roizman, 1986; Brown & Harland, 1987), but blocks antigen presentation to $CD8^+$ cytotoxic T lymphocytes, enabling the virus-infected cell to avoid lysis (York *et al.*, 1994). Vmw12 may therefore play a critical role in enabling HSV to persist and cause recurrent infections.

Vmw175 or ICP4 (encoded by IE gene 3) is continuously required for the expression of early and late genes and acts as a repressor of its own expression and that of Vmw110 and the LATs. Vmw175 is a multi-functional protein with domains involved in DNA binding, nuclear localisation, dimerization and transcriptional activation (DeLuca & Schaffer, 1988; Paterson & Everett, 1988a;b; Shepard *et al.*, 1989). In repression, the protein binds specifically to a DNA sequence incorporating the consensus ATCGTC. This binding site is found at its own transcriptional start site and at analogous locations in the Vmw110 and LAT promoters (Faber & Wilcox, 1986; Kristie & Roizman, 1986; Muller, 1987; Michael *et al.*, 1988; Roberts *et al.*, 1988; Michael & Roizman, 1993). In contrast, Vmw175 may not require specific binding sites

to activate transcription, since none has been identified in either early or late promoters (Everett, 1984; O'Hare & Hayward, 1987; Smiley *et al.*, 1992). Vmw175-mediated activation may be due to an ability to influence TATA-dependent assembly of cellular transcription factors via multiple protein-protein contacts (Gu & DeLuca, 1994).

Viruses with lesions in the IE gene 1 coding for Vmw110 (ICP0) are able to grow in tissue culture, albeit poorly at low multiplicity of infection, but are unable to reactivate latent virus, demonstrating that Vmw110 enables the lytic cycle to be initiated efficiently (Stow & Stow, 1986; Sacks & Schaffer, 1987; Russell *et al.*, 1987; Harris *et al.*, 1989; Cai *et al.*, 1993). In transfection experiments Vmw110 can activate gene expression from all three classes of viral promoters and a variety of cellular promoters, both by itself and in a synergistic fashion with Vmw175 (Everett, 1984, 1986a; O'Hare & Hayward, 1985a, b; Gelman & Silverstein, 1985, 1986; Quinlan & Knipe, 1985). However, in the context of the virus, Vmw110 is not sufficient to transactivate early gene expression in the absence of a functional Vmw175 protein (DeLuca *et al.*, 1985).

The essential viral protein Vmw63 (ICP27) is the product of the IE-2 gene (UL54) and is involved primarily in the switch from early to late gene expression (Sacks *et al.*, 1985; Rice & Knipe, 1988). It also augments viral DNA synthesis by stimulating expression of most of the essential DNA replication genes (McCarthy *et al.*, 1989; Uprichard & Knipe, 1996) and plays a role in the shutoff of host protein synthesis (section 1.3.2). Vmw63 exhibits transcriptional regulatory functions in transient expression assays, acting to repress Vmw175 and Vmw110-mediated activation of some early genes and to enhance their activating effects on late genes, depending on the target promoter (Everett, 1986b; Sekulovich *et al.*, 1988; Su & Knipe, 1989; Rice & Knipe, 1990). This may be due to an independent effect of Vmw63 on gene expression (Rice & Knipe, 1988; Samaniego *et al.*, 1995), or to Vmw63 modulating the activities of the Vmw110 and Vmw175 proteins by affecting posttranscriptional processing of Vmw110 (Hardwicke & Sandri-Goldin, 1994; Hardy & Sandri-Goldin, 1994) and posttranslational modification of Vmw175 (Rice & Knipe, 1988; Su & Knipe, 1989). In addition there is evidence that Vmw63 inhibits the nuclear localisation of both

Vmw110 and Vmw175 (Zhu *et al.*, 1994; Zhu & Schaffer, 1995), and that it can regulate the binding of Vmw175 to DNA (Samaniego *et al.*, 1995). Most regulatory effects of Vmw63, however, are thought to occur posttranscriptionally at the level of mRNA processing, in particular by stimulating utilisation of viral late gene poly(A) sites and inhibiting host cell splicing (McLauchlan *et al.*, 1992; Sandri-Goldin & Mendoza, 1992; Smith *et al.*, 1992; Phelan *et al.*, 1993; Hardy & Sandri-Goldin, 1994). It has recently been reported that Vmw63 is an RNA-binding protein which specifically binds to the 3' RNA-processing and poly(A) sequences of unstable intronless transcripts, resulting in mRNA stabilisation (Brown *et al.*, 1995). Repression of viral IE and cellular gene expression by Vmw63 appears to correlate with the presence of introns in these transcripts (Sandri-Goldin & Mendoza, 1992) and may be due to inhibition of splicing and/or nuclear export of intron-containing transcripts (Hardwicke & Sandri-Goldin, 1994; Hardy & Sandri-Goldin, 1994; Phelan *et al.*, 1996).

The functions of Vmw63 in the HSV replicative cycle are likely to be of central importance, since it is the only IE gene which is well conserved throughout the alpha, beta and gamma herpesvirus subfamilies.

Characterisation of HSV-1 mutants with lesions in the IE-4 (US1) gene, coding for Vmw68 (ICP22), has revealed that this protein is essential for viral growth only in certain types of cultured cells, implying that an analogous host function may be provided by other cell types (Post & Roizman, 1981). Hence Vmw68 may increase the range of host cells in which the virus is able to replicate. Since impaired growth correlated with reduced expression of a subset of late genes, a possible role in late gene regulation has been suggested (Sears *et al.*, 1985; Poffenberger *et al.*, 1993). The similar phenotypes of viral mutants with lesions in the US1 gene and in the UL13 gene, the latter encoding a putative protein kinase, suggest that phosphorylation of Vmw68, mediated directly or indirectly by the UL13 protein, is necessary for the expression of normal levels of a subset of late proteins in restrictive cells (Purves *et al*, 1993). A recent report demonstrates that Vmw68 is necessary for virus-induced posttranslational modification of the cellular RNA polymerase II, and that failure of a Vmw68 mutant to

modify RNA polymerase II was accompanied by reductions in viral late gene transcripton (Rice *et al.*, 1995).

b) early genes

Expression of early genes is dependent upon promoter *trans*-activation by the IE proteins (Honess & Roizman, 1974; Zhang & Wagner, 1987; Weinheimer & McKnight, 1987). Early genes show variation in their levels of synthesis and kinetics of expression, but all have transcription rates which peak either before or coincident with maximal rates of HSV-1 DNA synthesis. The differential levels of expression exhibited by genes belonging to the same temporal class may be explained by the diversity between their promoter sequences (Wagner, 1985). Mutational analysis of the promoter regulatory regions of many HSV-1 early genes has failed to detect specific sequences that mediate *trans*-activation by Vmw110 and Vmw175, although these promoters do contain homologies to "TATA" and "CCAATT" boxes and GC-rich elements (Eisenberg *et al.*, 1985; El Kareh *et al.*, 1985; Jones *et al.*, 1985). It has therefore been suggested that *trans*activation of early genes by the IE proteins may be mediated via their interactions with various cellular transcription factors which bind to these promoter elements (Everett, 1984; Eisenberg *et al.*, 1985; Coen *et al.*, 1986).

All seven of the HSV-1 proteins directly involved in viral DNA synthesis are expressed from genes belonging to the early class. A number of other early gene products are also involved in a less direct way in DNA replication.

Gene UL23 encodes a thymidine kinase (TK) whose activity may be essential for infection of non-dividing cells which do not themselves synthesize TTP (Jamieson *et al.*, 1974). The product of early gene UL50 is a deoxyuridine 5' triphosphatase (dUTPase), which catalyses the hydrolysis of dUTP to dUMP and pyrophosphate (Wohlrab & Francke, 1980; Caradonna & Cheng, 1981). This enzyme may provide dUMP for conversion into dTMP or may simply deplete intracellular concentrations of dUTP to prevent incorporation of uridine into replicating DNA.

Originally, the two genes encoding the large and small subunits of the ribonucleotide reductase (RR) enzyme were both classified as early genes, but the

expression of the large subunit under IE conditions and the presence of a TAATGARAT element in the promoter of its gene, UL39, indicate that it should more correctly be considered as an IE gene (Roizman & Batterson, 1985; Wymer *et al.*, 1989). Gene UL40, which codes for the small RR subunit, exhibits typical early gene characteristics. RR plays an important role in DNA precursor metabolism as it catalyses the reduction of ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates (Cohen, 1972; Thelander & Reicard, 1979). Like TK, the RR enzyme appears essential for infection of non-dividing cells - as well as certain cell types cultured above 38.5°C - where host pathways of dNTP synthesis may be inactivated (Goldstein & Weller, 1988a, b).

Enzymes with potential DNA repair functions include uracil-DNA glycosylase (UL2) which removes uracil residues arising either from mis-incorporation of dUTP into DNA or from deamination of cytosine residues within the DNA (Caradonna & Cheng, 1981), and alkaline nuclease (UL12) which exhibits both 5'-3' exonuclease and endonuclease activities (Morrison & Keir, 1968; Strobel-Fidler & Francke, 1980). Studies using an exonuclease null mutant have shown that the UL12 gene product is essential for viral growth but not for viral DNA replication (Moss, 1986; Weller *et al.*, 1990), and there are conflicting reports as to whether the enzyme localises in replication compartments within the nucleus (Randall & Dinwoodie, 1986; Thomas *et al.*, 1992). Proposed roles for this enzyme include a role in the cleavage of DNA concatemers during packaging into virion particles, provision of dNTPs for DNA synthesis and an involvement in resolving branched recombinational intermediates (see section 1.3.4).

c) **late genes**

Experiments with inhibitors of viral DNA synthesis and with DNA-negative HSV-1*ts* mutants have identified a class of genes (late genes) whose expression is dependent upon viral DNA synthesis, in addition to requiring functional IE and early gene products (Honess & Roizman, 1974; Holland *et al.*, 1980; Conley *et al.*, 1981; Pederson *et al.*, 1981).

Late genes also exhibit variation in their expression kinetics and can be sub-divided into two groups: leaky-late and true-late. Leaky-late transcripts are detectable prior to initiation of HSV-1 DNA synthesis, although they are not maximally synthesised until after the onset of viral DNA replication. Transcription of true-late genes is not detectable in the absence of viral DNA replication (Roizman & Batterson, 1985; Wagner, 1985).

Late gene promoters lack the upstream regulatory regions present in IE and early promoters, and no sequence-specific elements responsive to transactivation by viral gene products have been identified (Everett, 1984). Mutational analyses have defined the leaky-late promoters as an 80-120 bp pol II element, the true-late as a truncated 30 bp element, both containing a TATA-box and a mRNA transcriptional start site which, in combination with a *cis*-acting HSV-1 origin of replication, are sufficient for expression (Johnson & Everett, 1986a, b; Homa *et al.*, 1986). Both leaky- and true-late genes, however, have transcriptional regulatory elements present at and 3' of the cap site. These elements may be involved in blocking transcriptional shut-off following viral DNA replication or alternatively may allow newly synthesised DNA molecules to serve as transcription templates for late genes (Wagner, 1994).

It is unclear what role viral DNA replication plays in late gene expression. Johnson & Everett (1986a) followed replication of, and expression from, a plasmid containing ori_S sequences and the HSV-1 US11 late promoter linked to the rabbit ß-globin gene, intransfected cells superinfected with HSV-1. Between 8 and 12 hr post-adsorption, the copy number of replicated plasmids doubled, but ß-globin transcript accumulation increased 10-fold. From 12 hr on, plasmid-borne transcript accumulation increased proportionally with replicated plasmid copy number. Therefore, although an increased template copy number may contribute towards increased late gene expression, this does not fully explain the requirement for a replication origin in *cis*. It may be that structural changes in the DNA, arising during the replication process, are necessary to activate late gene expression (Johnson & Everett, 1986a).

Most of the viral structural proteins, including capsid proteins and glycoproteins, belong to the late class, although whether it is significant that some are leaky-late whilst others are true-late is unknown.

1.3.4 Properties of replicating DNA

Restriction enzyme terminal fragment analysis and pulsed field gel electrophoresis demonstrate that HSV DNA rapidly circularises following entry into the host cell nucleus (Poffenberger & Roizman, 1985; Garber *et al.*, 1993). This process probably occurs by direct ligation of the terminal "a" sequences (Davison & Wilkie, 1983; Poffenberger *et al.*, 1983) and may be facilitated by the complementary 3' single base overhangs present at the L and S termini (Mocarski & Roizman, 1982b). Since this can occur in the absence of *de novo* protein synthesis, it is likely that ligation of the DNA is carried out by a host cell enzyme or virion protein (Poffenberger & Roizman, 1985).

As described in section 1.4, seven of the early viral gene products participate directly in the replication of input HSV-1 genomes. The current model for HSV-1 DNA synthesis proposes that initial origin-dependent template amplification generates circular monomers by a theta form mechanism. Subsequently a phase of rolling circle replication ensues, with a replication fork moving continuously around the circular genome to produce concatemers consisting of head-to-tail repeats of the monomeric genome. The proposed switch from theta form to rolling circle replication for HSV-1 is similar to the events which occur during phage lambda replication (Enquist & Skalka, 1978). There is evidence that the gammaherpesvirus EBV also utilises both mechanisms to replicate: during latency, EBV DNA synthesis from oriP generates circular plasmid molecules, whilst during lytic replication oriLyt-dependent DNA synthesis generates long head-to-tail concatemers indicative of a rolling-circle mechanism (Hammerschmidt & Sugden, 1988).

Support for the above model of HSV-1 DNA synthesis comes from studies of replicative intermediates, which have shown that newly synthesised radio-labelled DNA is of high molecular weight and that the label can be chased into low molecular

weight species (Jacob & Roizman, 1977). In addition, restriction enzyme analysis shows a reduction of terminal fragments in newly replicated DNA, implying that replicative intermediates exist in an endless state, either as circular monomers or linear concatemers (Jacob *et al.*, 1979; Jongeneel & Bachenheimer, 1981). Initial replication via a theta form has been proposed since the rapid amplification (several hundredfold) of viral DNA seen in the first few hours after infection cannot be accounted for by a simple rolling-circle model, which would predict a linear increase in the yield of DNA (Hammerschmidt & Mankertz, 1991). Early replicative intermediates with lower sedimentation rates to those synthesised later may represent circular monomers generated by a theta form mechanism (Ben-Porat & Tokazewski, 1977).

Further evidence in support of a rolling circle replication mechanism comes from findings that concatemers of viral sequences can be generated from monomeric units of class I defective DNA (Vlazny & Frenkel, 1981) and that linear molecules consisting of tandem head-to-tail reiterations of plasmid DNA can be generated in cells transfected with a plasmid containing an HSV-1 origin of replication (ori_S), and superinfected with *wt* HSV-1 (Stow, 1982).

More direct evidence for a rolling circle mechanism in HSV-1 replication has come from *in vitro* systems. Using HSV-1 DNA replication proteins purified from HSV-infected cells Rabkin & Hanlon (1990) reconstituted rolling circle DNA synthesis on a preformed replication fork. A similar *in vitro* study showed that a complex composed of six of the HSV-1 replication proteins, purified from insect cells infected with recombinant baculoviruses, promoted rolling circle replication of circular plasmid templates (Skaliter & Lehman, 1994). This replication was not origin dependent and the origin binding protein, UL9, was not required. Indeed the presence of UL9 and ori_S inhibited DNA synthesis, suggesting that origin-dependent replication may not initially proceed by a rolling circle mechanism. Analysis of *ts* UL9 mutants demonstrates a requirement for functional UL9 in the initial phase of DNA syntheis (5 hpi) whereas abrogation of UL9 function at later times (10 hpi) had little effect on DNA synthesis (Blumel & Matz, 1995). This is consistent with a model in which UL9 acts primarily during the early stages of DNA synthesis as an initiator protein, and the reduced

requirement for UL9 function at late times is consistent with replication occurring by a rolling circle mechanism.

Gel electrophoresis of restriction enzyme-digested HSV-1 DNA demonstrated the presence of linear concatemers 48 hr post-infection, but digestion of DNA 8 hr post-infection indicated that DNA genomes were cross-linked and one cut per genome was not sufficient to release a linear fragment (Severini *et al.*, 1994). Neutral two-dimensional gel electrophoresis has recently provided direct evidence of branched structures in replicating HSV-1 DNA, the majority of which represented Y junctions such as replication forks, with some X junctions (either merging replication forks or recombination intermediates). This suggests that intracellular HSV-1 DNA exists as a network of replicating molecules held together by frequent replication forks and recombination sites (Severini *et al.*, 1996). The more complex structure of replicating DNA at early *versus* late times of infection, taking into account the high molecular weight and reduced termini of replicating DNA, could be explained by one of two models: formation by inter-molecular recombination of a branched network of replicating genomes as in T4 phage replication, or theta form replication coupled with intra-molecular recombination (Severini *et al.*, 1994). During late T4 phage DNA replication, strand invasion provides 3' hydroxyl primers for initiation of further DNA replication, generating new replication forks (Mosig, 1987). Alternatively theta-type amplification of a circular template followed by recombination between a replicated copy of an inverted repeat unit and an unreplicated copy would result in two forks chasing each other, generating concatemeric DNA (Zhang *et al.*, 1994). For either model, resolution of recombination structures is required in order to produce a linear genome for packaging, and during phage T4 replication the network is resolved by a debranching enzyme (endonuclease VII) to allow packaging of a linear genome (Mosig & Eiserling, 1988; Mizuuchi *et al.*, 1988). Recent evidence suggests that the alkaline exonuclease may perform a similar role during HSV infection. Alkaline exonuclease null mutants appear defective for the production of capsids able to mature into the cytoplasm (Shao *et al.*, 1993) These mutants are capable of synthesising *wt* levels of DNA, and undergo genomic inversion and recombination events, but replicative

intermediates of mutant viral DNA appear more nonlinear or branched than those of *wt* virus (Martinez *et al.*, 1996). Since the mutant replicated DNA is cleaved and encapsidated in the nucleus, it appears that the presence of aberrant DNA in capsids may be responsible for their failure to mature into the cytoplasm.

Late phase (48 hpi) replicated concatemeric DNA has been found to contain the two orientations of the U_L region in adjacent genomes (Severini et al, 1994). Genomic inversions have also been detected as early as 3 to 4 hpi (Zhang *et al.*, 1994), demonstrating that recombination occurs at a very high frequency throughout, and is intimately linked with, the replication process. Recombination resulting in segment inversion is not a prerequisite for DNA synthesis however, since viral mutants with noninvertable genomes are viable (Poffenberger & Roizman, 1985; Jenkins & Roizman, 1986) and origin-containing plasmids lacking inverted repeats can be replicated to produce concatemers (Stow, 1992). Initiation of recombination requires the generation of a single- or double-stranded end of DNA, and whilst these could be generated by the cleavage and packaging enzymes or by a cellular endonuclease (Wohlrab *et al.*, 1991), the early inversion events can occur independently of these two mechanisms (Zhang *et al.*, 1994; Sarisky & Weber, 1994; Smiley *et al.*, 1990). It could be that breaks occurring normally at an HSV replication fork may initiate strand invasion, leading to a recombination event (Martinez *et al.*, 1996).

1.3.5 DNA processing and assembly of virions

Following viral DNA replication the concatemeric products consisting of head-to-tail copies of the genome must be cleaved prior to or during packaging into capsids to give mature unit-length linear genomes. Studies using *ts* mutants indicate that the cleavage and packaging events are tightly coupled.

Proposals that the genomic terminal repeat sequences contained signals for the cleavage and encapsidation of DNA were derived from studies of defective genomes (Vlazny *et al.*, 1982) and origin-containing plasmids. It was demonstrated that a single copy of the "a" sequence was sufficient to direct cleavage and packaging, and was processed to generate two new termini, each bearing at least one copy of the "a"

sequence (Stow *et al.*, 1983; Varmuza & Smiley, 1985; Deiss & Frenkel, 1986). Two cleavage signals were identified within the "a" sequence (**Figure 3**), pac-1 in U_b and pac-2 in U_c (Varmuza & Smiley, 1985; Deiss *et al.*, 1986). These were predicted to drive cleavage events within the adjacent DR1 elements, to generate the S and L termini respectively. Although various mechanisms for cleavage and packaging have been proposed (Varmuza & Smiley, 1985; Deiss *et al.*, 1986) details of the process are still not well understood and a "packaging complex" has not been isolated.

Partial restriction enzyme digestion of HSV-1 DNA 48 hr post-infection produces a ladder of greater than unit length fragments generated by cleavage of concatemers, and two additional fragments shorter than unit length, corresponding to the left-end fragments of the linear genome in the two possible orientations of U_L (Severini *et al.*, 1994). This data is consistent with a model of rolling circle replication, where packaging occurs, as proposed by Varmuza & Smiley (1985) and Deiss & Frenkel (1986), from the L terminus of the genome, leaving the other end attached to the mass of replicating DNA.

The identities of the proteins which contribute directly to cleavage and packaging are unknown. Studies of HSV-1 null and *ts* mutants have demonstrated that all seven proteins required for capsid assembly (section 1.1.3c) are also essential for both cleavage and encapsidation of viral DNA (Desai *et al.*, 1993; Preston *et al.*, 1983; Sherman & Bachenheimer, 1988; Rixon *et al.*, 1988) illustrating the tight coupling between the cleavage of concatemeric DNA into unit-length genomes and the packaging of these genomes within capsids.

In addition, *ts* and null mutants with lesions mapping within the genes UL6, UL25, UL28, UL32 and UL33 synthesise viral DNA at the NPT but this DNA remains concatemeric and is not packaged, resulting in the accumulation of only type B capsids (Sherman & Bachenheimer, 1987; 1988; Addison *et al.*, 1984; 1990; Al-Kobaisi *et al.*, 1991; Tengelsen *et al.*, 1993; Patel *et al.*, 1996). Hence any of these genes could encode proteins directly involved in a "packaging complex".

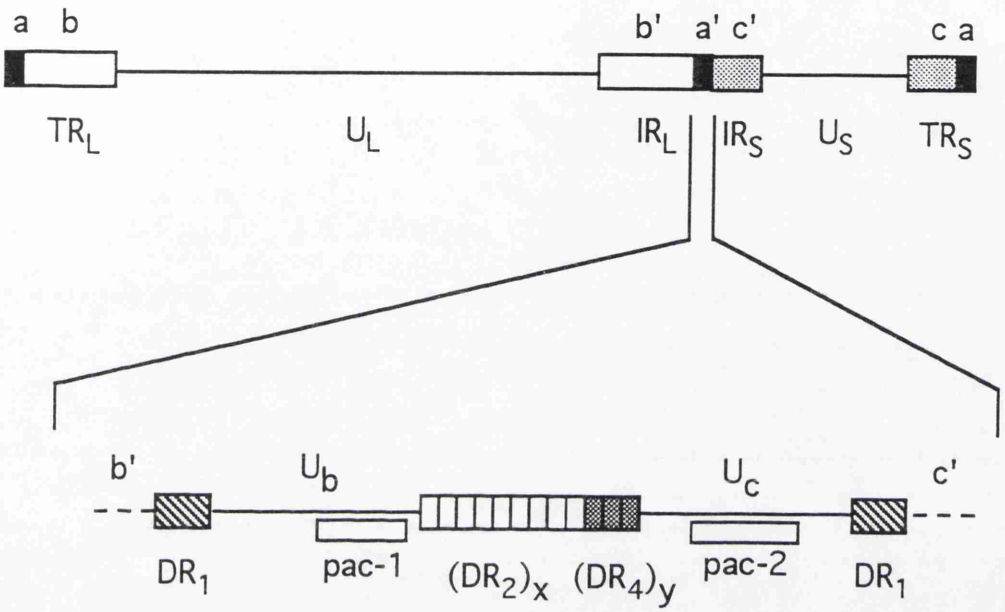
Davison (1992) reported that the UL15 protein may be related to T4 phage protein 17, a terminase involved in cleavage and packaging of phage DNA (Powell *et*

Figure 3. Structure of the HSV-1 "a" sequence.

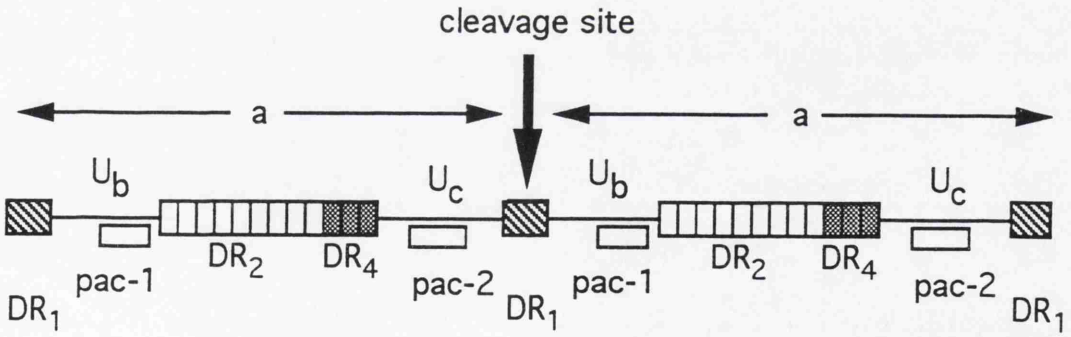
(a) The "a" sequence from the L-S junction (a') is shown. A direct repeat of 17-21 bp, DR_1, (striped box) is present at each end of the sequence, proximal to the IR_L and IR_S repeat sequences (b' and c'). 1-22 (x) copies of a 12 bp direct repeat, DR_2, (open box) and 0-3 (y) copies of a 37 bp direct repeat, DR_4 (shaded box) are located internally. Two unique regions U_b and U_c (solid lines) lie between DR_1 and DR_2/DR_4.

(b) In a double "a" (baac) junction, such as arises in viral concatemeric DNA, the tandemly reiterated "a" sequences share the intervening DR_1 element. Double "a" junctions are proposed to form the substrate for cleavage, which occurs within the central DR_1 element.

(a)


a b
b' a' c'
c a
TR$_L$
U$_L$
IR$_L$
IR$_S$
U$_S$
TR$_S$
b'
U$_b$
U$_c$
c'
DR$_1$
pac-1
(DR$_2$)$_x$
(DR$_4$)$_y$
pac-2
DR$_1$

(b)


cleavage site
a
a
U$_b$
U$_c$
U$_b$
U$_c$
DR$_1$
pac-1
DR$_2$
DR$_4$
pac-2
DR$_1$
pac-1
DR$_2$
DR$_4$
pac-2
DR$_1$

al., 1990; Rao & Black, 1988), and that the UL15 ORF arrangement and coding sequence is conserved among herpesviruses, including channel catfish herpesvirus. Subsequently a *ts* HSV-1 mutant with a lesion in the second exon of the UL15 gene was shown at the nonpermissive temperature to synthesise viral DNA and capsids, but failed to package the DNA (Poon & Roizman, 1993). Further studies demonstrated that the UL15 protein was located within a distinct region of the cell nucleus at 12 hpi, and that in cells infected with the *ts* UL15 mutant and maintained at the nonpermissive temperature, the viral DNA accumulating at 16 hpi was in an endless, concatemeric form (Baines *et al.*, 1994). The conclusion is that the UL15 protein is required at least for cleavage of viral DNA into mature, genomic length molecules, and possibly for packaging as well.

The final events occurring during the maturation and release of virions are not yet fully understood (for a review, see Rixon, 1993). Capsids are enveloped by budding through the inner membrane of the nucleus although this membrane may subsequently be lost and replaced by one acquired by budding into the Golgi. The viral envelope glycoproteins are matured during passage through the endoplasmic reticulum prior to release of virions from the infected cells. The site of tegumentation is not completely clear but is thought more likely to be cytoplasmic (following loss of the nuclear-derived membrane) than nuclear. The mature virion leaves the cell by exocytosis from Golgi-derived vesicles.

Section 1.4 MOLECULAR ASPECTS OF HSV-1 DNA REPLICATION

1.4.1 Cis-acting elements required for DNA replication

The presence of specific DNA replication origins within the viral genome was first deduced from electron microscopic studies which revealed replication bubbles within HSV-1 DNA (Friedman *et al.*, 1977). The approximate locations of these origins were derived primarily from studies of defective viral genomes which were generated during serial passage of HSV stocks at high m.o.i (Frenkel *et al.*, 1975).

Defective viral genomes, consisting of head-to-tail reiterations of small segments of viral DNA, were classified into two types according to the origin of the reiterated sequences: class I defective genomes consisting of repeat units from either end of the S component (Frenkel *et al.*, 1976; Graham *et al.*, 1978; Locker & Frenkel, 1979; Kaerner *et al.*, 1979) and class II genomes with repeat units containing sequences predominantly from the middle of U_L (Schroder *et al.*, 1975; Kaerner *et al.*, 1979; Locker *et al.*, 1982; Spaete & Frenkel, 1982).

Since these defective genomes were successfully replicated in the presence of helper virus, it was deduced that the reiterated units each contained a replication origin (Vlazny & Frenkel, 1981). The separate origins within the S and L segments were subsequently referred to as ori_S and ori_L, respectively.

a) **ori_S**

Direct evidence for the presence of an origin within class I defective DNA came from Vlazny and Frenkel (1981) who generated class I DNA concatemers in cells cotransfected with monomeric units of class I DNA and *wt* HSV-1 helper DNA. Stow (1982) developed an assay to further define the position of ori_S. In this assay, transfection of cells with plasmids containing fragments of *wt* HSV-1 DNA and superinfection with *wt* HSV-1 as helper resulted in amplification of those plasmids containing a functional origin of replication. This approach enabled the mapping of ori_S to a 995 bp fragment consisting of sequences entirely from the IR_S/TR_S repeats which flank U_S (Stow, 1982). A similar location was deduced independently by Mocarski & Roizman (1982a). Two identical copies of ori_S are therefore present within the HSV-1 genome.

Further deletion mapping of the 995 bp fragment identified a minimal 90 bp sequence containing all the *cis*-acting signals necessary for origin-dependent replication (Stow & McMonagle, 1983). Both copies of ori_S lie between the 5' ends of two divergently transcribed IE genes: IE-3 and either US1 or US12 in U_S (**Figure 2**) (Stow & McMonagle, 1983; Murchie & McGeoch, 1982). Although it has been proposed that the intervening region also contains a transcribed ORF, the status and function of this

transcript remain unclear (Hubenthal Voss *et al.*, 1987; Hubenthal Voss & Roizman, 1988).

The 90 bp ori_S sequence contains an almost perfect 45 bp palindrome, at the centre of which is an 18 bp stretch of A and T residues (**Figure 4**). An additional 25 bp to the left of the palindrome is also essential for DNA replication (Lockshon & Galloway, 1988). Disruption of the palindromic sequence in HSV-1 or in the highly homologous HSV-2 ori_S results in a loss of origin activity (Stow, 1985; Lockshon & Galloway, 1988).

The HSV-1 ori_S contains a high affinity binding site for the HSV-1 UL9 protein on each arm of the palindrome (**Figure 4**). Site I which has an approximately five fold higher affinity has the sequence CGTTCGCACT and site II has the sequence TGCTCGCACT, giving a consensus sequence YGYTCGCACT. This 10 bp motif is conserved in the origin sequences of HSV-1, HSV-2, VZV and EHV-1 (Stow & Davison, 1986; Lockshon & Galloway, 1988; Baumann *et al.*, 1989). Deletion of site I abolishes origin-dependent replication (Deb & Deb, 1989; Weir & Stow, 1990) whilst deletion of site II causes a 10- to 20-fold reduction in origin activity, although both mutated origins retain ability to bind UL9 at their unaltered site (Weir & Stow, 1990). This indicates that the presence of both UL9 binding sites is necessary for efficient origin activity (Weir & Stow, 1990). A third related sequence (site III: CGTTCTCACT) which lies adjacent to site I (**Figure 4**), has a much lower affinity for UL9 and its role in replication is not clear. Although binding of UL9 to site III could not be demonstrated (Weir & Stow, 1990), this motif may play an auxiliary role in DNA replication since deletion of site III causes a two- to three-fold decrease in origin activity (Lockshon & Galloway, 1988; Elias *et al.*, 1990; Weir & Stow, 1990; Hernandez *et al.*, 1991) and DNase I footprints over site III are produced by binding of UL9 to ori_S (Elias *et al.*, 1990).

Replacement of the central A+T rich region of HSV ori_S with GC-rich sequences also abolishes origin function (Stow, 1985; Lockshon & Galloway, 1988) whereas insertion of additional copies of the -AT- dinucleotide into the centre of the HSV-2 ori_S is only tolerated in terms of ability to support *in vivo* DNA replication if the

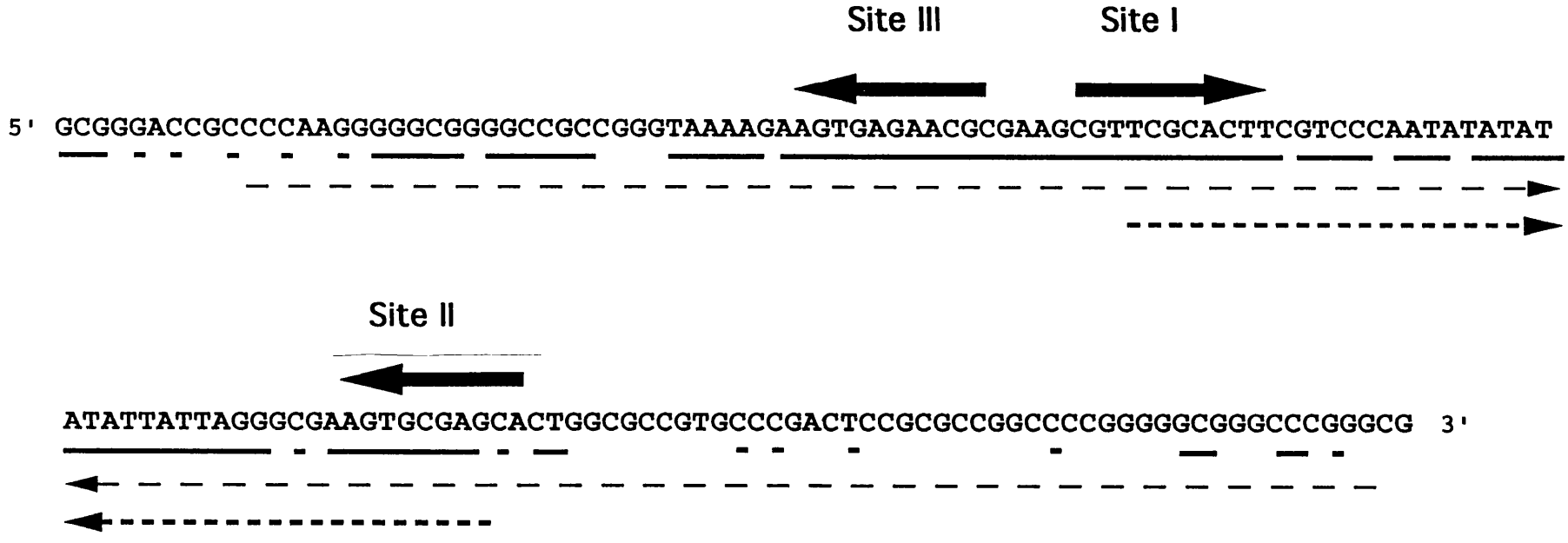


Figure 4. HSV-1 Ori_L and Ori_S sequences.

The sequence of ori_S is given. The solid bar below the sequence indicates bases which are conserved between ori_S and ori_L. The limits of the 144 bp palindrome of ori_L are indicated by the dashed arrows, while the dotted arrows show the limits of the 45 bp ori_S palindrome.The location and orientation of the10 bp motif present within Sites I, II and III, to which the UL9 origin-binding protein binds, are indicated by solid arrows.

number of dinucleotides inserted corresponds to one full helical turn or a multiple thereof (Lockshon & Galloway 1988). Even so, increasing the length of the AT-rich region by one full helical turn (*ie.* maintaining the helical phasing between binding sites I and II) does not result in full *wt* activity, indicating that the distance between the two binding sites is also crucial.

The GC-rich sequences on either side of this core 90 bp region enhance origin function in plasmid amplification assays (Stow & McMonagle, 1983; Wong & Schaffer, 1991). Various viral and cellular transcription factors are known to bind to the transcriptional regulatory elements surrounding the core origin, collectively promoting transcription from both the IE-3 and US1/US12 promoters, although it is not clear whether this is responsible for the enhancement of origin function (Preston *et al.*, 1984, 1988; Bzik & Preston, 1986).

HSV-1 ori_S thus resembles several other eukaryotic viral DNA replication origins in consisting of the core origin component essential for initiation of DNA replication, with an enhancer or promoter element acting as an auxiliary component (DePamphilis, 1988). It has been suggested that the assembly of transcription initiation complexes adjacent to the origin enhances origin function either by facilitating melting of the DNA duplex at the origin, or as a result of a direct interaction with replication proteins (Wong & Schaffer, 1991).

A more direct role for cellular proteins in the initiation of DNA replication at HSV-1 ori_S has been suggested by the demonstration of a specific complex formed between one or more cellular proteins, designated origin factor I (OF-I), and sites I, II and III of ori_S (Dabrowski *et al.*, 1994). The OF-1 binding site partially overlaps the UL9 binding site. Competition and binding analyses suggest that OF-I is a member of the CCAAT family of DNA-binding proteins, and muations within the OF-1 binding site impair replication of an ori_S-containing plasmid. It is possible that an interaction between UL9 and OF-I at ori_S is required during initiation of origin-dependent DNA synthesis (Dabrowski *et al.*, 1994).

The ori_S of VZV shares some homology with, and is in a position equivalent to, that of HSV-1. It contains a palindrome of similar length, although the central AT-rich

tract is longer in VZV. Two copies of a sequence identical to the 10 bp HSV-1 UL9 binding site and a third copy with 9 out of 10 bases matching are all located on one side of the AT-rich region in VZV (Stow *et al.*, 1990). Plasmids containing the VZV origin can be amplified by the HSV-1 *trans*-acting replication functions, suggesting that HSV-1 and VZV DNA replication are initiated by similar mechanisms (Stow & Davison, 1986), and the VZV origin-binding protein can substitute for HSV UL9 protein in a transient assay for origin-dependent DNA replication in insect cells (Webster *et al.*, 1995).

b) ori_L

Characterisation of the origin within class II defective genomes was hampered by the tendency for deletions to occur within these sequences. Direct evidence for the presence of an origin within class II defective DNA was provided by the demonstration that an amplicon, consisting of a class II repeat unit ligated to plasmid DNA, could be amplified in transfected cells upon superinfection with helper virus (Spaete & Frenkel, 1982). The deletion prone sequences were repaired by recombination with the helper virus.

Likewise it proved problematic to clone fragments spanning this region in *E. coli* without deletions occurring (Spaete & Frenkel, 1982; Gray & Kaerner, 1984; Weller *et al.*, 1985). The sequences were successfully cloned in an undeleted form using a yeast cloning vector, and a 425 bp fragment spanning the deletion-prone region was shown to be capable of origin function in a plasmid amplification assay when HSV-1 helper functions were supplied in *trans* (Weller *et al*, 1985). Analysis of the cloned sequence revealed the presence of a perfect 144 bp palindrome with striking homology to the palindrome and left hand flanking region of ori_S (**Figure 4**; Gray & Kaerner, 1984; Quinn & McGeoch, 1985; Weller *et al.*, 1985). The presence of such a long palindromic sequence probably accounts for the instability of ori_L in bacterial plasmids (Collins, 1981).

The HSV-1 ori_L lies within an untranscribed region of the genome between two divergently transcribed genes, UL29 and UL30, specifying the major DNA binding

protein and the DNA polymerase, respectively (**Figure 2**; Quinn & McGeoch, 1985). HSV-2 ori_L has also been cloned from a position homologous to its HSV-1 counterpart. Sequence analysis revealed it to be a 136 bp near-perfect palindrome, with 89% identity to HSV-1 ori_L (Lockshon & Galloway, 1986). The EHV-1 ori_L, however, is not located in an equivalent position to its HSV-1 counterpart, and VZV lacks an ori_L altogether (Telford *et al.*, 1992; Davison & Scott, 1986; Stow & Davison, 1986).

The significance of the presence of three origins of replication within the HSV-1 genome is unclear. The functional equivalence of ori_S and ori_L, and the requirement for only one origin of replication per genome (at least in tissue culture), have been demonstrated using deletion mutants. HSV-1 mutants lacking one copy of ori_S replicate normally (Longnecker & Roizman, 1986). The viability of mutants carrying deletions in ori_L of up to 150 bp indicates that ori_L is nonessential for viral DNA replication (Polvino-Bodnar *et al.*, 1987). Moreover, HSV-1 recombinants lacking both copies of ori_S are viable, indicating that ori_S is dispensable and that a single copy of either origin may suffice for the replication of HSV-1 DNA (Igarashi *et al.*, 1993). The functional equivalence of ori_S and ori_L has also been demonstrated in transient replication assays by cotransfection of two plasmids, one containing ori_S and the other ori_L (Lockshon & Galloway, 1988).

Whether ori_S and ori_L are both required for, and functionally equivalent in, HSV-1 infection *in vivo* is not known. The HSV-1 mutant lacking both copies of ori_S may exhibit a delayed transition from the early phase of replication to a late phase (Igarashi *et al.*, 1993), perhaps suggesting some degree of differential usage at different stages of viral replication. Based on the perfect symmetry of the ori_L but not the ori_S palindrome, Quinn and McGeoch (1985) suggested that initiation of replication might be bidirectional from ori_L, but asymmetric from ori_S. Since analysis of replicative intermediates has led to the proposition that initial HSV DNA amplification may occur by a mechanism similar to yeast 2μ plasmid-like DNA replication (Zhang *et al.*, 1994; section 1.3.4), and bidirectional initiation of replication is a prerequisite for 2μ circle amplification, it is possible that this type of replication initiates from HSV ori_L whereas

unidirectional replication could be directed by HSV ori_S. Alternatively, the presence of three origins may simply provide the virus with a selective advantage *in vivo*, reducing the time taken to replicate its genome.

1.4.2 Transacting functions essential for DNA synthesis

The initial identification of *trans*-acting functions essential for HSV-1 DNA synthesis was through the characterisation of temperature sensitive (*ts*) mutants defective in viral DNA synthesis. These mutants were found to belong to about 10 different complementation groups and were known to include mutations in the DNA polymerase gene and the ssDNA-binding protein gene (Parris *et al.*, 1978; Chu *et al.*, 1979; Chartrand *et al.*, 1980; Conley *et al.*, 1981; Purifoy & Powell, 1981; Weller *et al.*, 1983; Matz *et al.*, 1983; Honess *et al.*, 1984).

A separate approach, using a transient transfection assay, involved testing the ability of cloned HSV-1 DNA restriction fragments to supply the functions required to replicate a cotransfected HSV-1 origin-containing plasmid (Challberg, 1986). Five plasmids, containing fragments covering most of the HSV-1 genome, were initially shown to be capable of supporting amplification of the origin-containing plasmid. Systematic sub-cloning of these fragments, together with knowledge of the DNA sequence of the HSV-1 genome, led to the identification of seven HSV-1 genes which, when cotransfected with viral immediate-early genes to activate their expression, were both necessary and sufficient for origin-dependent DNA replication (Wu *et al.*, 1988; McGeoch *et al.*, 1988b). The seven genes are UL5, UL8, UL9, UL29, UL30, UL42 and UL52, and their locations within the HSV-1 genome are shown in **Figure 5.**

That the three immediate-early genes IE-1, IE-2 and IE-3 (encoding Vmw110, Vmw63 and Vmw175, respectively) were not directly required for origin-dependent DNA synthesis was confirmed by linking each of the seven genes to the HCMV major immediate-early promoter. Cotransfection of these constructs with an origin-containing plasmid resulted in constitutive expression of the seven gene products and efficient origin-dependent plasmid amplification (Heilbronn and zur Hausen, 1989).

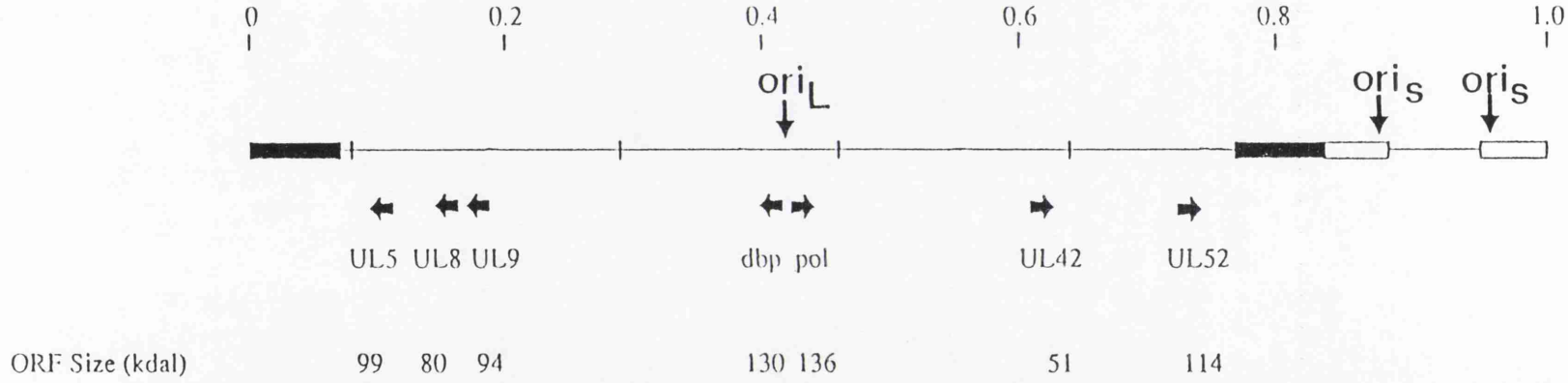


Figure 5. Location on the HSV-1 genome of genes encoding DNA replication proteins.

The HSV-1 genome is shown schematically with the inverted repeats as closed and open boxes flanking, respectively, the long and short unique regions (thin lines). The position and orientation of the ORFs encoding the seven HSV-1 proteins essential for viral origin-dependent DNA replication are shown by arrows, with the predicted molecular size (in kilodaltons) of each protein indicated below. Thepositions of the origins of replication, ori_L and ori_s, are also marked. Adapted from Olivo & Challberg, 1989.

Fine mapping of the lesions in HSV-1 *ts* mutants belonging to the DNA negative complementation groups indicated that these mutations were located within the same seven genes identified by the transient transfection assay, with the remaining complementation groups representing mutations within IE genes or intragenic complementation at the polymerase locus (Wu *et al.*, 1988; McGeoch *et al.*, 1988b; Zhu & Weller, 1988; Carmichael & Weller, 1989; Carmichael *et al.*, 1988; Weller *et al.*, 1983; Purifoy & Powell, 1981; Marchetti *et al.*, 1988; Goldstein & Weller, 1988c). This correlation between the two different approaches suggests that all of the HSV-1 genes essential for viral DNA replication in tissue culture have now been identified. It remains possible, however, that other viral gene products may be necessary for the replication of viral genomes *in vivo*.

Analysis of the biochemical activities of the replication gene products (sections 1.4.2a-d below) reveals that neither a topoisomerase nor DNA ligase activity is present. It therefore seems likely that one or more host-encoded proteins also play an essential role in viral DNA synthesis. Stow (1992) reported that insect cells infected with recombinant baculoviruses expressing the seven HSV-1 replication proteins supported the replication of a cotransfected plasmid containing an HSV-1 origin, indicating that any host function essential in mammalian cells for viral origin-dependent DNA replication must also be present in the infected insect cells. Skaliter and Lehman (1994) found that extracts prepared from similarly infected insect cells were capable of promoting rolling circle DNA replication of circular plasmid templates *in vitro*, and required neither the UL9 protein nor an HSV-1 origin. Although the six HSV-1 proteins that promoted rolling circle DNA replication could be isolated as a complex from insect cells by size-exclusion and ion-exchange chromatography, a mixture of the purified baculovirus-expressed UL30/UL42, UL29 and UL5/UL8/UL52 proteins failed to promote rolling circle DNA replication, unless an extract of mock-infected insect cells was added (Skaliter & Lehman, 1994). This also indicates that the complex capable of promoting rolling circle DNA replication may contain one or more essential host factors. Moreover recent results demonstrate that a drug which specifically inactivates cellular topoisomerase II, when present during the first 4 hpi, inhibits HSV-1 DNA

synthesis, and similarly inhibits the transient replication of an ori_S-containing plasmid (Hammarsten *et al.*, 1996). This suggests that topoisomerase II does play a vital role in HSV-1 DNA replication, possibly being involved in the decatenation of intertwined circular daughter molecules during the initial phase of replication (Hammarsten *et al.*, 1996).

The complete sequencing of the human herpesviruses VZV (Davison & Scott, 1986), HCMV (Chee *et al.*, 1990), HHV-6 (Gompels *et al.*, 1995) and EBV (Baer *et al.*, 1984) and the equine herpesvirus EHV-1 (Telford *et al.*, 1992) has led to the identification of homologous genes and positional equivalents to the HSV-1 DNA replication genes (**Table 1**). In addition, random DNA sequencing of the avian herpesviruses MDV and HVT has revealed homologues for several of the HSV-1 DNA replication proteins (Buckmaster *et al.*, 1988).

Although homologues of the six HSV-1 genes UL5, UL8, UL29, UL30, UL42 and UL52 have been found in alpha-, beta- and gamma-herpesviruses, UL9 homologues are only found amongst the alphaherpesviruses (*e.g.* HSV-1, HSV-2, VZV and EHV-1) and HHV-6 (a betaherpesvirus). The origin-binding proteins of HSV-1, VZV and EHV-1 have strikingly similar arrangements of helicase motifs and contain conserved sequences in the DNA-binding domain (Martin & Deb, 1994). The ability of these three proteins to bind sequence-specifically to their own viral origins as well as to each other's, recognising the same consensus sequence (Stow & Davison, 1986; Martin & Deb, 1994; Webster *et al.*, 1995), suggests that alphaherpesviruses share a common mechanism of initiation of DNA replication. Although HHV-6 is a betaherpesvirus that shares genetic colinearity with HCMV over much of its genome (Lawrence *et al.*, 1990; Neipel *et al.*, 1991), the origin of lytic replication in HHV-6 includes two 195 bp AT-rich imperfect direct repeats and an adjacent GC-rich motif (Dewhurst *et al.*, 1993, 1994; Inoue *et al*, 1994) which resembles alphaherpesviral origins much more closely than the large complex HCMV oriLyt structure (Anders *et al.*, 1992). In addition, an HHV-6 homologue of UL9, containing a well-conserved helicase domain but a divergent DNA-binding domain, was shown to bind in a sequence-specific manner to two sites within each AT-rich repeat (Inoue *et al.*, 1994). Despite sequence similarities

Table 1. HSV-1 genes required for DNA replication, and their herpesvirus homologues

(a) The seven HSV-1 genes whose products are required for HSV-1 origin-dependent DNA synthesis (Wu *et al.*, 1988; McGeoch *et al.*, 1988b).

(b) From McGeoch *et al.*, 1988a and 1988b.

(c) Refer to text (section 1.4.2 a-d).

(d) From Telford *et al.* (1992).

(e) From McGeoch *et al.* (1988b).

(f) From Chee *et al.* (1990).

(g) HHV-7 ORFs are named after, and are 40-75% identical to, their HHV-6 homologues (Nicholas, 1996; Gompels *et al.*, 1995).

(h) From McGeoch *et al.* (1988b) and Fixman *et al.* (1992).

(i) % amino acid identity between the gene product and the corresponding HSV-1 gene product.

(j) U74 shares sequence similarity with HCMV UL102 (Gompels *et al.*, 1995) which in turn contains a 55 amino acid region with sequence similarity to HSV-1 UL8 (Fixman *et al.*, 1992).

(k) BBLF2 and BBLF3 are spliced into a single transcript, specifying a protein which shares sequence similarity with HSV-1 UL8 over 55 amino acids (Fixman *et al.*, 1992).

(l) Positional equivalent. Functions in transcriptional transactivation but may also function in replication (Gompels *et al.*, 1995).

(m) Positional equivalent and functional homologue of HSV-1 UL42, but shares no sequence similarity (Fixman *et al.*, 1992). Also functions as a transcriptional activator (Zhang *et al.*, 1996)

α herpesviruses					ß herpesviruses		γ herpesvirus
HSV-1			Homologous EHV-1 gene (d)	Homologous VZV gene (e)	Homologous HCMV gene (f)	Homologous HHV-6/HHV-7 gene (g)	Homologous EBV gene (h)
Gene (a)	Predicted protein M_r (b)	Function of protein (c)					
UL5	98,710	helicase-primase complex (helicase)	57	55 (56.7%) (i)	UL105	U77	BBLF4 (34.0%)
UL8	79,921	helicase-primase complex	54	52 (28.3%)	UL102 (j)	U74 (j)	BBLF2/3 (k)
UL9	94,246	origin-binding protein	53	51 (44.8%)	none	U73	none
UL29	128,342	ssDNA binding protein	31	29 (50.2%)	UL57	U41	BALF2 (25.4%)
UL30	136,413	DNA polymerase (catalytic subunit)	30	28 (52.5%)	UL54	U38	BALF5 (33.3%)
UL42	51,156	DNA polymerase (accessory subunit)	18	16 (21.6%)	UL44	U27 (l)	BMRF1 (m)
UL52	114,416	helicase-primase complex (primase)	7	6 (37.6%)	UL70	U43	BSLF1 (23.7%)

in the core origins of HHV-6 and HSV-1, there are significant differences in the consensus origin binding sites (Inoue & Pellet, 1995) and their origin binding proteins and origins are not interchangeable (Inoue *et al.*, 1994), indicating a degree of divergence between alphaherpesviruses and HHV-6.

A transient cotransfection assay for HCMV oriLyt-dependent replication identified six essential HCMV genes encoding products corresponding to each of the essential DNA synthesis proteins of HSV-1 except UL9 (**Table 1**; Pari & Anders, 1993). An additional five loci were required to complement HCMV DNA replication, at least four of which cooperatively promoted expression of the replication genes (Iskenderian *et al.*, 1996). The contribution of the fifth locus, UL84, to *trans*activation of the replication genes is less clear, and although this protein shows no similarity to UL9, it remains possible that UL84 functions in an analogous role in HCMV lytic DNA replication (Iskenderian *et al.*, 1996). Similar cotransfection experiments to identify *trans*acting requirements for replication of EBV oriLyt have demonstrated an absence of a dedicated virally encoded origin-binding protein in lytic replication (Fixman *et al.*, 1995). Six proteins essential for DNA replication were identified, corresponding to HSV-1 proteins UL5, UL8, UL29, UL30, UL42 and UL52 (**Table 1**; Fixman *et al.*, 1992). There was also an absolute requirement for a seventh protein, Zta, a transcriptional transactivator which binds to sites within oriLyt and elsewhere in the EBV genome, and which can activate EBV lytic replication upon introduction into latently infected B cells in culture (Fixman *et al.*, 1995; Countryman & Miller, 1985). The six HSV replication proteins (minus UL9) can replace the six EBV homologues and support EBV oriLyt-dependent DNA synthesis in the presence of Zta (Fixman *et al.*, 1995), demonstrating that these proteins are functionally equivalent in the two viruses and that EBV does not require a UL9 homologue. Two additional EBV transactivators, Rta and Mta, play less direct roles in DNA replication, their presence contributing to efficient replication. The minimally defined EBV oriLyt includes two transcriptional elements containing Zta and Rta binding sites - the promoter and leader for the BHLF1 gene and an enhancer element - which flank two short AT-rich palindromes (Hammerschmidt & Sugden, 1988). The HCMV oriLyt also contains

multiple transcription factor binding sites, more reminiscent of a cellular replication origin (Anders *et al.*, 1992; Anders & Punturieri, 1991). The variability in lytic origin structure and lack of origin-binding protein conservation between the herpesvirus subfamilies suggest that the precise mechanism of initiating DNA replication may not be as crucial as the regulation of the initiation event itself.

Unique to gamma-1 herpesviruses is the EBNA-1 protein, which binds to multiple sites within the origin of latent replication, oriP (Yates *et al.*, 1984; Rawlins *et al.*, 1985; Hearing *et al.*, 1992). EBNA-1 is the only virally encoded protein required for replication of episomal EBV genomes, which occurs once per cell cycle in latently infected B cells (Yates *et al.*, 1985). There is no requirement for a latency-specific origin in neurotrophic alphaherpesviruses since neurons do not undergo cell division.

The seven HSV-1 DNA replication gene products comprise four functionally separate proteins or protein complexes: a heterodimeric DNA polymerase (encoded by genes UL30 and UL42), a monomeric ssDNA-binding protein (encoded by UL29), a homodimeric origin-binding protein helicase (encoded by UL9), and a heterotrimeric helicase-primase complex (encoded by genes UL5, UL8 and UL52).

a) DNA polymerase holoenzyme (UL30 and UL42)

The induction of a novel DNA dependent DNA polymerase activity in HSV-1 infected cells was first described over 30 years ago (Keir & Gold, 1963). Genetic analysis of thermolabile and drug resistant mutants demonstrated that the novel polymerase activity was virally encoded and essential for viral DNA synthesis (Purifoy *et al.*, 1977; Hay & Subak-Sharpe, 1976; Purifoy & Powell, 1981). The polymerase mutations were subsequently mapped to the region of the genome containing the UL30 ORF (Knipe *et al.*, 1979; Chartrand *et al.*, 1979; 1980; Coen *et al.*, 1984). Sequence analysis of this region predicted the UL30 gene to encode a polypeptide of 1235 residues, approximately 136 kDa (Gibbs *et al.*, 1985; Quinn & McGeoch, 1985). Measurements of the DNA polymerase activity expressed from the UL30 ORF by *in vitro* transcription and translation (Dorsky & Crumpacker, 1988), in yeast cells (Haffey

et al., 1988), and by recombinant baculoviruses (Marcy *et al.*, 1990) demonstrated that the UL30 protein is sufficient for catalysis.

In addition to the DNA polymerase activity, the UL30 protein also exhibits a proof-reading 3'-5' exonuclease action (Knopf, 1979; Marcy *et al.*, 1990) as well as a 5'-3' exonuclease activity capable of functioning as an RNase H (Crute & Lehman, 1989; Marcy *et al.*, 1990). The latter is likely to function in the removal of RNA primers.

Mutations conferring altered sensitivity to a variety of anti-viral drugs such as acyclovir, aphidicolin, phosphonoacetic acid and phosphonoformic acid, all of which are dNTP or pyrophosphate analogues, were mapped to four highly conserved regions within the C-terminal half of the DNA polymerase protein, spanning residues 690 to 1100. These regions, proposed to constitute a nucleotide binding site for polymerisation, show marked homology between the herpesvirus DNA polymerases and with the DNA polymerases of vaccinia virus, adenovirus 2 and bacteriophage ϕ29 (Gibbs *et al.*, 1985, 1988; Larder *et al.*, 1987; Tsurumi *et al.*, 1987; Wong *et al.*, 1988). The UL30 protein has been classified as a member of the eukaryotic DNA polymerase α family (Miller *et al.*, 1988). Others have suggested that it is more closely related in terms of sequence, structure and catalytic properties to DNA polymerase δ (Gottlieb *et al.*, 1990).

The N-terminal half of UL30 can be subdivided into the 5'-3' exonuclease or RNase H domain (residues 65 to 390) and the 3'-5' exonuclease domain spanning residues 390 to 660 (Haffey *et al.*, 1990). Residues 171 to 214 exhibit modest homology to the 5'-3' exonuclease domain of *E. coli* pol I (Becker, 1988). Sequence alignments and site-directed mutagenesis indicate the 3'-5' exonuclease region contains three sets of residues (Exo I, II, and III) involved in metal or ssDNA binding, which are highly conserved in both the *E. coli* pol I-like and α-like DNA polymerases (Bernad *et al.*, 1989; Hall *et al.*, 1995).

A 54-65 kDa protein was frequently found to copurify with DNA polymerase activity from HSV infected cells (Powell & Purifoy, 1977; Knopf, 1979; Vaughan *et al.*, 1985). This protein was identified as the product of HSV-1 gene UL42, an abundant dsDNA binding protein of infected cells (Parris *et al.*, 1988; Gallo *et al.*, 1988). It was

subsequently demonstrated that in HSV-1 infected cells the DNA polymerase enzyme exists predominantly as a heterodimer comprising a catalytic UL30 and an accessory UL42 subunit (Crute & Lehman, 1989; Gottlieb *et al.*, 1990). The UL42 protein has been reported to stimulate the DNA polymerase activity of UL30 on an activated calf thymus DNA template (Gallo *et al.*, 1989) and to increase polymerase processivity on singly primed single-stranded circular templates by enhancing the affinity for primer termini and presumably 3' termini of nascent strands (Gottlieb *et al.*, 1990; Hernandez & Lehman, 1990). It has been suggested that the role of UL42 in the DNA polymerase complex is to act as a clamp, decreasing the probability that the polymerase dissociates from the template after each cycle of catalysis (Gottlieb *et al.*, 1990).

Mapping analysis using *in vitro* transcription/translation and immunoprecipitation demonstrated that the C-terminal 228 amino acids of UL30 were sufficient to bind UL42 (Digard & Coen, 1990). This region is distinct from other regions of the protein involved in substrate binding and catalysis (Larder *et al.*, 1987; Haffey *et al.*, 1990). Finer scale mapping identified a region of about 35 amino acids at the extreme C-terminus of UL30 as dispensable for polymerase activity but crucial for binding of, and stimulation by, UL42, and indicated that this interaction was necessary for viral DNA synthesis both in the context of the viral genome and in a plasmid amplification assay (Digard *et al.*, 1993a, Stow, 1993). The importance of the extreme C-terminal 27 amino acids of UL30 in its physical interaction with UL42 was confirmed using anti-idiotype antibodies, a competition ELISA and inhibition of the interaction by peptides (Marsden *et al.*, 1994).

Mutational analyses suggest that UL42 contains an N-terminal domain comprising approximately the first 340 amino acids which includes both UL30-binding and DNA-binding functions and is sufficient to increase polymerase processivity (Digard *et al.*, 1993b; Gao *et al.*, 1993; Monahan *et al.*, 1993; Tenney *et al.*, 1993), and indeed can complement replication of a UL42 null mutant (Digard *et al.*, 1993b). The C-terminal region of UL42 seems to be non-essential for replication in tissue culture (Digard *et al.*, 1993b; Gao *et al.*, 1993). The UL30-binding and DNA-binding activities are separable, since an insertion mutation at position 160 destroyed heterodimerisation

without affecting DNA binding (Digard *et al.*, 1993b). A separate region within the N-terminal domain was found to be required for stimulation of polymerase activity but was not essential for physical interaction with UL30 (Monahan *et al.*, 1993).

Recently, two mutants with linker insertions at positions 203 and 206 of UL42 have been reported to be unable to bind to double-stranded DNA whilst retaining their ability to bind UL30 (Chow & Coen, 1995). These mutants were severely impaired in their ability to stimulate long-chain DNA synthesis *in vitro* and to complement growth of a UL42 null virus. This distinguishes UL42 from many other 'sliding clamp' processivity factors (Stukenberg *et al.*, 1991; Krishna *et al.*, 1994), in that UL42 apparently requires an intrinsic DNA binding activity for its function in processivity.

UL42 is at least 20-fold more abundant than UL30 in HSV infected cells (Gottlieb *et al.*, 1990), and uncomplexed UL42 binds with high affinity to double-stranded DNA in a sequence-independent manner (Gallo *et al.*, 1988; Bayliss *et al.*, 1975). This suggests that uncomplexed UL42 protein may have a specific additional role in viral DNA synthesis. Experiments showing that transformed cell lines expressing only 5% the *wt* levels of UL42 can complement UL42 null mutant replication to *wt* levels however argue against this independent function for UL42 (Johnson *et al.*, 1991).

b) Major DNA binding protein (UL29)

The major DNA binding protein (mDBP, also known as ICP8) was first recognised as an abundant 130 kDa protein induced in HSV-1 infected cells (Honess & Roizman, 1973; Powell & Courtney, 1975). Genetic studies using *ts* mutants identified this protein as the product of HSV-1 gene UL29, and demonstrated that it was necessary for viral DNA replication (Conley *et al.*, 1981; Weller *et al.*, 1983; Littler *et al.*, 1983; Dixon *et al.*, 1983; Godowski & Knipe, 1983; Leinbach *et al.*, 1984; Holland *et al.*, 1984).

mDBP binds preferentially and cooperatively to ss-DNA with no detectable sequence specificity, suggesting that its function, analogous to that of the *E. coli* SSB protein, may be to bind to and stabilise regions of ss-DNA at the replication fork

(Bayliss *et al.*, 1975; Powell & Purifoy, 1976; Powell *et al.*, 1981; Knipe *et al.*, 1982; Lee & Knipe, 1983; Ruyechan, 1983; Ruyechan & Weir, 1984). It has also been reported that mDBP reduces the melting temperature of poly(dA-dT) DNA *in vitro*, suggesting a role in facilitating strand separation (Powell *et al.*, 1981). In addition, the ability of saturating amounts of mDBP to melt small fragments of duplex natural DNA in an efficient, highly cooperative and non-directional manner is appropriate for a role in which mDBP destabilises duplex DNA during origin unwinding and fork movement (Boehmer & Lehman, 1993a).

DNA binding by UL29 *ts* mutants is thermolabile *in vivo* (Lee & Knipe, 1983; Leinbach & Casto, 1983) and *in vitro* (Ruyechan *et al.*, 1986), indicating this is an essential property of mDBP. The predicted sequence of mDBP consists of 1196 amino acids (Quinn & McGeoch, 1985). Correlation of several reports delineating the region absolutely required for the interaction of mDBP with ss-DNA places it between residues 564 and 849 (Leinbach & Heath, 1988; Gao *et al.*, 1988; Gao & Knipe, 1989; Wang & Hall, 1990), with residues 803-849 forming a possible consensus DNA-binding motif shared with other prokaryotic and viral DNA-binding proteins (Wang & Hall, 1990). In addition, mutations that disrupt a zinc finger consensus sequence between residues 499 and 512 of mDBP greatly affect its solubility, reduce its DNA-binding and abolish its ability to function in viral growth (Gao *et al*, 1988; Gao & Knipe, 1989). Atomic absorption analysis indicates that mDBP is indeed a zinc metalloprotein, and that the zinc is required for structural integrity of the protein (Gupte *et al.*, 1991). Although zinc-binding motifs are common to many DNA binding proteins (Berg, 1986) it remains unclear whether in mDBP this motif participates in interactions contributing to cooperative DNA binding and/or to stabilisation of the 'core' DNA-binding region (Gao & Knipe, 1989).

Several of the UL29 *ts* mutants were found to specify products which were either defective in their localisation to the cell nucleus but retained their DNA-binding activity, or capable of entering the nucleus but unable to bind ss-DNA, suggesting that several regions of mDBP, including residues 100-150, 326-586, and 965-1196, contribute to nuclear localisation and that these regions are distinct from those required

for DNA binding (Lee & Knipe, 1983; Gao *et al.*, 1988; Gao & Knipe, 1989; Knipe, 1989). Since deletion of the very C-terminus abolished nuclear localisation, Gao & Knipe (1993) replaced the C-terminal 28 residues of mDBP with the SV40 large T-antigen nuclear localisation signal. They found that despite localising to the nucleus and displaying DNA-binding *in vitro*, this mutant failed to support viral DNA synthesis and viral growth, but could be complemented by a mutant with a lesion in the N-terminal region of mDBP. This demonstrated that the very C-terminus is required for an additional function involved in viral DNA replication separable from nuclear localisation and DNA binding. An N-terminal deletion mutant with a similar phenotype has also been described (Gao & Knipe, 1989).

Recent work has identified several other functions of mDBP. mDBP stimulates the DNA polymerase holoenzyme (Ruyechan & Weir, 1984; Gottlieb *et al.*, 1990; Hernandez & Lehman, 1990), the helicase activity of UL9 (Fierer & Challberg, 1992; Boehmer *et al.*, 1993) and is required for complete unwinding of duplex DNA by the helicase/primase complex (Crute & Lehman, 1991). These effects are driven by the preferential binding of mDBP to ss-DNA and the ability of mDBP to interact directly with other viral replication proteins (see section 1.4.3).

It has also been suggested that since mDBP promotes homologous pairing, strand transfer and renaturation of complementary DNA strands, it may contribute to the high level of homologous recombination that occurs during HSV replication (Bortner *et al.*, 1993; Dutch & Lehman, 1993).

Studies of *ts* and deletion mutants have indicated a role for mDBP in regulating viral gene expression (Godowski & Knipe, 1986; Gao & Knipe, 1991). A mutant mDBP in which residues 1082-1169 (lying between the DNA-binding domain and the C-terminal nuclear localisation signal) were deleted exhibited *trans*dominant inhibition of viral replication by inhibiting normal functions of mDBP, resulting in reduced viral DNA replication but a relatively greater reduction in late gene expression (Gao & Knipe, 1991). This suggests a direct role for mDBP in stimulation of late gene expression, possibly by binding to progeny DNA and keeping the promoter regions open for transcription, or by interacting specifically with late promoters or the proteins

which recognise them (Gao & Knipe, 1991). An ability to activate transcription is exhibited by other ssDNA-binding proteins, including bacteriophage T4 gene 32 protein and *E. coli* SSB protein (Gauss *et al.*, 1987; Haynes & Rothman-Denes, 1985). Three more T4-encoded DNA polymerase accessory proteins stimulate T4 late gene expression, such that moving replication forks act as mobile enhancers (Herendeen *et al.*, 1989, 1990).

Stable interactions between mDBP and the viral alkaline nuclease have been demonstrated by their coprecipitation from, and colocalisation in, HSV-infected cells (Thomas *et al.*, 1992). A coordinated action of alkaline nuclease and mDBP might be important in resolving branched products of recombination (Martinez *et al.*, 1996). An additional interaction between mDBP and UL37, a tegument protein of unknown function, has been reported (Shelton *et al.*, 1994).

In addition to the above interactions with viral proteins, the HSV mDBP may also interact with cellular proteins of the nuclear matrix to promote assembly of nuclear structures involving both viral and cellular DNA replication proteins (reviewed by Knipe, 1989). The intranuclear location of mDBP varies according to the status of viral DNA replication: prior to viral DNA synthesis (2.5-3.5 hpi) mDBP exhibits a focal punctate distribution within the nucleus in structures referred to as prereplicative sites (Quinlan *et al.*, 1984; de Bruyn Kops & Knipe, 1988). If viral DNA replication is allowed to proceed, a few larger foci of mDBP staining appear at approximately 3.5 hpi, increase in size, and form randomly distributed globular structures termed replication compartments, in which the mDBP is bound to viral DNA . If viral DNA replication is blocked, mDBP remains associated with the nuclear matrix in the punctate foci (Quinlan *et al.*, 1984). A subset of other viral and cellular proteins colocalise with prereplicative sites (see section 1.4.3), and there is evidence that assembly of these sites requires functional mDBP (Knipe, 1989).

c) Origin binding protein (UL9)

Transient replication assays first demonstrated that the UL9 gene product was essential for HSV-1 origin-dependent DNA replication (Wu *et al.*, 1988; McGeoch *et*

al., 1988b). Confirmation of the involvement of UL9 in viral DNA synthesis came from the mapping of lesions in several DNA negative mutants to the UL9 gene locus (Carmichael *et al.*, 1988; Malik *et al.*, 1992).

Previously, filter binding studies had shown that a protein present in HSV-1 infected cells could bind specifically to the viral origin of replication ori_S (Elias *et al.*, 1986). This protein, of apparent M_r 83 kDa, had been shown by DNase I 'footprinting' to protect two sites, each approximately 18 nucleotides long, within the ori_S sequence (Elias & Lehman, 1988). The origin-binding protein exhibited a ten-fold greater affinity for Site I, which overlaps with the left end of the ori_S palindrome, than for Site II which lies in the opposite orientation on the right arm of the palindrome and which differs from the Site I sequence in three of its 18 bases (**Figure 4**).

It was subsequently demonstrated that this origin binding protein (OBP) is encoded by the UL9 gene of HSV-1 (Olivo *et al.*, 1988; Weir *et al.*, 1989). Complexes between labelled ori_S-containing DNA and partially purified ori_S-binding activity were immunoprecipitated from HSV-infected cells using antisera directed against the then uncharacterised products of the four DNA replication genes UL5, UL8, UL9 and UL52. The antiserum against the UL9 protein precipitated the greatest amount of ori_S (Olivo *et al.*, 1988). The UL9 gene product was then expressed in insect cells using a recombinant baculovirus and was found to comigrate with the protein immunoprecipitated by anti-UL9 serum from HSV-infected cells (Olivo *et al.*. 1988). The UL9 protein expressed in insect cells was shown by immunoprecipitation to bind to both ori_S and ori_L, and DNase I footprint analysis showed protection of the same sites as described previously by Elias *et al.* (1986).

Weir *et al.* (1989) independently confirmed that UL9 encodes the origin binding protein by using retardation assays which identified specific complexes between ori_S sequences and a protein from *wt* HSV-1 infected cells. These complexes were specifically supershifted by antiserum directed against the UL9 gene product. In addition, an HSV-1 recombinant capable of expressing UL9 in the absence of the other six DNA replication proteins specified origin binding activity as measured in a gel retardation assay.

* Fierer & Challberg, 1992).

UL9 is a delayed early gene, the product of which is expressed in low abundance in HSV-1 infected cells and localises to the nucleus, exhibiting a globular pattern of immunofluorescence similar to that of UL29 (Olivo *et al.*, 1989). UL9 is not thought to be subject to posttranslational modifications (Olivo *et al.*, 1989; Fierer & Challberg, 1992). The UL9 protein exists as a homodimer in solution (Bruckner *et al.*, 1991;

Several experimental approaches including DNase I footprinting, chemical modification and the use of mutated origin sequences were used to define the UL9 recognition sequence. This was found to be CGTTCGCACT at site I and TGCTCGCACT at site II, giving a consensus YGYTCGCACT (Koff & Tegtmeyer, 1988; Deb & Deb, 1989; Elias *et al.*, 1990). Deletion of either site affects origin-dependent replication although mutated origins retain ability to bind UL9 at their unaltered site (Deb & Deb, 1989; Weir & Stow, 1990). This indicates that the presence of both UL9 binding sites is necessary for efficient origin activity (Weir & Stow, 1990). Elias *et al.* (1990) reported evidence of cooperative interactions between UL9 proteins bound to sites I and II in that a mutated site I with significantly impaired UL9 binding when tested in isolation was shown to bind UL9 when linked to a site II sequence. The DNase I footprint analysis also suggested that this cooperative interaction between bound UL9 proteins led to a conformational change, possibly bending, of the DNA in the central AT-rich region (Elias *et al.*, 1990).

The AT-rich region in the centre of the palindrome has indeed proved to be important in viral replication. Insertion of increasing numbers of AT dinucleotides has an oscillating effect on DNA replication depending on the number of helical turns inserted (Lockshon & Galloway, 1988). Nuclease and chemical footprinting studies by Koff *et al.* (1991) demonstrated that UL9 loops the AT-rich DNA between binding sites I and II and distorts the looped DNA helix. This looping and distortion occurs regardless of whether helical phasing is disrupted by insertion of additional AT bases, although differences in phasing lead to different patterns of helical distortion which may result in the region being unable to support formation of an initiation complex (Koff *et al.*, 1991). Surprisingly, ori_S constructs where the spacer sequence has been altered by half a helical turn support the formation of more stable complexes with UL9,

but nevertheless fail to support DNA replication, providing further evidence that properly spaced binding sites are required for UL9-induced conformational changes at the origin (Gustafsson *et al.*, 1994). Moreover the observation that formation of UL9 complexes on *wt* ori_S DNA is inefficient suggests that additional factors may be needed to promote the assembly of functional UL9-DNA complexes capable of initiating DNA synthesis (Gustafsson *et al.*, 1994).

Expression of various portions of UL9 as fusion proteins in *E. coli* revealed that the C-terminal 317 amino acids of UL9 retained specific DNA-binding activity to sites I and II of ori_S (Weir *et al.*, 1989). This DNA-binding domain was subsequently narrowed down to 269 amino acids, consisting of residues 564 to 832 of the UL9 protein (Deb & Deb, 1991). This domain was proposed to contain a helix-turn-helix motif and a pseudo-leucine zipper, both indispensable for DNA binding (Deb & Deb, 1991). However, mutations within the helix-turn-helix motif do not impair DNA-binding (Arbuckle & Stow, 1993) or replicative function (Martinez *et al.*, 1992), and the pseudo-leucine zipper is not well conserved in the VZV and EHV-1 OBP sequences (Arbuckle & Stow, 1993). The isolated DNA-binding domain of UL9 can bind to viral origins and exerts a transdominant inhibition of viral DNA replication (Stow, 1992; Perry *et al.*, 1993; Stow *et al.*, 1993). Measurements from double-label gel shift assays indicate that two UL9 DNA-binding domains bind to site I, and two bind to site two (Fierer & Challberg, 1995), in agreement with predictions that each site consists of two inverted overlapping binding sequences for UL9 (Koff & Tegtmeyer, 1988; Hazuda *et al.*, 1991). and that two UL9 dimers bind to ori_S, one each at Site I and Site II (Fierer & Challberg, 1992).

Studies comparing *wt* and truncated UL9 proteins demonstrated that the N-terminal portion contains elements required for dimerisation and cooperative binding of UL9 dimers to sites I and II of ori_S (Hazuda *et al.*, 1992; Elias *et al.*, 1992). Integrity of the leucine zipper element between amino acids 150-171 of UL9 is essential for this cooperativity (Hazuda *et al.*, 1992). Moreover, DNAse I footprint analysis indicates that weak interactions between UL9 and site III are stabilised by the cooperative interactions between UL9 proteins complexed at ori_S (Elias *et al.*, 1992).

The amino terminal regions of UL9 and its homologues contain several sequence motifs characteristic of a superfamily of putative helicases (Gorbalenya *et al.*, 1989). Subsequent experiments demonstrated that UL9 is capable of functioning as a DNA helicase and DNA-dependent ATPase (Bruckner *et al.*, 1991). The helicase activity of UL9 moves 3' to 5' on the strand to which it is bound. It can displace a short 5' tailed oligonucleotide annealed to ss M13 DNA, but in the presence of the ssDNA-binding protein UL29 both the rate and extent of DNA unwinding are increased (Fierer & Challberg, 1992; Boehmer *et al.*, 1993; Dodson & Lehman, 1993). There is also genetic evidence that the helicase activity of UL9 is essential for viral DNA replication: point mutations within the six conserved helicase motifs render UL9 incapable of complementing a UL9 null mutant in transient assays of viral DNA replication (Martinez *et al.*, 1992). The UL9 helicase activity does not require the presence of the origin binding domain, since a truncated protein comprising amino acids 1-535 of UL9 has DNA helicase and a novel ssDNA-binding activity (Abbotts & Stow, 1995).

UL9 bound to the origin of replication (ori_S) has recently been demonstrated by electron microscopy to use its intrinsic helicase activity to achieve local unwinding of the DNA helix, forming partially single-stranded stem-loop structures (Makhov *et al.*, 1996a). A similar examination of UL9 and mDBP (UL29) bound to DNA lacking HSV-1 origins suggests that the resultant unwinding process differs greatly from mechanisms involving progressive unwinding at Y-shaped replication forks that move along the DNA (Makhov *et al.*, 1996b). It seems likely that local unwinding of the DNA at origins of HSV-1 replication, and an ability to initiate assembly of a multi-protein pre-initiation complex (see section 1.4.3), are the vital roles of UL9 in viral DNA synthesis.

d) Helicase primase complex (UL5, UL8 and UL52)

The products of the genes UL5, UL8 and UL52 had not been identified prior to reports that they were essential for viral origin-dependent DNA replication. Knowledge of their DNA sequences enabled specific antisera to be raised against predicted carboxy-terminal oligopeptides and against the whole proteins overexpressed in *E. coli*.

Using these antibodies, proteins with estimated M_rs close to those predicted for the products of the UL5, UL8 and UL52 genes were identified in HSV-1 infected cells by immunoblotting and immunoprecipitation (Zhu & Weller, 1988; Olivo *et al.*, 1989).

Indirect immunofluorescence indicated that the three proteins, which were expressed in very low abundance in infected cells, localised to the nucleus where they exhibited a diffuse distribution (Olivo *et al.*, 1989) with additional discrete globular regions of UL5 around the nuclear periphery (Zhu & Weller, 1988).

Since synthesis of the gene products was not inhibited by phosphonacetic acid (PAA) which specifically blocks viral DNA synthesis, UL5, UL8 and UL52 were presumed to be regulated as early genes (Olivo *et al.*, 1989).

To further study these proteins, the products of the UL5, UL8 and UL52 ORFs were expressed in insect cells using baculovirus vectors, yielding proteins which were immunoprecipitable with the appropriate anti-peptide sera and which comigrated with the corresponding products from HSV-1-infected cells (Olivo *et al.*, 1989).

First indications of the functions of the UL5, UL8 and UL52 gene products in viral origin-dependent DNA replication came from the purification of a complex from HSV-1-infected cells which exhibited DNA helicase activity (Crute *et al.*, 1989; 1988). The helicase activity was demonstrated by displacement of an oligonucleotide annealed to single-stranded M13 DNA with a 3' unpaired tail, implying that the direction of translocation on the M13 DNA was 5' -> 3' (Crute *et al.*, 1988). This activity was purified as a complex (M_r approximately 440 kDa) consisting of three polypeptides with molecular weights of 120 kDa, 97 kDa and 70 kDa, which were recognised by the previously described UL5, UL8 and UL52 specific antisera. In addition to DNA helicase activity, the complex exhibited DNA-dependent ATPase, DNA-dependent GTPase and DNA primase activities (Crute *et al.*, 1989).

Substantiation that the UL5, UL8 and UL52 proteins functioned as a helicase-primase complex was provided by triply infecting *S.f.* cells with recombinant baculoviruses each expressing one of the three gene products (Dodson *et al.*, 1989; Calder & Stow, 1990). A fully active enzyme was assembled *in vivo* in the infected insect cells and, when purified to near homogeneity, this recombinant complex

exhibited DNA-dependent ATPase, DNA-dependent GTPase, DNA helicase and DNA primase activities that were essentially identical to the enzyme isolated from HSV-1-infected cells. A 2-fold lower specific activity for the recombinant enzyme was not considered significant (Dodson *et al.*, 1989). In agreement with Crute *et al.* (1989), the enzyme complex was found to consist of three polypeptides immunoreactive to UL5, UL8 or UL52 anti-peptide sera, with apparent M_rs of 97 kDa, 70 kDa and 120 kDa, respectively. The M_r of the complex was, however, estimated to be approximately 270 kDa, a figure that, although much lower than that estimated previously, was more consistent with the complex consisting of three single polypeptides, each the product of one of the three ORFs.

Further characterisation of the helicase-primase complex isolated from HSV-1-infected cells showed that it was indeed a heterotrimer, consisting of the UL5, UL8 and UL52 polypeptides in a 1:1:1 ratio (Crute & Lehman, 1991). Determination of the molecular weight of the enzyme gave a value of 263 kDa, close to the values of 287 kDa or 293 kDa predicted by SDS-PAGE or sequence analysis, respectively.

The primase activity of this complex was shown to catalyse the synthesis of oligoribonucleotide primers 8-12 nucleotides in length, while the helicase activity could, in the presence of ATP and the HSV-1-encoded ssDNA-binding protein UL29, completely unwind nicked circular duplex DNA at the rate of about two nucleotides/second (Crute & Lehman, 1991). Although the primase was found to be active only over a narrow range of conditions, other enzymatic activities involved in HSV-1 DNA synthesis showed substantial activity under similar conditions, including the DNA polymerase activity of UL30/UL42, the helicase activity of UL5/UL8/UL52 and the origin-binding and helicase activities of UL9.

The role of the HSV-1 helicase-primase in viral DNA replication was therefore predicted to involve coordinate unwinding of the DNA and priming of Okazaki fragment synthesis at the viral replication fork.

None of the individual subunits of the helicase-primase complex has been shown to exhibit appreciable enzymatic activity when expressed alone. However, coexpression experiments using recombinant baculoviruses showed that a partially

purified extract from insect cells doubly infected with baculoviruses expressing the UL5 and UL52 gene products did exhibit DNA-dependent ATPase and DNA helicase activities (Calder & Stow, 1990). The UL5/UL52 subassembly purified from such cells was stable and also exhibited DNA-dependent GTPase and DNA primase activities (Dodson & Lehman, 1991). Two sites capable of hydrolysing nucleoside triphosphates in a DNA-dependent manner have been proposed to reside within the UL5/UL52 subassembly (Crute *et al.*, 1991).

These findings showed that expression of the UL8 protein, although essential for viral origin-dependent DNA replication, appeared not to be required for the enzymatic activities associated with the helicase-primase complex. This raised questions as to the role of the UL8 protein in DNA replication. Further investigations aimed at answering this will be described later in this section, but first the UL5/UL52 subassembly will be discussed in more detail.

Homologues of the UL5 and UL52 proteins have been identified in other herpesviruses belonging to all three subfamilies (see **Table 1**). Clearly identifiable homologues of UL5 but not UL52 also occur in the databases amongst non-herpesviral proteins. Examination of the sequence of the UL5 protein revealed the presence of a consensus sequence: G--g-GKt (completely conserved residues in uppercase letters and partially conserved ones in lowercase, McGeoch *et al.*, 1988b) associated with ATP-binding sites in many proteins (sequence A, Walker *et al.*, 1982). This consensus sequence was also found in the VZV and EBV homologues of UL5, suggesting that the UL5 protein contains a functional ATP-binding site (McGeoch *et al.*, 1988b).

More detailed computer alignments of protein sequences containing the ATP-binding motif resulted in the identification of two major superfamilies of established and putative helicases from *E. coli*, yeast, RNA viruses, herpesviruses and mammalian cells (Hodgman, 1988; Lane, 1988; Gorbalenya & Koonin, 1989). The UL5 protein was assigned to helicase superfamily 1, along with its homologues in VZV, HCMV and EBV (Hodgman, 1988; Gorbalenya & Koonin, 1989). This superfamily is characterised by 6 or 7 shared motifs, with motifs I and II corresponding to the A and B sequences of the ATP-binding motif derived by Walker *et al.* (1982). The products of the HSV-1

UL9 gene and its homologue in VZV (gene 51) were assigned to a different but distantly related superfamily (putative helicase superfamily 2; Gorbalenya *et al.*, 1989).

The importance of the six conserved helicase motifs for the function of the UL5 protein was confirmed by mutagenesis of the most highly conserved residues within each motif and examination of the resultant proteins for ability to complement a UL5-null mutant (*hr*99, Zhu & Weller, 1992a) in a transient assay of HSV-1 origin-dependent DNA replication (Zhu & Weller, 1992b). All six conserved motifs were found to be essential to the function of UL5 protein in viral DNA replication, whereas similar amino acid substitutions outside the conserved motifs were tolerated. In addition, mutations in motifs I and IV, when introduced into viral genomes, did not affect the ability of UL5 to form specific interactions with UL8 and UL52, indicating that at least some regions involved in catalytic activity are unlikely to be responsible for protein-protein interactions within the helicase-primase complex (Zhu & Weller, 1992b).

Despite the evidence suggesting that UL5 is primarily responsible for the helicase activity of the complex, UL5 expressed in the absence of UL52 (using recombinant baculovirus infection of insect cells) does not exhibit intrinsic ATPase or DNA helicase activity (Calder & Stow, 1990; Sherman *et al.*, 1992). Complex formation with UL52 may possibly stimulate the helicase activity of UL5 by converting it to an active conformation.

Since UL5 contains all six conserved helicase motifs and the UL5/UL52 subassembly exhibits both helicase and primase activities, UL52 seemed likely to confer the primase activity. However, the low solubility of UL52 when expressed alone and the lack of non-herpesviral homologues in the databases made it difficult to gain experimental evidence for the assignment of this function.

Klinedinst and Challberg (1994) investigated the functional contribution UL52 makes to the activities of the tripartite complex by introducing a single aspartate to glutamine substitution (D628Q) within a 24 amino acid region of UL52 that showed high conservation with its counterparts in VZV, EHV-1, HCMV and EBV, and contained a region with limited similarity to a six amino acid motif present in known

phage, yeast and mouse primases. A heterotrimeric complex purified from insect cells coinfected with recombinant baculoviruses expressing *wt* UL5 and UL8 and the mutated UL52 exhibited specific ATPase and DNA helicase activities comparable to the wild-type complex and functioned as well as the wild-type complex in an assay of leading-strand replication which assessed the ability of the complex to support displacement DNA synthesis (rolling circle replication) from a preformed replication fork in conjunction with UL29 and UL30/UL42.

To assess whether the complex could participate in RNA-primed DNA synthesis the production of double-stranded circular molecules from an unprimed single-stranded DNA template in the presence of UL29, UL30/UL42 and the *wt* or mutant heterotrimer was measured. In this assay the complex incorporating mutant UL52 exhibited less than 12% of the activity of wild-type UL5/UL8/UL52. The mutant complex was also shown directly to be incapable of synthesising RNA primers. UL52 thus contributes at least some, if not all, of the sequences required for primase activity (Klinedinst & Challberg, 1994).

More recently, Dracheva *et al.* (1995) used multiple sequence alignment of HSV-1 UL52 with its homologues from seven other herpesviruses to identify a conserved region which resembled the putative metal-binding site found in both prokaryotic and eukaryotic primases. This conserved sequence contained residues Asp^{628} and Asp^{630} (designated the HSV-1 primase DXD motif) and corresponded to the conserved region identified by Klinedinst & Challberg (1994). Dracheva *et al.* (1995) showed that alteration of either of the Asp residues virtually abolished the primase activity of the UL5/UL8/UL52 complex without affecting helicase function, whereas mutation of a nearby conserved residue (Asn^{624}) had little effect on priming activity. They concluded that the UL52 protein contains at least part of the primase catalytic site and suggested that the DXD motif may be involved in ribonucleotide triphosphate binding.

The experimental evidence therefore supports the assignment of UL5 as a helicase and UL52 as a primase, albeit that each activity is dependent on the presence

of the other subunit. Since the presence of UL8 is not essential for either enzymatic activity, the role of UL8 in viral DNA replication appeared unclear.

In an attempt to clarify the contribution of UL8 towards the enzymatic activities of the complex, Sherman *et al.* (1992) developed the coupled primase-polymerase or 'lagging strand' assay described above and found that although the UL5/UL52 subassembly could synthesise primers on UL29-coated single-stranded M13 DNA, these primers were not elongated by the UL30/UL42 polymerase if UL8 was absent. Lagging-strand DNA synthesis could, however, be restored by the addition of partially purified UL8 to the system. Since the rate of primer synthesis and the length of primers produced were unaffected by the absence of UL8, it was concluded that UL8 was critical for the efficient utilisation of primers. In addition, the same requirement for UL8 in lagging-strand synthesis was observed when *E. coli* DNA polymerase I was substituted for the HSV-1 DNA polymerase, hence it was proposed that UL8 was more likely to act by stabilising primers on the template DNA, rather than through an interaction with the viral DNA polymerase complex. Furthermore, the absence of UL8 had no effect on elongation by *E. coli* Pol I of primers synthesised by UL5/UL52 on a poly(dT) template (Sherman *et al.* 1992). The low sequence complexity of poly(dT) presumably removes the requirement for any factors which stabilise primer-template associations because primer reassociation is rapid.

In contrast, when Tenney *et al.* (1994) examined the activity of the UL5/UL52 subassembly in coupled primase-polymerase assays, they detected significant levels of primer extension using either M13 or øX174 ssDNA as template. This activity was, however, stimulated in a dose-dependent manner by addition of UL8 to UL5/UL52 to reconstitute the heterotrimer. Changing the polymerase from HSV-1 to modified T7 DNA polymerase affected neither the activity of UL5/UL52 nor its stimulation by UL8. Direct assays of primer synthesis indicated that addition of UL8 to the UL5/UL52 subassembly increased the amount of primers synthesised, but not their size or distribution. Nevertheless, stimulation of primer synthesis did not account for all of the increased activity seen in the coupled primase-polymerase assay, even though the amount of primer annealed to template DNA and the fraction extended by DNA

polymerase correlated with the amounts synthesised. Likewise, addition of UL8 to UL5/UL52 did not affect the stability of the complex nor its affinity for template DNA. The conclusion was therefore that the UL5/UL52 subassembly contains *in vitro* primase activity and that addition of UL8 increases the rate of UL5/UL52 primer synthesis.

The relatively smaller stimulatory effect of UL8 on UL5/UL52 in the direct primer synthesis assay compared to the coupled primase-polymerase assay suggests that UL8 is unlikely to function solely to increase primase activity. In fact the UL8 protein appears to be involved in interactions with several other HSV-1 DNA replication proteins, as will be discussed in section 1.4.3.

A further role for UL8 within the helicase-primase, namely to facilitate localisation of the complex to the nucleus, has been indicated by immunofluorescence studies of recombinant *ts* herpes simplex viruses individually expressing the UL5, UL8 and UL52 gene products in the absence of other DNA replication proteins (Calder *et al.*, 1992). The results demonstrated that co-expression of all three components of the helicase-primase complex was required for efficient nuclear localisation, and suggested that since UL5 and UL52 form an enzymatically competent complex, a possible role of UL8 may be to facilitate nuclear uptake.

In support of the requirement for all three proteins in nuclear localisation of the complex, McLean *et al.* (1994) have demonstrated by means of baculovirus expression vectors and coimmunoprecipitation studies that UL8 can bind separately to both the UL5 and UL52 proteins. Taken with the previous finding that UL5 and UL52 form a stable interaction (Calder & Stow, 1990; Dodson & Lehman, 1991), this indicates that within the complex each protein interacts directly with the other two.

1.4.3 The replication complex

The two assemblies of HSV-1 replication proteins discussed above constitute the virally-encoded polymerase and helicase-primase activities. The heterodimeric polymerase resembles other prokaryotic and eukaryotic polymerases of the alpha family in having a catalytic subunit and an accessory factor to increase processivity, although UL42 is notable amongst processivity factors in its requirement for intrinsic DNA-

binding activity (Chow & Coen, 1995). The combination of primase with a helicase in HSV-1 resembles the pairing of these activities in prokaryotes, rather than the pairing of primase with DNA polymerase common in eukaryotes (Kornberg & Baker, 1992b). Mapping and analysis of the predominant site of primer synthesis by the HSV-1 helicase-primase on øX174 virion DNA showed that the template sequence requirements for HSV primer synthesis are specific, resembling characterised template-specific primases of *E. coli*, T4, T7 and the mammalian polymerase-α-primase complex, although the HSV primase function is more tolerant of sequence alterations (Tenney *et al.*, 1995).

Aside from the interactions between the two subunits of the HSV-1 DNA polymerase and the three components of the helicase-primase complex, it is likely that further interactions between the seven HSV-1 replication proteins, as well as with cellular proteins, are necessary to coordinate viral DNA replication. Several interactions are implicated by the reported stimulatory effects of particular replication proteins on other components of the replicative machinery, and direct physical evidence exists for some of these proposed interactions.

Using an anti-UL8 monoclonal antibody McLean *et al.* (1994) have coimmuno-precipitated UL9 with the other three components of the helicase-primase complex. Moreover, following a dual infection, UL8 and UL9 were also coprecipitated. Thus the origin-binding protein can interact directly with the helicase-primase complex and this interaction is mediated by the UL8 subunit. This suggests that UL8 may play a role in positioning the helicase-primase complex at the replication origins, where its primase activity is likely to be a key step in initiating DNA synthesis. It is possible that the helicase activity of the UL5/UL8/UL52 complex also contributes to the initial opening of the origins. The region of UL9 involved in the interaction with UL8 was shown to reside within the N-terminal 535 amino acids (McLean *et al.*, 1994).

Protein affinity and gel-filtration chromatography have demonstrated a tight physical interaction between the C-terminal DNA-binding domain of UL9 protein and the major ss-DNA-binding protein UL29 (Boehmer & Lehman, 1993b). It seems likely that the previously reported stimulation of the UL9 DNA helicase activity by mDBP

(Boehmer *et al.*, 1993) results from this association. Further experiments using UL9 mutants revealed that the C-terminal 27 residues of UL9 were important for physical and functional interaction with mDBP, but were not required for origin-binding or helicase activity (Boehmer *et al.*, 1994).

Taken together, these reports begin to define initiation events at the HSV-1 origin, where UL9 binds to the origin and interacts via its C-terminus with mDBP and via its N-terminal domain with the helicase-primase, forming an initiation complex. Recent evidence confirms that the interaction between the C-terminal domain and mDBP serves to position the latter protein with high precision at an origin of DNA replication (Gustafsson *et al.*, 1995). The ability of mDBP to destabilise duplex DNA and stimulate the helicase activity of UL9 suggests that the concerted action of UL9 and mDBP may unwind the origin, possibly aided by the helicase activity of the UL5/UL8/UL52 complex, whose primase activity would subsequently synthesise primers for DNA synthesis.

Preliminary results from two groups suggest that UL8 may be involved in further structural and functional interactions within the replication complex. One group reports that UL8 facilitates stimulation of the helicase-primase by mDBP *in vitro* (Tenney *et al.*, 1995), and there is also evidence for an interaction between UL8 and UL30, the catalytic subunit of the viral DNA polymerase (G. McLean, personal communication). It is conceivable that interactions between the helicase-primase, the polymerase holoenzyme and mDBP are required to coordinate processive and discontinuous DNA synthesis with unwinding of the DNA duplex at the replication fork and the synthesis of primers on the lagging strand.

As mentioned previously (section 1.4.2 b), in the absence of viral DNA replication HSV mDBP localises to small punctate foci in the nucleus termed prereplicative sites, and relocates upon initiation of DNA synthesis to larger, randomly distributed globular structures called replication compartments, in which mDBP is bound to viral DNA (Quinlan *et al.*, 1984). Bromodeoxyuridine (BrdU) immunofluorescence microscopy revealed that cellular DNA replication sites are relocated upon HSV-infection to the viral prereplicative structures, and that this

redistribution requires functional mDBP (de Bruyn Kops & Knipe, 1988). Several cellular replication proteins, including topoisomerase II, single-stranded DNA binding protein, PCNA (an accessory factor to polymerase ∂), DNA ligase I and polymerase α, have also been shown to redistribute upon HSV-1 infection, colocalising with HSV-1 mDBP and viral DNA in replication compartments (Ebert *et al.*, 1994; Wilcock & Lane, 1991). At least some of these cellular proteins can be envisaged to play a role in HSV-1 DNA replication, in particular the topoisomerase and ligase, and possibly DNA polymerase α (recently reported to interact with and be stimulated by UL9: Lee *et al.*, 1995), and it may be that redistribution of these by the virus causes the whole cellular replication complex, or replicase, to be transported. The retinoblastoma protein and p53 relocate to the same sites of viral replication, possibly because alteration of the function of these anti-oncogene products is necessary for the virus to overcome quiescence in the host cell (Wilcock & Lane, 1991).

UL9, UL30 and UL42 possess an intrinsic ability to localise to the nucleus and colocalise with mDBP in replication compartments during productive infection (Olivo *et al.*, 1989; Goodrich *et al.*, 1990). The helicase-primase complex has also recently been shown to colocalise with mDBP in replication compartments (Lukonis & Weller, 1996). Thus all seven virally-encoded replication proteins, along with components of the cellular replication machinery, localise to the same compartments in infected cells, confirming their designation as sites of viral DNA synthesis.

The precise composition and function of prereplicative sites, formed when viral DNA replication is blocked by HSV-specific polymerase inhibitors or by mutation of the polymerase complex, require elucidation. Goodrich *et al.* (1990) reported that when DNA synthesis was blocked by the HSV polymerase inhibitor phosphonoacetic acid (PAA), a proportion of UL30 accumulated in prereplication sites occupied by mDBP, but UL42 was more diffusely localised throughout the nucleus. Bush *et al.* (1991) confirmed that UL30, at least, colocalises to a large degree with mDBP in prereplicative sites, whereas UL30 expressed transiently in the absence of mDBP remains diffusely distributed throughout the cell nucleus. Thus it appeared that mDBP was necessary but not sufficient to direct accumulation of other viral replication proteins to the

prereplicative sites. Recently, immunofluorescence assays using epitope-tagged proteins have shown that the components of the helicase-primase complex also colocalise with mDBP in prereplicative sites in the presence of PAA (Lukonis & Weller, 1996).

mDBP staining is nuclear and diffuse in cells infected with either UL8, UL5, UL52 or UL9 null mutants, implying that these four proteins may play a role in prereplicative site formation. However, in the presence of HSV polymerase inhibitors (PAA, acyclovir, hydroxyurea or aphidicolin), greater than 50% of the cells infected with the same null viruses contain prereplicative sites which colocalise with BrdU incorporation. Cells containing diffuse nuclear mDBP do not incorporate BrdU. This suggests that active cellular DNA synthesis or presence of S phase factors, and not the presence of UL5, UL8, UL52 or UL9, is a prerequisite for formation of prereplicative sites in these cells (Lukonis & Weller, 1996; Liptak *et al.*, 1996).

In contrast, cells infected with a polymerase or UL42 null mutant in the presence and absence of drug inhibitors exhibit colocalisation of mDBP and BrdU in prereplicative sites (Lukonis & Weller, 1996; Liptak *et al.*, 1996). Moreover, inhibition of HSV DNA replication by subtle mutation of UL5 or UL9 is not sufficient to induce prereplicative site formation unless the viral polymerase is also inhibited (Lukonis & Weller, 1996). Thus it may be that presence of an active polymerase holoenzyme prevents formation of prereplicative sites.

In cells transfected with the mDBP gene in the presence and absence of PAA, the distribution of mDBP is diffuse, confirming that other viral components are required for formation of prereplicative sites (Liptak *et al.*, 1996). Interestingly, in cells transfected with the genes encoding mDBP, UL5, UL8 and UL52, mDBP is localised to punctate structures resembling prereplicative sites in approximately 40% of the cells, whereas mDBP alone or mDBP in the absence of any one component of the helicase-primase exhibits a diffuse nuclear distribution (Liptak *et al.*, 1996).

It remains unclear whether prereplicative sites are formed by the coassembly of proteins UL5, UL8, UL52, UL9 and mDBP, followed by binding of UL30 and UL42, serving as precursors to replication compartments (Liptak *et al.*, 1996), or whether

prereplicative sites are formed by the association of viral replication proteins with sites of cellular DNA synthesis merely as a result of inactivating polymerase holoenzyme function, and are not the same sites at which viral DNA replication proceeds (Lukonis & Weller, 1996). Whilst it has been demonstrated that replication compartments colocalise with major sites of DNA synthesis in infected cells (Randall & Dinwoodie, 1986; de Bruyn Kops & Knipe, 1988), and that these sites of HSV-1 DNA replication are located in a nonrandom distribution apparently dependent upon preexisting structural elements with the infected cell nucleus (de Bruyn Kops & Knipe, 1994), it has recently been shown that prereplicative sites, as defined by punctate mDBP-positive staining, do not stain for viral DNA by *in situ* hybridisation (Maul *et al.*, 1996). Moreover the input viral DNA (3 hpi) is found instead at the periphery of preexisting nuclear domains called ND10. A striking association between ND10 and viral replication sites, defined by *in situ* hybridisation with viral DNA and immunofluorescent staining of mDBP, can be seen at 7 hpi (Maul *et al.*, 1996). ND10 are discrete matrix-bound nuclear sites of unknown function, with a diameter of 0.3-0.5 μm and an average frequency of ten per nucleus (Ascoli & Maul, 1991). ND10-associated proteins (Sp100 and PML) are dispersed during HSV-1 infection (Maul *et al.*, 1993), a process which requires functional Vmw110 (Everett & Maul, 1994). Hence the association of viral replication sites with ND10 can be observed using Vmw110-deficient viruses (Maul *et al.*, 1996). Although some mDBP staining was associated with ND10 at sites occupied by viral DNA, the numerous punctate foci of mDBP staining, which define prereplicative sites, were not associated with viral DNA or ND10, suggesting that prereplicative sites do not generally develop into replication sites (Maul *et al.*, 1996).

Section 1.5 AIMS OF THE WORK PRESENTED IN THIS THESIS

When the work presented in this thesis was begun, little was known about the function of the UL8 protein in the context of the helicase-primase complex, aside from it being essential for efficient nuclear localisation of the complex (Calder *et al.*, 1992) and for HSV-1 origin-dependent DNA replication (Wu *et al.*, 1988; McGeoch *et al.*, 1988b). Understanding the roles of the UL8 protein in HSV-1 DNA replication could be important for the development of antivirals against herpesviruses, all of which possess proteins homologous to UL8.

The main aim of the work presented here was thus to relate the structure of the UL8 protein to its known functions by creating UL8 deletion mutants and assessing their ability to replace *wt* UL8 in facilitating nuclear localisation of the helicase-primase complex and in supporting HSV-1 origin-dependent DNA synthesis in transient transfection assays.

The *wt* and mutated UL8 proteins were initially expressed from plasmids in transfected cells, under the control of a strong constitutive promoter. A plasmid-based assay for intracellular localisation of the helicase-primase complex was therefore developed, to circumvent the requirement for constructing recombinant herpesviruses expressing the mutated UL8 proteins. Monoclonal antibodies raised against UL8 (Parry, 1993) were characterised and employed to investigate further the properties of the mutated UL8 proteins.

Subsequent to starting these studies, UL8 was demonstrated to coprecipitate with the UL5 protein and with the UL52 protein from cells infected with appropriate recombinant baculoviruses (McLean *et al.*, 1994). Biochemical assays of protein complexes purified from recombinant baculovirus-infected cells had also shown that UL8 influences the rate of primer synthesis (Tenney *et al.*, 1994) and efficiency of primer utilisation (Sherman *et al.*, 1992) during DNA synthesis.

Selected mutations were therefore used to generate recombinant baculoviruses, permitting expression of sufficient quantities of the UL8 proteins for use in experiments aimed at identifying regions of UL8 essential for interaction with UL5 and UL52.

CHAPTER 2: MATERIALS AND METHODS

Section 2.1 MATERIALS

2.1.1 Chemicals and Reagents

Most analytical grade chemicals were purchased from either **BDH Ltd.** or **Sigma Chemical Co. Ltd.** Exceptions to this included:

Aldrich Chemical Co. Ltd.	-dimethyl sulphate (DMS)
Beecham Research	-ampicillin (Penbritin)
Bio-Rad Laboratories	-ammonium persulphate
	-Coomassie brilliant blue
	-gelatin
	-TEMED (N,N,N',N'-tetramethylethylenediamine)
DuPont Ltd.	-En^{3}Hance
Fluka Chemicals Ltd.	-formamide
Hayman Ltd.	-absolute alcohol 100
Melford Laboratories Ltd.	-caesium chloride
Pharmacia Ltd.	-DEAE-sephacel
	-dATP, dCTP, dGTP and dTTP

2.1.2 Miscellaneous Materials

The suppliers of miscellaneous materials were as follows:

Amersham International plc.	-Hybond-N nylon (0.45 μm)
	-Rainbow coloured protein m.w. markers (range 14.3k-200k)
Boehringer Mannheim	-agarose (multi-purpose)
Chance Propper Ltd.	-glass coverslips (16 mm diameter)
Citifluor Ltd.	-CITIFLUOR (mountant)
Kodak Ltd.	-autoradiographic film (XS1)
	-Ektachrome Elite 400 35 mm colour slide film
	-TriX-pan 400 35 mm film (black & white)
	-X-OMAT duplicating film
Medicell International Ltd.	-dialysis tubing
Pharmacia Ltd.	-Sephadex G-50
Polaroid (UK)	-Polaroid-667 film
Promega (Protoblot system)	-colour development solution (NBT & BCIP)
	-Tween 20 (polyoxethylene sorbitan monolaurate)
Schleicher & Schuell	-nitrocellulose (0.45 μm)
Wacker Chemicals	-wacker saline GF 38
Whatman International Ltd.	-Whatman 3 mm paper

2.1.3 Solutions

β-galactosidase staining solution	5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 1 mg/ml X-gal, in PBS/A
Bal31 buffer (5x)	60 mM $CaCl_2$, 60 mM $MgCl_2$, 1 M NaCl, 5 mM EDTA, 100 mM Tris.HCl (pH 8.0)
boiling mix	6% SDS, 30% stacking gel buffer, 30% glycerol, 210 mM ß-mercaptoethanol, 0.3% bromophenol blue
buffer E	100 mM Tris.HCl (pH 8.0), 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.5 mM PMSF
chloroform/isoamyl alcohol	chloroform:isoamyl alcohol, 24:1 (v/v)
CLB (cell lysis buffer)	1 mM EDTA, 10 mM Tris.HCl (pH 7.5), 0.6% SDS
Denhardt's reagent (50x)	1% polyvinylpyrrolidine, 1% Ficoll 400, 1% BSA
destaining solution (gel)	10% methanol, 5% glacial acetic acid in H_2O
elution buffer	1 mM EDTA, 500 mM NH_4OAc (pH 7.0), 0.1% SDS
fixing solution (gel)	methanol: H_2O: glacial acetic acid, 50:50:7 (v/v)
formamide/TBE dyes	80% formamide, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue, in 0.5 x TBE
Gel Soak I	0.6 M NaCl, 0.2 M NaOH
Gel soak II	0.6 M NaCl, 1.0 M Tris.HCl (pH 8.0)
H (hybridisation) buffer (final concentrations)	6 x SSC, 5 x Denhardt's reagent, 0.05% SDS, 50 µg/ml denatured CT DNA, 20 mM Tris.HCl (pH 7.5), 1 mM EDTA
HBS (lipofection)	150 mM NaCl, 20 mM hepes.NaOH (pH7.4)
HeBS (transfection)	137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.5 mM D-glucose, 21 mM hepes.NaOH (pH 7.05)
methylcellulose medium	3% carboxymethylcellulose sodium salt in H_2O
NT buffer (10x) (nick translation)	0.5 M Tris.HCl (pH 7.5), 0.1 M $MgCl_2$, 10 mM DTT, 0.5 mg/ml BSA
PBS/A	170 mM NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.2)
PBS/ABC	PBS/A supplemented with 6.8 mM $CaCl_2$ and 4.9 mM $MgCl_2$
PBS/FCS	PBS/A supplemented with 1% FCS
permeabilisation buffer	10% sucrose and 0.5% Nonidet-P40 in PBS/A
prehybridisation buffer	6 x SSC, 5 x Denhardt's solution, 0.1% SDS, 20 µg/ml denatured CT DNA

resolving gel buffer (4x)	1.5 M Tris.HCl (pH 8.9), 0.4% SDS
sample buffer	1 vol. boiling mix and 2 vol. H_2O
SE (soluble extract) buffer	100 mM NaCl, 20 mM Tris.HCl (pH 8.0), 10% glycerol
stacking gel buffer (4x)	0.488 M Tris.HCl (pH 6.7), 0.4% SDS
20 x SSC	3 M NaCl, 0.3 M trisodium citrate
STET buffer	50 mM EDTA, 10 mM Tris.HCl (pH 8.0), 8% sucrose, 0.5% Triton X-100
sucrose reagent	250 mM sucrose, 50 mM Tris.HCl (pH 8.0), 2 mM $MgCl_2$,
1 x tank buffer (SDS-PAGE)	52 mM Tris base, 53 mM glycine, 0.1% SDS
TBE	90 mM Tris base, 89 mM boric acid, 1 mM EDTA
TBE dyes (5x)	50% sucrose and 0.25% bromophenol blue in 5 x TBE
TBS	10 mM Tris.HCl (pH 8.0), 150 mM NaCl
TBST	0.05% Tween 20 in TBS
TE	10 mM Tris.HCl (pH 7.5), 1 mM EDTA
transfer (Towbin) buffer	192 mM glycine, 25 mM Tris.HCl (pH 8.3), 20% (v/v) methanol
Triton reagent	62.5 mM EDTA, 50 mM Tris.HCl (pH 8.0), 0.5% Triton X-100
trypsin	0.25% trypsin in TS
TS	25 mM Tris.HCl (pH 7.4), 140 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 1mg/ml dextrose, 100 units/ml penicillin, 100 µg/ml streptomycin
TSB (transformation and storage buffer)	10 mM $MgCl_2$, 10 mM $MgSO_4$, 10% (w/v) PEG (m.w. 3350) and 5% (v/v) DMSO in L-broth pH 6.1
TSB/20 mM glucose	20 mM glucose in TSB
versene	0.6 mM EDTA and 0.02% phenol red in PBS/A

2.1.4 Enzymes

Restriction enzymes, nuclease Bal31, proteinase K and hexanucleotide mix were supplied by **Boehringer Mannheim** and T4 DNA ligase from **Life Technologies Ltd.**

T4 DNA polymerase, *E. coli* DNA polymerase I and T4 polynucleotide kinase were from **New England Biolabs**; DNA polymerase (Klenow fragment) was made in the Institute by Dr E. Telford.

Lysozyme (from chicken egg white), pronase (protease type XIV from *S. griseus*), DNase I, RNase A (type 1A) and RNase T_1 (grade IV) were manufactured by **Sigma.**

2.1.5 Radiochemicals

All radio-labelled compounds were supplied by **Amersham International plc.**

	Specific Activity
5' [α-^{32}P] deoxyribonucleoside triphosphates	3000 Ci/mmol (10 µCi/µl)
[^{35}S]-L-methionine	800 Ci/mmol (15 µCi/µl)

2.1.6 Cells and Tissue Culture Media

Baby hamster kidney 21 clone 13 (BHK) cells (MacPherson & Stoker, 1962) were maintained in ETC10 (Glasgow Modified Eagle's Medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 10% tryptose phosphate broth and 10% newborn calf serum).

Spodoptera frugiperda (*S.f.*) cells (strain IPLB-SF-21; Vaughn *et al.*, 1977) were maintained in TC100/5 (TC100 medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 5% foetal calf serum).

Tissue culture reagents were obtained from **Life Technologies Ltd.** and included Glasgow Modified Eagle's Medium (GMEM); Optimem 1; TC100 medium; TC100 medium lacking methionine and bacto-yeast extract; NBCS; penicillin and streptomycin. FCS was supplied by **Biological Industries.**

The following solutions were also used in conjunction with cultured cells:

Eagle's wash	(GMEM with 100 units/ml penicillin and 100 µg/ml streptomycin)
EC5	(GMEM supplemented with the above antibiotics and 5% NBCS)
BHK storage medium	(GMEM with the above antibiotics, 25% NBCS and 10% glycerol)
TC100 wash	(TC100 with 100 units/ml penicillin and 100 µg/ml streptomycin)
S.f. storage medium	(TC100 with the above antibiotics, 10% FCS and 10% DMSO)
Optimem pH6	(Optimem 1 with the pH adjusted to 6.0 using HCl)

2.1.7 Viruses

The temperature sensitive mutant HSV-1 *ts*K *syn*$^+$ virus (Marsden *et al.*, 1976) and the recombinant viruses *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 and *ts*K/UL52 (Calder *et al.*, 1992) were obtained from Dr N. D. Stow.

The recombinant baculoviruses AcUL5, AcUL8, AcUL52 (Calder & Stow, 1990) and AcUL9 (Stow, 1992) were also provided by Dr N. D. Stow.

AcPAK6, containing the *lac Z* gene under the control of the polyhedrin promoter, flanked by two additional *Bsu*36I sites (BacPAK6, Kitts & Possee, 1993), was used in the construction of recombinant baculoviruses (as described in Methods) and was supplied by Dr N. D. Stow.

2.1.8 Bacterial Strains and Culture Media

Plasmids were propagated in *E. coli* strain DH5 (Hanahan, 1985). The bacteria were grown in L-broth (10 g/l NaCl, 10 g/l bactopeptone, 5 g/l yeast extract) or on LB-agar plates (1.5% w/v agar in L-broth), both supplemented with ampicillin to 50 µg/ml when appropriate.

2.1.9 Plasmids

The following plasmids were provided by Dr N. D. Stow.

Plasmid pCMV10 contains the major immediate-early (IE) promoter of human cytomegalovirus (HCMV), a polylinker multiple cloning site, and simian virus 40 (SV40) RNA-processing sequences (Stow *et al.*, 1993).

Plasmids pE5, pE8, pE9, pE29, pE30, pE42 and pE52 contain HSV-1 genes UL5, UL8, UL9, UL29, UL30, UL42 and UL52 respectively, inserted into the *Bam*HI site (except *Eco*RI site for pE9, and *Xba*I site for pE29 and pE30) of the pCMV10 multiple cloning region (Stow *et al.*, 1993).

Plasmid pM2 is a similar construct containing the *Escherichia coli* ß-galacto-sidase gene *lac Z* under the control of the HCMV IE promoter (essentially identical to pM1 in Stow *et al.*, 1993). This plasmid was used as a control for checking transfection efficiencies.

Plasmid p9CT is a mutated version of pE9 in which part of the UL9 gene has been deleted such that it specifies a product consisting of the N-terminal 10 and C-terminal 317 amino acids of *wt* UL9 protein (Stow *et al.*, 1993).

Plasmid pS1 consists of the vector pAT153 with a 435-bp insert encoding a functional HSV-1 ori_S replication origin (Stow & McMonagle, 1983).

Plasmid pTZ19U (Mead *et al.*, 1986) is a standard cloning vector containing a multiple cloning site and conferring ampicillin resistance. It has vector sequences in common with pS1 and was used as a probe in the plasmid amplification assay.

Plasmid pAcYM1 is a transfer vector (Matsuura *et al.*, 1987) containing the *Eco*RI i fragment of AcNPV, including upstream promoter sequences, the untranslated leader, and the A of the initiation codon of the AcNPV polyhedrin gene, but lacking the rest of the polyhedrin open reading frame. The plasmid contains a unique *Bam*HI site into which genes can be cloned for expresssion.

2.1.10 Antibodies

The following antibodies were used for the detection of proteins:

Rabbit antisera raised against synthetic deca- or octa-peptides from the C-termini of the UL5, UL9 and UL52 proteins (Olivo *et al.*, 1989) were provided by Dr M. D. Challberg.

Rabbit antiserum 064, raised against a synthetic peptide corresponding to amino acids 2-16 of the UL8 protein (Parry *et al.*, 1993) and rabbit antisera 094 and 105, raised against whole purified UL8 protein (Parry, 1993), were provided by Dr M.E. Parry.

Mouse ascitic fluid containing monoclonal antibodies (MAbs) 0801 through to 0820, raised against purified UL8 protein (Parry *et al.*, 1993) as described by McLean *et al.* (1994), were also provided by Dr M.E. Parry.

Secondary conjugated antibodies and their suppliers were as follows:

Sigma Chemical Co.: Goat anti-rabbit IgG (whole molecule) FITC (fluorescein)-conjugated antibody

Goat anti-mouse IgG (whole molecule) FITC (fluorescein)-conjugated antibody

Goat anti-mouse IgG (whole molecule) TRITC (rhodamine)-conjugated antibody

Promega Ltd.: Goat anti-rabbit IgG (crystallisable fragment) alkaline phosphatase-conjugated antibody

Goat anti-mouse IgG (heavy & light chain) alkaline phosphatase-conjugated antibody

Section 2.2 METHODS

2.2.1 Tissue culture and preparation of virus stocks

a) Tissue culture of BHK cells

Cells were grown in ETC10 in 175 cm^2 tissue culture flasks, at 37°C in an atmosphere of 5% carbon dioxide (CO_2) in air. Confluent monolayers were washed once with versene and once with versene:trypsin (1:1 by vol.) before being resuspended in 10 ml ETC10 for routine passaging. Cells remained viable at 4°C for up to five days, and were discarded after 30 passages.

b) Tissue culture of *S.f.* cells

Cells were grown in TC100/5 in 175 cm^2 tisue culture flasks, at 28°C in ungassed incubators. Cells were routinely passaged by banging the flask to dislodge the cells and resuspending them in 10 ml TC100/5. Cells remained viable at 4°C for up to two days.

c) **Long-term storage**

The cells from one flask were resuspended in 5-10 ml storage medium, divided into 1 ml aliquots, and cooled slowly to -70°C before being stored in liquid nitrogen (-190°C).

d) **Preparation of HSV-1 *ts*K and *ts*K recombinant virus stocks**

BHK cell monolayers in 850 cm^2 roller bottles were infected with virus at a m.o.i. of 0.02 pfu per cell in 40 ml EC5. Infected cell cultures were maintained at 31°C (the permissive temperature) for approximately 3 days until extensive cytopathic effect (cpe) was evident. The infected cells were harvested by shaking them into the medium, and pelleted at 2000 rpm for 5 min at 4°C (Beckman GPR centrifuge). Each cell pellet was resuspended in 5 ml EC5 and cell-associated virus (CAV) was released by two rounds of sonication. Each supernatant was transferred to a fresh tube and spun at 10000 rpm for 150 min at 4°C (Sorvall GSA rotor). The pelleted cell-released virus (CRV) was gently resuspended in 5 ml EC5. CAV and CRV were stored in aliquots at -70°C.

e) **Preparation of AcNPV *wt* and recombinant virus stocks**

S.f. cell monolayers grown in 175 cm^2 flasks were infected when 50% confluent with *wt* or recombinant virus at a m.o.i. of 5 pfu per cell. The virus was added in a total volume of 10 ml TC100/5 and adsorbed for 1 hr at room temperature. Following addition of a further 40 ml TC100/5, infected flasks were incubated at 28°C for 3-4 days, until most of the cells had detached from the plastic. Infected cells were shaken into the medium and pelleted at 2000 rpm for 5 min at 4°C (Beckman GPR centrifuge). If required the cell pellet was processed as described below. The supernatant virus was either left unconcentrated, or was concentrated by centrifugation at 4°C at 12000 rpm for 180 min (Sorvall GSA rotor) or 18000 rpm for 90 min (Sorvall SS34 rotor). The virus pellet was resuspended in 5 ml TC100/5 per flask and sonicated. Virus stocks were divided into aliquots and stored at 4°C or -70°C.

Preparation of total infected cell proteins from *S.f.* cells: The cell pellet obtained from the process described above was washed twice in TS and then resuspended in 1 ml TS. A sample containing total proteins for analysis by SDS-PAGE or Western blotting was made by mixing one vol. cells in TS with 3 vols. water and 2 vols. boiling mix. Total protein samples were stored at -20°C. Washed cell pellets were stored at -70°C.

f) Sterility checks

Sterility checks were performed by streaking virus preparations onto blood agar plates and incubating at 37°C for 5 days (HSV-1 stocks) or 28°C for 4 days (AcNPV stocks).

g) Titration of HSV-1 *ts*K virus stocks

Virus stocks were serially diluted 10-fold in PBS/ABC containing 8% NBCS. 90% confluent BHK cell monolayers in 35 mm plates were inoculated with 0.1 ml aliquots of virus dilutions 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}. After 1 hr adsorption time at 31°C (permissive temperature) the inoculum was removed and the cells were overlaid with methylcellulose medium to prevent secondary plaque formation. Incubation was continued at 31°C for 4 days, then 2 ml Giemsa stain was added to each plate. After 30 min at room temperature the plates were rinsed to remove stain and methylcellulose, and plaques counted using a dissecting microscope.

h) Titration of AcNPV stocks

Baculovirus stocks were titrated on *S.f.* cell monolayers in 35 mm plates as described by Brown & Faulkner (1977). Serial ten-fold dilutions of virus were prepared in TC100/5. The growth medium was removed from the plates and the monolayers were inoculated with 0.1 ml volumes of diluted virus. After 1 hr at room temperature, the inoculum was removed and replaced with 1.5 ml of overlay medium (equal volumes of molten 3% (w/v) low gelling temperature agarose (Seaplaque) and TC100/5 prewarmed to 37°C). 1.5 ml of TC100/5 was added as a liquid overlay to the solidified agarose and the plates were incubated at 28°C for 4 days. 0.5 ml of neutral red stain (a 1:25 dilution of 0.5% w/v neutral red stock) was then added and the plates were returned to 28°C. 24 hr later the plaques were counted.

2.2.2 Transfection of cells with plasmid DNA

a) Preparation of liposomes

Liposomes containing 1 mg/ml DOPE and 0.4 mg/ml DDAB (Rose *et al.*, 1991) work well with both BHK C13 cells and *S.f.* cells, and were prepared as follows.
1.0 ml DOPE (10 mg/ml in chloroform) and 0.4 ml DDAB (10 mg/ml in chloroform) were mixed in a sterile glass universal and the chloroform was evaporated off using nitrogen gas followed by 30 min in a lyophiliser. The lipids were resuspended in 10 ml sterile H_2O by sonication in a water bath sonicator. Liposomes were generated from this lipid suspension by sonication using a probe sonicator (15 sec bursts every min for 20 min, until no further clearing of the suspension occurred). Liposomes were stored at 4°C. The optimum volume of liposomes used to lipofect a 35 mm plate of cells was

determined using a range of liposome volumes and a fixed amount of pM2 plasmid DNA (containing the *lac Z* gene). 24 hr post-lipofection, plates were fixed and stained for ß-galactosidase expression, and the number of positive cells counted.

b) Lipofection of BHK cells for immunofluorescence studies and SDS-PAGE analysis

Approximately 1.2-1.5 x 10^5 BHK cells were seeded in 35 mm plates one day prior to lipofection. For each plate, 1.5-2.0 µg plasmid DNA was mixed with 85 µl HBS and 0.5 ml Optimem 1 by gentle vortexing. 10 µl of liposomes diluted in 0.5 ml Optimem 1 was added, and mixed again. After 10 min the BHK monolayers were washed with Eagle's wash, drained and the liposome mix was added. After 4 hr incubation at 37°C, the cells were overlaid with 1 ml EC5 per plate and incubation was continued at 37°C.

c) Calcium phosphate transfection of BHK cells for transient replication assays (Stow and Wilkie, 1976)

Approximately 1 x 10^6 BHK cells were seeded in 35 mm plates one day prior to transfection. 0.5 ml HeBS containing 11 µg calf thymus (CT) carrier DNA and 0.625 µg of each of the indicated plasmids was mixed rapidly with 35 µl 2M $CaCl_2$ and allowed to form a precipitate for 5 min. The medium was removed from the plate of cells and 0.4 ml of $CaPO_4$ precipitate was added to the cells. After 40 min at 37°C, the cells were overlaid with 3 ml EC5 and incubation was continued at 37°C. 4 hr post-transfection the cells were 'boosted' with 22.5% DMSO in HeBS for 4 min, washed with Eagle's wash and returned to 37°C with 3 ml EC5 per plate.

2.2.3 Generation and isolation of recombinant baculoviruses

The preparation of AcPAK6 DNA for transfection and the purification of baculovirus recombinants were performed essentially as described by O'Reilly *et al.* (1992).

a) Preparation of AcPAK6 DNA

Four 175 cm^2 flasks of *S.f.* cells infected with AcPAK6 were harvested and the infected cells pelleted from the medium at 5000 rpm for 5 min at 4°C (Sorvall GSA rotor). The supernatant virus (CRV) was pelleted by centrifugation at 18000 rpm for 120 min at 4°C (Sorvall SS34 rotor). The virus pellet was resuspended in 2 ml TE by sonication. After addition of SDS to 0.6% and proteinase K to 200 mg/ml, the virus suspension was incubated at 50°C for 2 hr. Following extraction with phenol and with chloroform/isoamyl alcohol, the DNA was dialysed against TE and stored at -20°C.

Viral DNA was cut with *Bsu*36I (5 units enzyme/μg DNA), extracted sequentially with phenol and chloroform/isoamyl alcohol, ethanol precipitated and redissolved in TE. Complete digestion was confirmed by cutting a sample with a second restriction enzyme (*Eco*RI) and running the products alongside *Eco*RI-cut AcPAK6 DNA on a 1% agarose mini-gel.

b) Transfection of cells to generate AcNPV recombinants

Bsu36I-cleaved AcPAK6 viral DNA (as prepared above) and uncut transfer vector DNA (pAcYM1 with mutated UL8 sequences inserted) were cotransfected into *S.f.* cells using liposomes as follows.

Approximately 1.5 x 10^6 *S.f.* cells were seeded in 35 mm plates one day prior to lipofection. 1 μg Bsu36I-cleaved AcPAK6 DNA and 3 μg transfer vector DNA in 0.5 ml Optimem pH6 were mixed with 15 μl liposomes in 0.5 ml Optimem pH6. After 10 min the cell monolayers were washed once with Optimem pH6, drained and the liposome mix was added. After 4 hr at 28°C, the cells were overlaid with 2 ml TC100/5 and incubation was continued at 28°C for 2-3 days. The supernatant virus (CRV) was then removed from the plates and recombinant viruses isolated as described below.

c) Amplification of AcNPV recombinants

Approximately 3 x 10^5 *S.f.* cells in linbro wells were inoculated with 0.5 ml of supernatant virus from the above lipofection and incubated at 28°C for 3-5 days. The supernatant virus was then removed from the linbro wells and half of it (0.5 ml) was used to infect a second round of *S.f.* cells in linbro wells. 3-5 days p.i. the supernatant virus was removed and stored at -70°C, ready for purification of recombinant viruses (see below). The infected cells were gently washed with TS, lysed in sample buffer, and the infected cell proteins analysed by SDS-PAGE and Western blotting, to confirm the presence of recombinants.

d) Purification of AcNPV recombinants

Recombinant viruses were purified by performing limiting dilutions on microtitre wells of *S.f.* cells. The titre of linbro well supernatant virus (from above) was estimated to be approximately 5 x 10^6 pfu/ml.

A 10^{-2} dilution of linbro well supernatant virus was made (10 μl in 1 ml TC100/5). 20 μl of this diluted virus was added to 1.5 ml TC100/5 in a bijou and serial three-fold dilutions were performed in bijoux, resulting in eight 1 ml volumes of TC100/5 containing between an estimated 666 pfu and 0.333 pfu of virus per bijou. 7.8 x 10^5 *S.f.* cells in 1.6 ml TC100/5 were added to each bijou. Twelve 200 μl aliquots from each bijou were plated out across the wells of a microtitre tray, so that the wells of row A contained an estimated 51 pfu/well and the wells of row H 0.025 pfu/well.

After 3 days at 28°C, 80 µl supernatant from each well was removed, mixed with 0.6 x 10^5 *S.f.* cells in suspension (100 µl) and plated out into corresponding positions in a fresh microtitre well tray. Following a further 3 days at 28°C, the microtitre wells were screened for cpe. The supernatants from 6-8 infected wells of the greatest dilutions yielding an average of less than one pfu/well (*i.e.* not more than 4 infected wells per row - see theoretical note below) were each removed into 1 ml TC100/5.

0.5 ml of this diluted microtitre well supernatant was mixed with 3 x 10^5 *S.f.* cells in suspension and plated out in linbro wells. After 3 days at 28°C, the supernatants were harvested and stored at -70°C, and the cells were taken up in 1 ml TS, spun at 2000 rpm for 5 min (Beckman GPR centrifuge) and resuspended in 60 ml sample buffer. 20 µl was electrophoresed on a 9% SDS-polyacrylamide gel to check for the expression of recombinant protein. Virus stocks were prepared by further passaging the supernatants from wells in which expression of the desired protein was detected.

Theoretical note (adapted from O'Reilly *et al.*, 1992):
Virus particles in solution are distributed according to the Poisson distribution.
Hence, the proportion (p) of cultures receiving no infectious units is $p = e^{-\mu}$,
(where μ is the mean concentration of infectious units in the dilution),
the proportion receiving one or more infectious particles is $1 - e^{-\mu}$,
and the fraction receiving only one infectious unit is $\mu e^{-\mu}$.
The ratio of pure cultures to the total number of cultures infected is $\mu e^{-\mu} / (1 - e^{-\mu})$.
If we require that 95% of all infected cultures are initiated by a single infectious unit, then $\mu e^{-\mu} / (1 - e^{-\mu}) = 0.95$ which, when solved, gives $\mu = 0.094$.
The proportion of wells remaining uninfected is $e^{-\mu} = 0.91$.
Thus, to be 95% confident that any infected well received only one infectious unit, only 10% of the wells should show infection (or 1 out of 12 wells).
Likewise, to be 90% confident that any infected well received only one infectious unit, no more than 2 out of 12 wells should show infection, and to be 80% confident, no more than 4 out of 12 wells should show infection.

2.2.4 Staining of cells

a) Staining for ß-galactosidase expression in transfected cells

Transfected BHK cell monolayers, usually in 35 mm plates, were rinsed briefly with PBS/A, drained and fixed for 5 min in a solution of 2% formaldehyde/0.2% glutaraldehyde in PBS/A (2 ml per plate). After rinsing the cells with PBS/A to remove the fixative solution, 1 ml of ß-galactosidase staining solution was added per plate and the cells were incubated at 37°C. 12-24 hr later the stain was removed and water was

added. Cells expressing ß-galactosidase appeared blue and the number of blue cells in representative fields of view under the microscope were counted.

b) Immunofluorescent staining of proteins in infected or transfected cells

Infection. Approximately 1.2 x 10^5 BHK cells were seeded in 35 mm plates one day prior to infection. 1 x 10^7 pfu of *ts*K or *ts*K-recombinant virus in a total volume of 0.1 ml Eagle's wash was added to each plate at 37°C, and the plates were incubated at 38.5°C in 5% CO_2 in air for 45 min, with gentle agitation every 15 min. The inoculum was removed and cells were washed once with Eagle's wash (prewarmed to 38.5°C) after which 2 ml of prewarmed EC5 was added and incubation was continued at 38.5°C. 7-10 hr post-infection cells were fixed (see below).

Transfection. Sparse monolayers of BHK cells in 35 mm plates were transfected with plasmid DNA using liposomes (for up to 3 plasmids/plate) or $CaPO_4$ precipitation (for more than 3 plasmids/plate) as described in section 2.2.2. 24-30 hr post-transfection the monolayers were fixed.

Fixation. Cell monolayers were briefly washed twice with PBS/A, drained and fixed for 2 min in 2 ml/plate of 50% acetone/50% methanol. After 3 more washes with PBS/A, the plates were drained ready for addition of antibody.

Staining. Immunofluorescent staining was adapted from a method of Randall & Dinwoodie (1986). The fixed cell monolayers were briefly air dried before addition of 50 µl of primary antibody, diluted in PBS/FCS, to a marked 16 mm diameter circle of cells in the centre of each plate. After 1 hr incubation at room temperature, the monolayers were washed four times with PBS/A and briefly air dried again. 50 µl of a 1:80 dilution of secondary antibody (goat anti-rabbit or anti-mouse IgG antibody as appropriate, conjugated with a fluorescent marker) was added to the same marked circle of cells and incubated for 1 hr at room temperature. The monolayers were washed once with permeabilisation buffer and four times with PBS/A, air dried and mounted in CITIFLUOR under 16 mm glass coverslips.

Plates were viewed under the x25, x40 and x50 objectives of a Nikon Microphot-SA fluorescence microscope using red and green filters as appropriate, and photographed on Kodak TriX-pan 400 black and white 35 mm film or Kodak Ektachrome Elite 400 35 mm colour slide film.

2.2.5 Manipulation and propagation of DNA

a) Restriction enzyme digestion of DNA

Restriction enzyme digestions were performed using commercial restriction enzymes and buffers. Usually 1 µg DNA was incubated with 1-5 units enzyme and the

appropriate buffer in a final volume of 25 µl for 3-4 hr at the recommended temperature.

b) Deletion of plasmid DNA with the nuclease Bal31

Approximately 35 µg of plasmid pE8 DNA was cut with an excess (2.5 units/µg DNA) of *Xba*I (for C-terminal truncations) or *Eco*RI (for N-terminal truncations), serially extracted with phenol and chloroform/isoamyl alcohol, ethanol precipitated and resuspended in 190 µl H_2O. This linearised pE8 DNA was incubated at 31°C with 2 units of nuclease Bal31 and 45 µl of Bal31 buffer. At various times (2-30 min for smaller deletions, 10-60 min for larger deletions), aliquots of 30-40 µl were removed into phenol containing 20 mM EGTA and vortexed to stop the digestion. Following sequential extractions with phenol and chloroform/isoamyl alcohol, the samples were ethanol precipitated and resuspended in 22 µl H_2O. The course of the Bal31 digestion was checked by cutting a 0.5 µg sample from each timepoint with 5 units *Xho*I (for C-terminal truncations) or *Sma*I (for N-terminal truncations) and electrophoresing the products through a 1% agarose mini-gel.

c) Fill-in synthesis of DNA

The following method was used to generate blunt ended DNA from DNA with 3' or 5' overhanging ends as a result of restriction enzyme digestion.

10 µg DNA was incubated with 3 units of T4 DNA polymerase, 3 µl of 10x T4 pol buffer and 0.33 mM concentrations of dATP, dCTP, dGTP and dTTP in a total volume of 30 µl, for 30 min at 37°C. After serial extractions with phenol and chloroform/isoamyl alcohol, the DNA was ethanol precipitated and resuspended in 20 µl H_2O.

d) Ligation of DNA fragments

For ligation of an oligonucleotide linker or a DNA fragment to a linearised plasmid, the ratios were usually calculated so that the 5' ends of the linker or fragment were in a 10-100 fold molar excess compared to the 5' ends of the plasmid. In addition, the plasmid was usually linearised in the presence of calf intestinal phosphatase, to prevent recircularisation during the ligation. The concentration of total DNA in the ligation mix was 50-100 µg/ml.

The DNAs were mixed with 4 µl of 5x ligation buffer and 1 unit of T4 DNA ligase in a total volume of 20 µl, and incubated at room temperature overnight. If necessary, the ligase was then inactivated at 70°C for 10 min prior to digestion with an appropriate restriction enzyme.

DNA was sequentially extracted with phenol and chloroform/isoamyl alcohol, ethanol precipitated and resuspended in H_2O or TE, prior to transformation of *E. coli*.

e) Preparation and transformation of competent *E. coli* (Chung *et al.*, 1989)

E. coli DH5 (Amp^s) bacterial cells were cultured in L-broth at 37°C to an optical density (OD_{600}) of 0.3-0.6. Cells were pelleted from 20 ml of culture at 2500 rpm for 10 min at 4°C (Beckman GPR centrifuge) and resuspended in 2 ml TSB at 4°C. After 10 min incubation on ice, the cell suspension was divided into 100 µl aliquots in polypropylene tubes. Aliquots not required immediately were stored at -70°C.

Approximately 100 pg plasmid DNA was added per tube of competent cells and the tubes were incubated on ice for 5-30 min. 0.9 ml TSB/20 mM glucose was added per tube and the tubes were incubated at 37°C for 1 hr with shaking, to allow expression of the plasmid ß-lactamase gene conferring Amp^r. 100 µl aliquots of transformed cells were plated out at zero, 10^{-1} and 10^{-2} dilutions on LB-agar/ampicillin, and incubated at 37°C overnight. Colonies were picked and shaken at 37°C overnight in 5 ml of L-broth containing 50 µg/ml ampicillin, after which small scale plasmid preparations were made.

f) Small scale plasmid preparation (mini-prep) (Sambrook *et al.*, 1989)

Cells were pelleted from 1 ml of transformed bacterial culture by centrifugation at 12000 rpm for 10 sec (MSE Microfuge). The pellet was resuspended in 100 µl STET buffer by vortexing, and 16 µl of fresh lysozyme (10 mg/ml H_2O) was added. The samples were boiled for 50 sec and immediately centrifuged at 12000 rpm for 10 min at room temperature (MSE Microfuge). The pellets were removed, and the DNA was precipitated from the supernatant with 100 µl isopropanol for 5 min at -20°C, followed by centrifugation at 12000 rpm for 5 min (MSE Microfuge). The DNA pellet was dried and resuspended in 100 µl TE containing RNase A (5 µg/ml) and RNase T_1 (10 units/ml). Mini-prep plasmid DNA was stored at -20°C and 5 µl samples were used for restriction enzyme analysis.

g) Large scale plasmid preparation

Starter cultures were prepared by inoculation of 5 ml L-broth containing 50 µg/ml ampicillin with either a colony picked from an LB-agar/ampicillin plate or 10 µl of bacterial stock (stored in 7% DMSO at -70°C). Starter cultures were shaken at 37°C for approximately 8 hr, then added to 400 ml L-broth containing 50 µg/ml ampicillin, in 2 litre conical flasks. The flasks were shaken at 37°C overnight.

Bacterial cells were pelleted from the medium at 8000 rpm for 10 min at 4°C (Sorvall GS3 rotor), washed in 8 ml TE and re-pelleted at 5000 rpm for 5 min at 4°C (Sorvall SS34 rotor). Cell pellets were resuspended in 2 ml sucrose reagent by vortexing, 400 µl of fresh lysozyme (20 mg/ml H_2O) were added and the cells were incubated at 4°C for 1-2 hr. 0.8 ml of 0.25 M EDTA and 3.2 ml Triton reagent were added and incubation was continued at 4°C for a further 15 min, after which the cells

were pelleted by centrifugation at 35000 rpm for 30 min at 4°C (Sorvall Ti50 or T865 rotor). The supernatant volume was adjusted to 9 ml with H_2O and 9 g caesium chloride (CsCl) were added to give a final density of 1.55-1.60 g/ml. Following the addition of 200 µl ethidium bromide (10 mg/ml) the mixture was transferred to cellulose nitrate tubes and centrifuged at 44000 rpm for 16 hr at 15°C (Sorvall TV865 rotor). DNA bands were visualised under long wave UV light and the lower band containing supercoiled plasmid DNA was extracted using a hypodermic needle and a 2 ml syringe. Ethidium bromide (EtBr) was removed by extraction with equal volumes of isoamyl alcohol (usually four times) until no fluorescence was detectable under long wave UV light. Caesium chloride was removed by dialysing the DNA preparation against two 2-4 litre volumes of TE buffer for a total of 16 hr at 4°C. Plasmid DNA preparations were stored at -20°C.

The quality of plasmid DNA preparations was checked by linearising 1 µl samples with a restriction enzyme and electrophoresing the products through a 1% agarose mini-gel. Plasmid DNA was quantified by UV absorption at 260 nm, using the calculation that an absorbance value of 1.0 corresponds to 50 µg/ml ds DNA.

2.2.6 Gel electrophoresis of nucleic acids

a) Non-denaturing agarose gels

Horizontal slab gels (21.5 cm x 16.5 cm) or minigels (10 cm x 7 cm) of 0.6-1.0% agarose in 1 x TBE containing 0.5 µg/ml EtBr were prepared. DNA samples were mixed with a 1/4 volume of TBE dyes, loaded and electrophoresed at 30 V overnight or 60 V for 5 hr (slab gels), or 50-60 V for 2-3 hr (minigels).

b) Purification of DNA from non-denaturing agarose gels

DNA fragments which had been resolved by electrophoresis through an agarose gel were visualised under long wave UV light and the required DNA band was excised from the gel. The gel slice was sealed inside dialysis tubing along with 1-2 ml 1 x TBE (without EtBr), placed in 1 x TBE at 90° to the current flow, and electroeluted at 200 V for 2 hr. The DNA-containing TBE buffer was removed from the tubing, centrifuged at 12000 rpm (MSE Microfuge) for 2 min to remove residual gel fragments, and applied to a 0.4 ml bed volume DEAE sephacel column. The column was washed before and after loading the DNA sample with 5 ml TE containing 0.1 M NaCl. The DNA was eluted from the column in two 0.4 ml aliquots of TE containing 1 M NaCl, precipitated with 2.5 volumes of ethanol, and resuspended in TE. DNA fragments which were being prepared for sequencing were ethanol precipitated in the presence of sonicated CT DNA and resuspended in H_2O.

c) Non-denaturing polyacrylamide gels

For the resolution of low molecular weight fragments of DNA, 1.5 mm thick vertical slab gels (18 cm x 22 cm) of 8% polyacrylamide (acrylamide:bisacrylamide 39:1, polymerised with 0.01 vol. 10% ammonium persulphate and 0.001 vol. TEMED) were run in 1 x TBE. Samples were loaded as for agarose gels, the gels electrophoresed at 150 V for 2-3 hr, and stained in 1 x TBE containing 1 µg/ml EtBr for 15 min.

d) Denaturing polyacrylamide gels

The products of DNA sequencing reactions were resolved on denaturing 16% polyacrylamide gels, run in 1 x TBE. An 'instagel' mix containing 16% deionised acrylamide (acrylamide:bisacrylamide 29:1) and 8.3 M urea in 1 x TBE was made and stored in the dark at 4°C. 0.35 mm thick gels were prepared with 75 ml instagel and polymerised with 100 µl 25% ammonium persulphate and 75 µl TEMED in long vertical glass plate sandwiches, in which the front plate was coated with repelcote and the back plate with Wacker saline solution. 5 µl reaction samples in formamide/TBE dyes were heated to 100°C for 2 min and immediately loaded on to the gel. Electrophoresis was at 1 Watt/25 cm^2, until the bromophenol blue was approximately 1/3 of the way down the gel, so that sequences close to the labelled nucleotide could be read.

2.2.7 Preparation and radioactive labelling of DNA

a) Synthesis and purification of oligonucleotides

Oligonucleotides were synthesised within the Institute by Dr J McLauchlan and Mr Ricky van Deursen using a Cruachem PS250 DNA synthesizer, and were supplied following elution in 1 ml ammonia. After being unblocked by incubation at 55°C for 5 hr, the DNA was lyophilised overnight, resuspended in 50 µl H_2O and divided into two 25 µl aliquots. One aliquot was stored at -70°C and the other was lyophilised again for 2 hr. The DNA pellet was resuspended in 25 µl of 90% formamide in 1 x TBE, heated to 100°C for 1 min and loaded into 3 wells of a small vertical denaturing polyacrylamide gel (8% acrylamide with acrylamide:bisacrylamide 29:1 and 8 M urea in 1 x TBE). 5 µl of formamide/TBE dyes were loaded in an adjacent well and the gel was electrophoresed in 1 x TBE at 200 V for 4-6 hr until the bromophenol blue was approximately 2/3 of the way down the gel. The gel was transferred onto clingfilm and the oligonucleotide bands were visualised over a silica gel thin layer chromatography plate under a hand-held short wave UV lamp. The bands were excised and shaken end over end at 4°C overnight in 700 µl elution buffer (500 mM NH_4OAc, 0.1% SDS and 1 mM EDTA) in 1.5 ml Eppendorf tubes. The DNA-containing buffer was transferred to a fresh tube and centrifuged at 12000 rpm for 5 min (MSE Microfuge) to remove

fragments of gel. The supernatant was extracted sequentially with phenol/chloroform (1:1) and chloroform/isoamyl alcohol, and the DNA was ethanol precipitated and resuspended in 50 µl H_2O. The concentration of the recovered DNA was estimated from its absorbance value at 260nm, taking an A_{260} value of 1 as equivalent to 20 µg/ml ss DNA.

The following complementary single-stranded oligonucleotide linkers, containing an *Eco*RI cohesive end, an *Nco*I site and an ATG codon for initiation of translation, were synthesised:

17mer: $^{5'}$AATTCGCCACCATGGGG$^{3'}$

13mer: $^{3'}$GCGGTGGTACCCC$^{5'}$

b) <u>Phosphorylation of oligonucleotide linkers</u>

The above synthetic oligonucleotides were phosphorylated prior to ligation. Commercial oligonucleotide linkers, containing an *Xba*I restriction site and stop codons in all three frames, were also supplied unphosphorylated.

5 µg of oligonucleotide or 2.5 µg of *Xba*I linker were incubated with 10 units of T4 polynucleotide kinase in a total volume of 25 µl of kinase buffer, containing 7 mM DTT and 1 mM ATP, at 37°C for 30 min. After deactivating the kinase at 70°C for 10 min, the DNA was allowed to cool slowly to room temperature.

c) <u>Annealing oligonucleotides to form linkers</u>

Following phosphorylation, the synthetic 13mer and 17mer were annealed to form a linker. 4 µg of the 13mer and 5.2 µg of the 17mer were mixed together (total vol. approximately 40 µl), heated to 100°C for 2 min and incubated at 37°C for 30 min to allow annealing. Annealed linker was stored at -20°C.

d) <u>Preparation of CT DNA ($CaPO_4$ transfections)</u>

20 mg of calf thymus DNA (type I, Sigma) was dissolved in 10 ml TE and stored in aliquots at -20°C.

e) <u>Preparation of sonicated CT DNA (DNA sequencing)</u>

Calf thymus DNA (type I, Sigma), used as a competitor in sequencing reactions, was dissolved in H_2O at a concentration of 7.5 mg/ml, by sonication, which was continued until the solution was only slightly viscous. SDS was added to 1% and NaCl to 300 mM and the DNA was de-proteinised by serial extractions with phenol and chloroform/isoamyl alcohol. Following ethanol precipitation, the DNA was resuspended in 5 ml H_2O by vortexing, the absorbance at 260 nm was measured, and the DNA concentration was adjusted to 2 mg/ml. Sonicated CT DNA was stored at 4°C.

f) Preparation of denatured CT DNA (Southern blots)

Calf thymus DNA, used in pre-hybridisation and hybridisation buffers, was prepared by dissolving the DNA in sterile H_2O at a concentration of 2 mg/ml, followed by boiling for 15 min to denature it. Denatured CT DNA was stored at 4°C.

g) 3' end labelling of linearised plasmid DNA

Approximately 25 µg of linearised plasmid DNA was mixed with 10 µCi of [α ^{32}P]dGTP and 2 mM dATP/dCTP/dTTP (or 10 µCi of [α-^{32}P]dATP and 2 mM dCTP/dGTP/dTTP) in a final volume of 70 µl nick translation (NT) buffer together with 1 unit of Klenow fragment DNA pol I. After incubation at 14°C for 30 min 2 mM cold dGTP (or dATP) was added for a chase period of 10 min. If required the DNA polymerase was inactivated by heating to 70°C for 5 min.

h) Labelling of plasmid DNA by nick translation

250 ng pTZ19U DNA was labelled by nick translation (Rigby *et al.*, 1977) with 20 µCi each of [α-^{32}P]dGTP and [α-^{32}P]dCTP, using 1 unit of *E. coli* DNA polymerase I, 10^{-7} mg/ml DNase I, 50 µM cold dATP and 50 µM cold dTTP in a 34 µl final volume of NT buffer. After 75 min incubation at 16°C, the DNA was extracted once with phenol and applied to a Sephadex G-50 column in order to separate labelled plasmid DNA from unincorporated nucleotides.

i) Labelling of plasmid DNA by random priming

The method used was as described by Feinberg & Vogelstein (1983, 1984).
2 µg pTZ19U DNA was linearised with *Hin*dIII, extracted with phenol and chloroform/isoamyl alcohol, ethanol precipitated, resuspended in 1 ml H_2O and stored at -20°C until required. Linearised pTZ19U DNA was denatured by boiling for 5 min and was immediately transferred to ice. 24 ng of denatured DNA was mixed with 20 µCi [α-^{32}P]dGTP, 20 µCi [α-^{32}P]dTTP, 0.05 mM cold dATP, 0.05 mM cold dCTP, 3 µl hexanucleotide mix (Boehringer Mannheim) and 1-2 units of Klenow fragment DNA polymerase I in a final volume of 30 µl. Following incubation at 37°C for 30 min, the mixture was phenol extracted and applied to a Sephadex G-50 column in order to separate labelled plasmid DNA from unincorporated nucleotides.

2.2.8 Plasmid DNA sequencing

DNA sequencing by chemical cleavage (Maxam and Gilbert, 1980) was performed to determine the end points of the UL8 gene truncations produced by nuclease Bal31 digestion.

a) Preparation of DNA for sequencing

C-terminal truncations: 25 µg plasmid DNA was cut with a combination of *Eco*RV and *Xba*I, mixed with TBE dyes and loaded over 3 wells of a non-denaturing 1% agarose gel. Following electrophoresis at 35 V overnight, the UL8 gene fragment was excised and the DNA was recovered from the gel as described previously (section 2.2.6b). The ethanol-precipitated DNA was resuspended in 20 µl H_2O and was 3' end labelled at the *Xba*I site with [α-^{32}P]dGTP. The labelled DNA was extracted with phenol and applied to a Sephadex G-50 column in order to separate it from unincorporated nucleotides. The DNA was ethanol precipitated in the presence of CT DNA (2 µg) and resuspended in 40 µl H_2O containing 4 µg CT DNA. Sequencing reactions were then carried out.

N-terminal truncations: 25 µg plasmid DNA was cut with *Eco*RI, serially extracted with phenol and chloroform/isoamyl alcohol, ethanol precipitated and resuspended in 60 µl H_2O. The DNA was 3' end labelled with [α-^{32}P]dATP after which the DNA polymerase was heat inactivated. Following digestion with *Xba*I, the DNA was mixed with TBE dyes and loaded over 3 wells of a non-denaturing 1% agarose gel. The gel was electrophoresed at 35 V overnight and the UL8 gene fragment was excised and purified from the gel. The eluent from the DEAE sephacel column was ethanol precipitated in the presence of 4 µg CT DNA and resuspended in 60 µl H_2O containing 4 µg CT DNA. Sequencing reactions were then carried out.

b) Sequencing reactions

Sequencing reactions were carried out essentially as described (Maxam and Gilbert, 1980; procedures 10-13) except for the G+A reaction (procedure 11), which was carried out as follows: 10 µl of [α-^{32}P]-end-labelled DNA was mixed with 10 µl H_2O and chilled to 0°C. 50 µl of 90% formic acid was added and the reaction was incubated at 25°C for 10 min. Following the addition of 180 µl of 0.3 M sodium acetate/0.1 mM EDTA containing 25 µg/ml tRNA, the DNA was precipitated twice with ethanol, incubated with piperidine and lyophilised as described in procedure 10.

In order to determine the sequence of bases close to the ^{32}P label, the base modification reactions were incubated at 25°C for the following times: 4 min (G reaction); 10 min (G+A reaction); 15 min (C+T reaction); 15 min (C reaction).

After the final lyophilisation, the DNA was resuspended in 10 µl of formamide/TBE dyes, heated to 100°C for 2 min and loaded on a denaturing 16% polyacrylamide gel. Following electrophoresis the gel and back plate were covered tightly with clingfilm and exposed to autoradiographic film at -70°C.

2.2.9 Plasmid amplification assay

In order to assess the ability of mutated UL8 proteins to support DNA replication, BHK cell monolayers were transfected with pS1 (an origin-containing plasmid) and plasmids expressing either *wt* or mutated UL8 proteins and the other 6 replication proteins, using the calcium phosphate precipitation method described in section 2.2.2c. The cells were then processed as follows.

a) Preparation of total cellular DNA from transfected BHK cells

30-36 hr post-transfection the medium was removed from the cells and CLB containing 0.5 mg/ml protease (Sigma grade XIV) was added (2 ml/35 mm plate). Following incubation at 37°C for 3-5 hr, the cell lysate was extracted sequentially with phenol and chloroform/isoamyl alcohol, and the nucleic acids were precipitated with ethanol and resuspended in 200 µl TE containing 1 µg RNase A and 10 units RNase T_1.

For transient replication assays, a 20 µl sample of DNA was digested with *Eco*RI and *Dpn*I, separated by electrophoresis through a 0.8% agarose gel, and Southern blotted.

b) Southern blot transfer of DNA to nitrocellulose

Restriction enzyme-digested DNA was electrophoresed through a 0.8% agarose gel in 1 x TBE. The gel was denatured by gentle agitation in Gel Soak I for 1 hr, then neutralised in Gel Soak II for 1 hr. The DNA was blotted overnight onto a pre-soaked nylon (Hybond-N) membrane using 6 x SSC, a wick of Whatman 3 mm chromatography paper, and a weighted stack of absorbent paper towelling (Southern, 1975). The nylon was air-dried and the DNA was cross-linked using 120 mJ cm^{-2} UV light prior to hybridisation.

c) DNA:DNA hybridisation

The membrane was incubated with 100 ml pre-hybridisation buffer in a sealed plastic bag in a shaking water bath at 68°C for 1 hr. The probe was prepared by denaturing 10^7-10^8 cpm [^{32}P]-labelled DNA with 0.15 M NaOH (final vol. 1.2 ml) for 10 min at room temperature. The denatured probe was neutralised with HCl, 8.6 ml H mix (prewarmed to 68°C) were added, and this solution was immediately used to replace the pre-hybridisation buffer. The membrane was incubated with the denatured probe at 68°C overnight, washed three times in 2 x SSC/0.1% SDS at 68°C for a total of 1 hr, rinsed with H_2O, air dried and exposed to autoradiographic film at -70°C.

2.2.10 Analysis of proteins

a) Preparation of total cell proteins from transfected cells

Approximately 30 hr post-transfection, cell monolayers were washed briefly with PBS/A, drained and lysed in sample buffer (150-300 µl per 35 mm dish). Protein samples were stored at -20°C.

b) Extraction of soluble proteins from transfected cells

24-30 hr post-transfection, the medium was removed from the cell monolayers and the cells were scraped into TS buffer (1 ml per 35 mm plate). The cells were pelleted (6000 rpm, 2 sec, MSE Microfuge), washed with SE buffer and resuspended in 100 µl SE buffer at 4°C. Nonidet-P40 was added to 1% and the samples were incubated on ice for 15 min. Cell lysates were centrifuged at 60000 rpm for 30 min (Beckman TLA100.2 rotor) and the supernatant (soluble fraction) was stored at -70°C. Samples were mixed with boiling mix prior to SDS-PAGE and Western blot analysis.

c) SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Usually a 9% polyacrylamide resolving gel (acrylamide:bisacrylamide 39:1) was prepared in 1x running gel buffer in vertical gel plates and on top of this a 5% polyacrylamide stacking gel (acrylamide:bisacrylamide 19:1) in 1x stacking gel buffer was polymerised. Large gels were prepared in 18 cm x 22 cm plates, and mini-protein gels were prepared using Bio-Rad mini-gel plates. For co-immunoprecipitation experiments large 8.5% polyacrylamide resolving gels with 5% polyacrylamide stacking gels were prepared.

Protein samples were boiled in sample buffer for 2-3 min and immediately loaded onto the gel. Gels were electrophoresed in 1x Tank Buffer at 15-20 mA overnight (large gels) or 120 V for 90 min (mini-gels).

Gels were either fixed and stained using 0.2% Coomassie brilliant blue in fixing solution for 20-45 min, destained and dried at 80°C under vacuum onto Whatman filter paper, or the proteins were transferred from the gel to nitrocellulose and detected using antibodies (sections d and e below).

For co-immunoprecipitation experiments, gels were fixed for 30 min, treated with En^{3}Hance (according to the manufacturer's instructions), dried at 80°C under vacuum and exposed to autoradiographic film at -70°C.

The apparent molecular weights of proteins on both SDS-polyacrylamide gels and nitrocellulose blots were estimated by comparison to prestained molecular weight markers (M_r 25,000-127,000; Sigma).

d) Transfer of proteins to nitrocellulose by Western blotting

(Electroblot transfer, Towbin *et al.*, 1979)

Proteins were analysed by Western blotting following electrophoresis through SDS-polyacrylamide mini-gels. The gel was placed on Whatman 3 mm paper presoaked in transfer buffer. The top surface of the gel was covered with a nitrocellulose membrane and more 3 mm paper (both presoaked in transfer buffer), and the whole assembly was inserted into a Bio-Rad Mini Trans-blot Cell according to the manufacturer's instructions. Electro-blotting was carried out in transfer buffer at 70 V for 60 min at 4°C. Nitrocellulose membranes were air dried and stored in sealed plastic bags at -20°C.

e) Protein detection using antibodies

Nitrocellulose membranes were gently agitated in blocking buffer (3% gelatin in TBS) at 37°C for 30 min (after 15 min the buffer was replaced with fresh blocking buffer and incubation was continued). After two 5 min washes in TBST, the membranes were sealed in small plastic bags along with the primary antibody (diluted 1:2000 in TBST/1% gelatin/0.01% sodium azide) and gently agitated for 2 hr at room temperature. After two more 5-10 min washes in TBST the membranes were sealed in small plastic bags with the appropriate anti-IgG-alkaline phosphatase conjugate (diluted 1:7500 in TBST). After 30-45 min of gentle agitation at room temperature the membranes were removed from the bags and washed twice in TBST for 5-10 min and once in TBS for 5-10 min. The membranes were immersed in colour development solution according to the manufacturer's instructions (Promega Protoblot system). Once the required colour had developed, the membranes were rinsed in several changes of H_2O, air dried and stored in the dark prior to photography.

f) Preparation of infected cell extracts for co-immunoprecipitation

(McLean *et al.*, 1994)

Approximately 8 x 10^5 *S.f.* cells were seeded per 4 cm^2 well in 12-well plates one day prior to infection. Cells were either mock-infected or infected in various combinations with 10 pfu per cell of AcUL5 and AcUL52 and 5 pfu per cell of recombinant AcNPVs expressing *wt* (AcUL8) or mutated (Ac033, Ac165, Ac280 and Ac8N1) UL8 proteins. Following adsorption for 1 hr at room temperature, 2 ml TC100/5 were added per well and incubation was continued at 28°C for 24 hr. The medium was then replaced with 500 µl/well of TC100 medium lacking methionine, bacto-yeast extract and yeast hydrolosate and containing 60 µCi/ml [^{35}S]-L-methionine, and the cells were returned to 28°C. 40 hr p.i. the cells were scraped into the medium, pelleted at 6000 rpm for 12 sec (MSE Microfuge), washed once with TC100 wash and

resuspended in 400 µl buffer E at 0°C. After 15 min incubation on ice, the extracts were clarified at 60000 rpm for 15 min at 4°C (Beckman TLA100.2 rotor).

Extracts that were stored at -70°C at this stage were centrifuged again (60000 rpm for 15 min at 4°C; Beckman TLA100.2 rotor) immediately before use in co-immunoprecipitation experiments to remove any proteins which had been precipitated by the freezing process.

g) **Co-immunoprecipitation** (McLean *et al.*, 1994)

Labelled extracts (200 µl) were mixed constantly with 1.0 µl of anti-UL8 monoclonal antibody (MAb0811 or MAb0817) for 4-5 hr at 4°C. 70 µl of a 50% (vol/vol) preparation of protein A-sepharose beads (Sigma) in buffer E were added and mixing continued for a further 60 min. The beads were pelleted (12000 rpm for 15 sec, MSE Microfuge) and washed twice in buffer EB (buffer E containing 2 mg/ml BSA), once in buffer EN (buffer E containing 500 mM NaCl) and four times in buffer E. To reduce non-specific background the beads were then transferred to fresh tubes, before being pelleted and resuspended in 100 µl sample buffer. After heating the samples to 100°C for 2 min and pelleting the beads, the supernatants were analysed by SDS-PAGE using an 8.5% polyacrylamide gel. The gel was fixed, treated with En^{3}Hance, dried and exposed to autoradiographic film at -70°C.

CHAPTER 3: RESULTS

Section 3.1 DELETION MUTAGENESIS OF THE UL8 GENE

3.1.1 Introduction

The data presented in sections 3.1 and 3.4 are concerned with the mutagenesis of the UL8 gene and the analysis of polypeptides expressed from the resulting plasmid constructs.

The starting plasmid for mutagenesis was pE8 (**Figure 6b**) which consists of the UL8 ORF-containing *Mst*II-*Sst*I fragment (nucleotides 20492-17850 of the HSV-1 genome; McGeoch *et al.*, 1988a) cloned using *Bam*HI linker oligonucleotides into the *Bam*HI site of plasmid pCMV10 (Stow *et al.*, 1993). The plasmid pCMV10 is a pUC-based vector, containing HCMV major IE promoter sequences, a multiple cloning site, and SV40 splicing and polyadenylation signals (**Figure 6a**). This plasmid also contains the ß-lactamase gene, and therefore can be selected for in bacterial cells by ampicillin resistance. In mammalian cells high levels of expression can be obtained from genes inserted into the multiple cloning site.

3.1.2 Construction of pCMV10S

A modified vector, pCMV10S, was first constructed by inserting the 14 bp *Xba*I linker, containing termination codons in all three frames (5'-CTAGTCTAGACTAG-3'), into pCMV10 at the unique *Xba*I site. The use of this vector ensured that when 3'-truncated UL8 genes ligated to the same termination codon linker were cloned into this *Xba*I site, the termination codons in all three reading frames were retained.

pCMV10S was constructed as follows. *Xba*I-linearised pCMV10 DNA was made blunt-ended by fill-in synthesis using T4 DNA polymerase, recircularised in the presence of the phosphorylated 14 bp *Xba*I linker and used to transform competent *E. coli* DH5 bacteria. Colonies were selected for ampicillin resistance and small scale plasmid preparations were screened for the presence of linear plasmid following

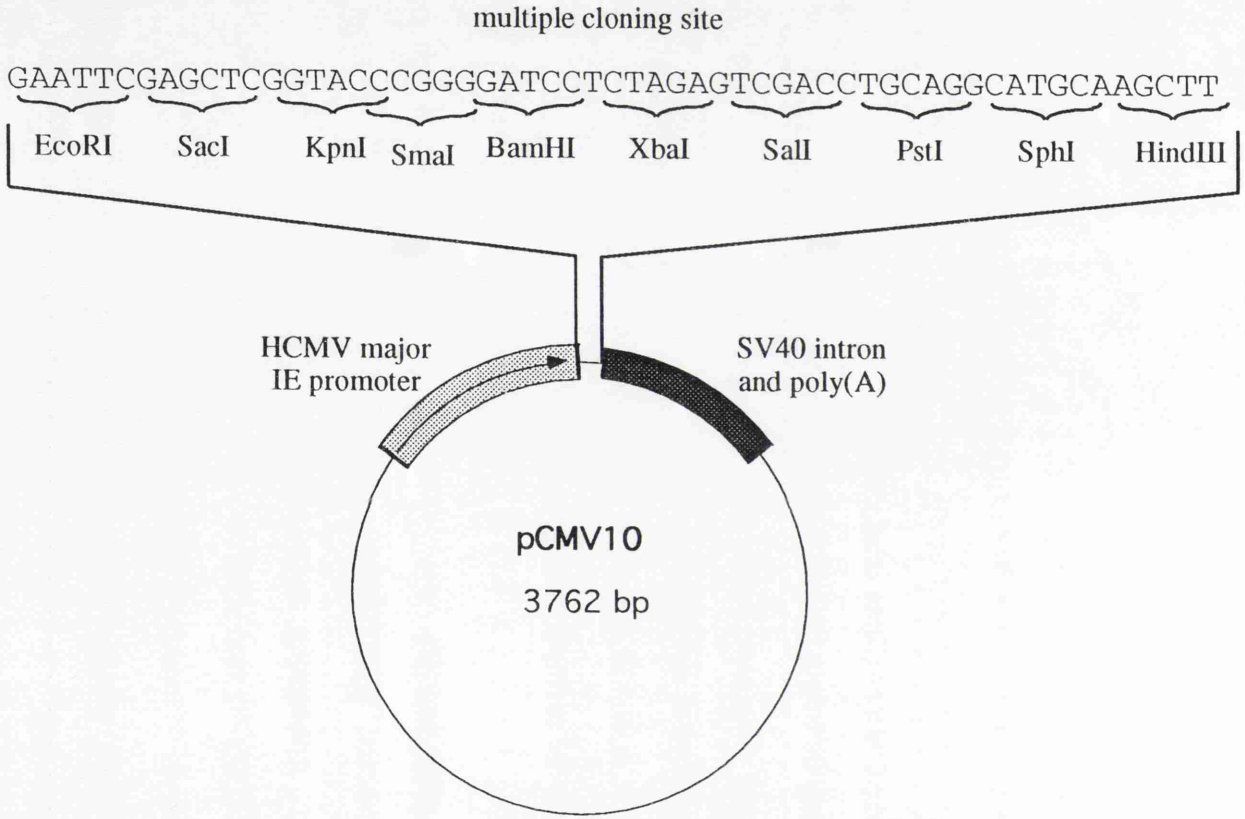


Figure 6a. Plasmid map of pCMV10.
The locations of the HCMV major immediate-early promoter ( ) and SV40 splicing and polyadenylation signals ( ), and the location and sequence of the multiple cloning site, are indicated.

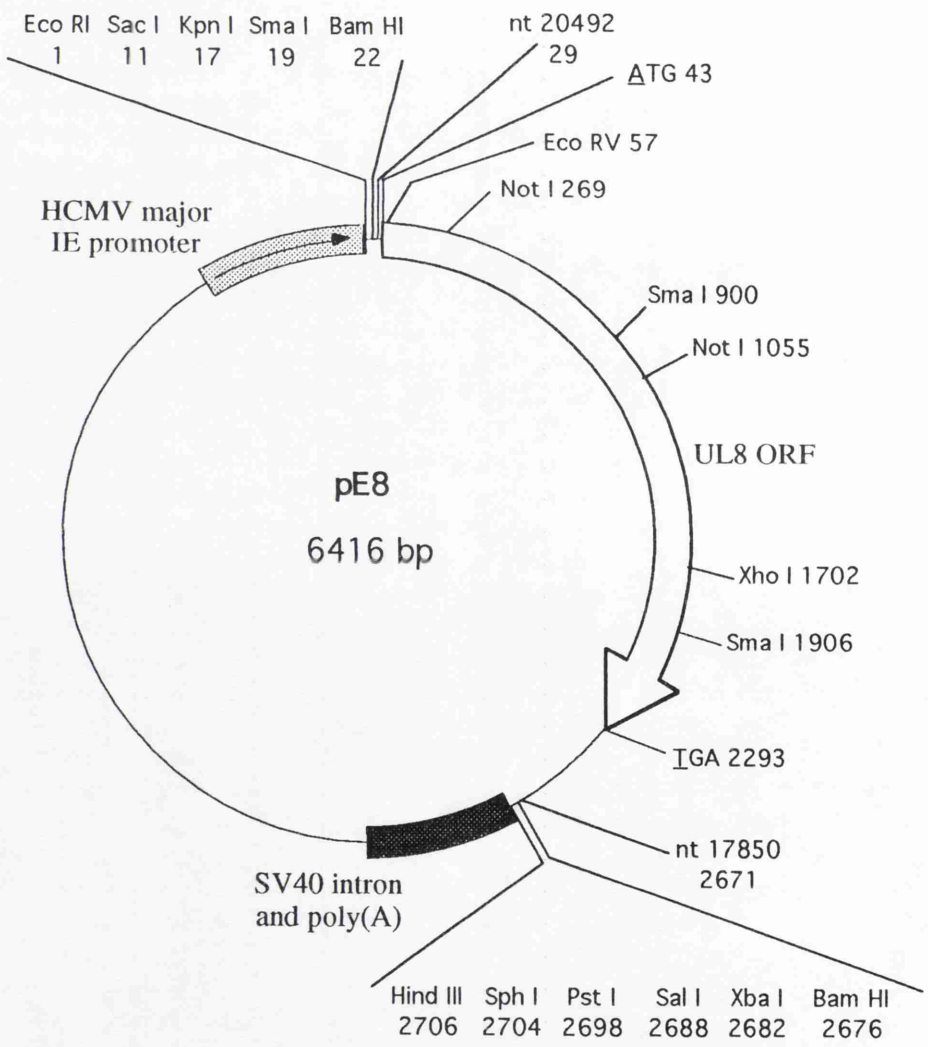


Figure 6b. Plasmid map of pE8.
pE8 consists of nucleotides (nt) 20492-17850 from the HSV-1 genome, inserted into the *Bam*HI site of pCMV10. The locations of the HCMV major immediate-early promoter ( ), UL8 open reading frame ( ), and SV40 splicing and polyadenylation sequences ( ) are shown. The positions of the first nucleotide of the UL8 initiation and termination codons and of selected restriction enzyme sites are also indicated.

digestion with *Xba*I. Plasmids containing an *Xba*I site were selected and analysed for the presence of the 14 bp linker. DNA was digested with *Eco*RI and *Hin*dIII and the products resolved on an 8% polyacrylamide gel (**Figure 7**). A shift in size of the small fragment from 51 bp (as in **lane 8**) to 69 bp (as in **lane 7**) indicated that a single linker had been inserted. Colony 227 (**lane 7**) was chosen from several colonies containing such an insert and a large scale preparation of pCMV10S vector DNA was prepared from it.

3.1.3 Construction and characterisation of UL8 C-terminal truncation mutants

The following strategy was used to construct a set of plasmids based on pE8, with deletions extending into the 3' end of the UL8 ORF.

Plasmid pE8 was linearised at the unique *Xba*I site downstream of the UL8 gene fragment and digested with exonuclease Bal31 for various times. This resulted in the production of families of DNA fragments with deletions extending to varying degrees into the UL8 3' non-coding region and ORF at one end, and into the SV40 sequences at the other end. The extent of the Bal31 deletions was assessed by taking a sample from each timepoint, digesting with *Xho*I, and resolving the products on an agarose gel (**Figure 8**). Cleavage of pE8 with *Xba*I and *Xho*I results in two fragments of 980 bp and approximately 5.4 kb in size (**lane 1**). The smaller fragment contains the last 590 bp of the UL8 ORF and 379 bp of 3' untranslated sequences. The reduction in size of the 980 bp fragment was used as an indicator of the degree of Bal31 digestion. For instance, by comparison with *Hin*fI-cleaved pAT153 DNA marker fragments (**lane 8**), Bal31 digestion for 10 min (**lane 2**) was estimated to remove up to approximately 380 bp from the smaller fragment. Three pools of DNA fragments were obtained with estimated deletions of up to 100 bp, 300 bp and 500 bp into the UL8 ORF.

The Bal31-treated DNA was ligated to 14 bp phosphorylated *Xba*I linker oligonucleotides containing termination codons in all three frames (5'-CTAGTCTAGACTAG-3'). Following ligation, the DNA samples were digested with a combination of *Xba*I, *Bam*HI and *Pvu*II. *Xba*I removed multiple linkers and

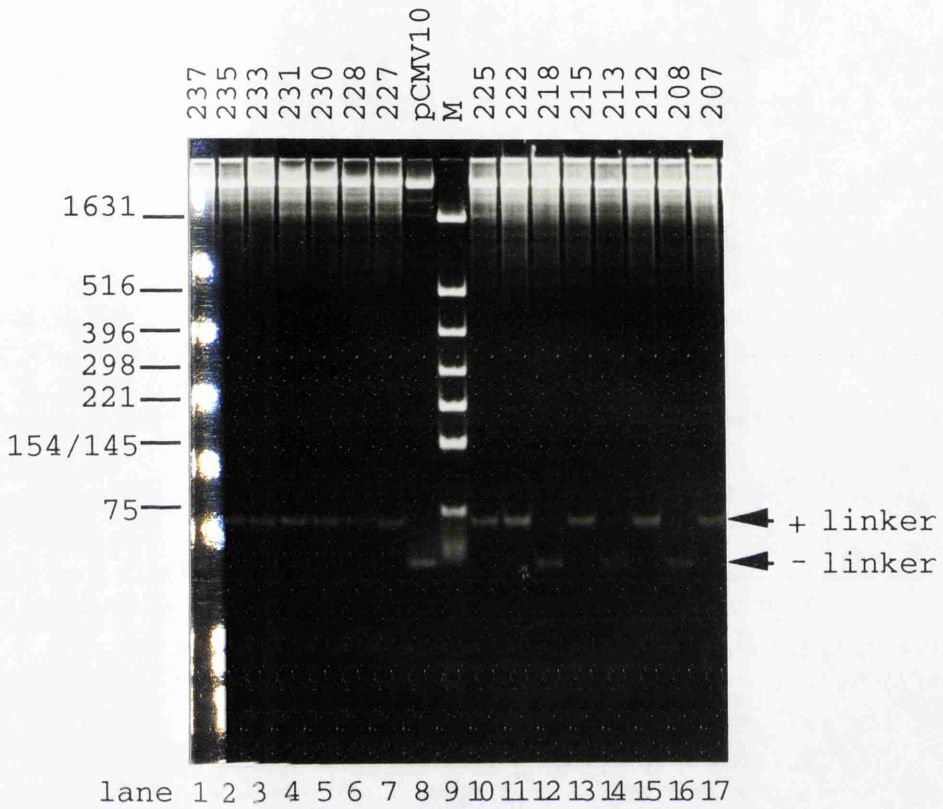


Figure 7. Screening for the presence of a 14 bp *Xba*I linker inserted into plasmid pCMV10.

Plasmid DNA from the colonies indicated (lanes 1-7 and 10-17) was digested with *Eco*RI and *Hin*dIII and the products were resolved on an 8% polyacrylamide gel with *Eco*RI plus *Hin*dIII-cleaved pCMV10 DNA (lane 8) and *Hin*fI-cleaved pAT153 DNA as marker (M, lane 9). The sizes of the marker DNA fragments are indicated in bp. A shift in size of the small *Eco*RI-*Hin*dIII fragment from 51 bp (marked "- linker") to 69 bp (marked "+ linker") indicates insertion of a single *Xba*I linker into pCMV10.

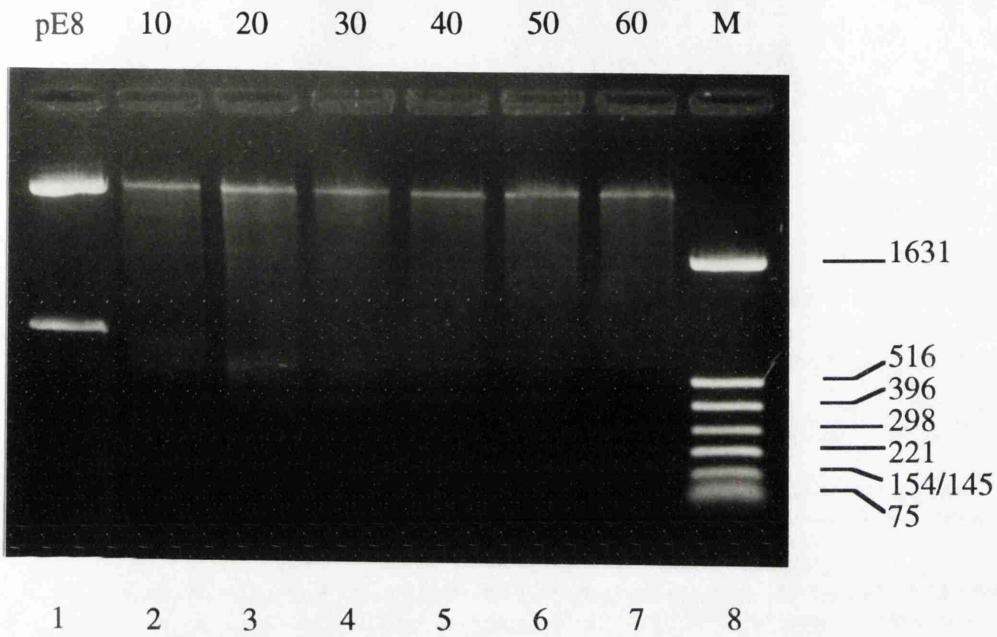


Figure 8. Bal31 digestion of *Xba*I-linearised pE8 DNA.

*Xba*I-linearised pE8 DNA was digested with Bal31 for various times. An aliquot from each timepoint (lanes 2-7, time in minutes) was cleaved with *Xho*I and electrophoresed through a 1% agarose gel with *Xba*I plus *Xho*I-cleaved pE8 (lane 1) and *Hin*fI-cleaved pAT153 DNA (M, lane 8). The sizes of the pAT153 *Hin*fI fragments are indicated. A reduction in the size of the 980 bp fragment (lane 1) with increasing time of digestion is apparent.

created a 'cohesive' end, *Bam*HI cleaved at the 5' end of the UL8 ORF, and *Pvu*II cleaved the pCMV10 vector to reduce the probability of recircularisation of the vector sequences. The products of this triple digest were ligated to pCMV10S DNA which had been cleaved with *Xba*I and *Bam*HI in the presence of calf intestinal phosphatase (CIP). The complete sequence of the 14 bp linker should therefore be regenerated upon joining of the vector and insert at their *Xba*I termini, resulting in the presence of stop codons in all three frames at the 3' end of each UL8 insert.

The products of this ligation were used to transform competent *E. coli* DH5 cells and small scale plasmid preparations were made from ampicillin resistant colonies. Plasmids that yielded two fragments when cleaved by *Bam*HI and *Xba*I were selected and digested with *Eco*RV and *Xba*I to confirm that the smaller fragment contains UL8 coding sequences (*Eco*RV cleaves 15 bp into the UL8 ORF).

The approximate sizes of the deletions were estimated following digestion with *Sma*I and *Xba*I as illustrated in **Figure 9**. Cleavage of pE8 with *Sma*I and *Xba*I results in four fragments of 776, 881, 1006 and approximately 3750 bp in size (**Figure 9a, lane 2**). The 776 bp fragment contains the 3' end of the UL8 ORF and downstream untranslated sequences, the 1006 bp fragment contains the middle section of the UL8 ORF and the 881 bp fragment contains the 5' end (**Figure 9b**). The 3750 bp fragment consists of pCMV10 vector sequences. The smallest fragment generated from colony 354 DNA was approximately 170 bp in size (**Figure 9a, lane 3**), indicating a deletion extending an estimated 216 bp into the 3' end of the UL8 ORF (**Figure 9b**). In **lanes 4** and **5** the fragment containing the middle section of the UL8 ORF had been reduced in size from 1006 bp (**lane 2**) to approximately 575 bp (**lane 4**) and 330 bp (**lane 5**), indicating that approximately 817 bp and 1062 bp of the UL8 ORF had been delcted in plasmids from colonies 307 and 370 respectively. In **lane 6**, the 776 and 1006 bp fragments were absent and the 881 bp fragment had been reduced to aprroximately 795 bp, indicating that 1478 bp had been deleted from the 3' end of the UL8 ORF in plasmid DNA from colony 332.

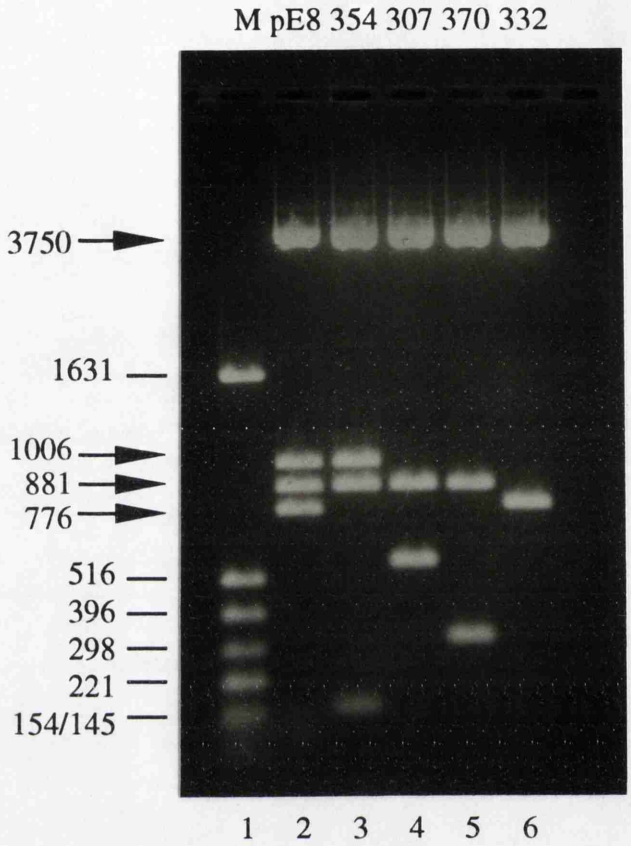
Colonies 332, 370, 307, 354, 436, 433, 411 and 447 were chosen, exhibiting a representative range of deletions, and large scale plasmid preparations were made

Figure 9. Estimating the sizes of UL8 deletions by restriction enzyme mapping.

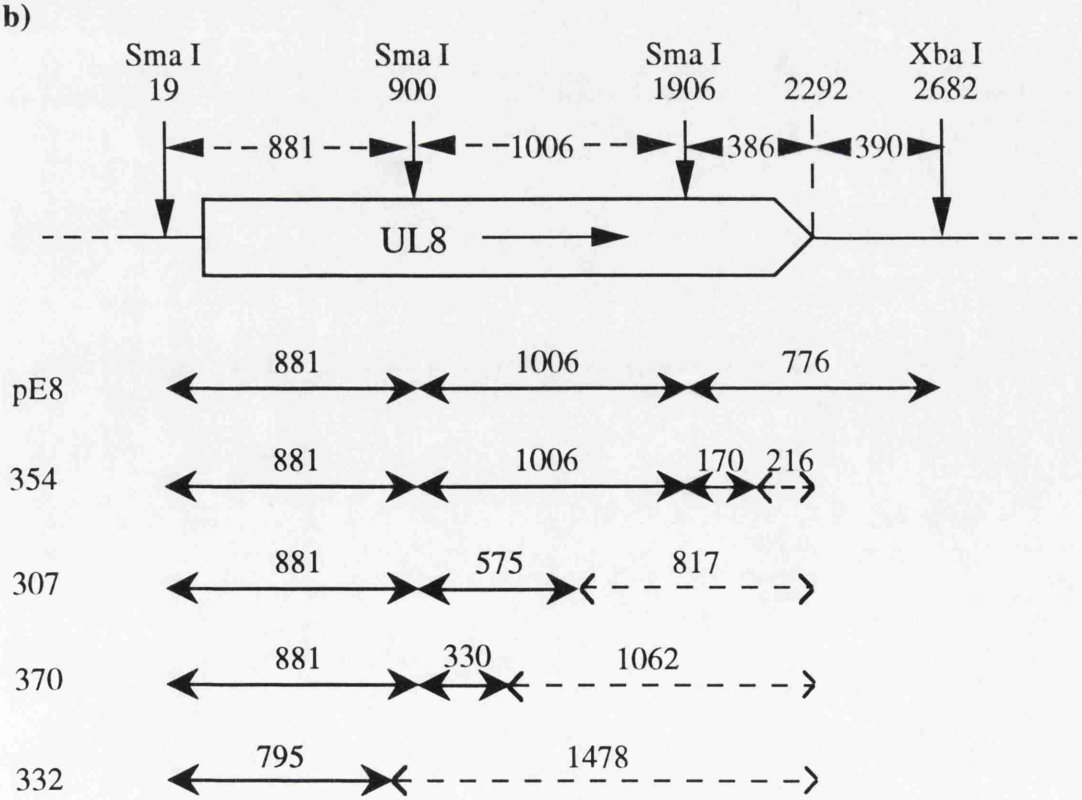
a) Plasmid DNA from the colonies indicated (lanes 3-6) was digested with *Sma*I plus *Xba*I and subjected to agarose gel electrophoresis with *Sma*I plus *Xba*I-cleaved pE8 DNA and *Hin*fI-cleaved pAT153 DNA (M). The sizes of the pE8 and pAT153 fragments are indicated in bp.

b) Diagram of part of plasmid pE8, showing the UL8 ORF and relevant restriction enzyme sites with the fragment sizes indicated in bp. The *Xba*I-*Sma*I fragment (776 bp) is shown in two parts to indicate the position of the 3' end of the UL8 ORF. Below this are shown the UL8 sequence-containing fragments (bold lines) obtained following digestion of the plasmids indicated with *Sma*I plus *Xba*I. The sizes of fragments were estimated from the gel shown in **a)**. The dotted lines indicate the region of the UL8 ORF deleted.

a)


M pE8 354 307 370 332
3750
1631
1006
881
776
516
396
298
221
154/145
1 2 3 4 5 6

b)


Sma I
19
Sma I
900
Sma I
1906
2292
Xba I
2682
881
1006
386
390
UL8
pE8
881
1006
776
354
881
1006
170
216
307
881
575
817
370
881
330
1062
332
795
1478

from these. **Figure 10** shows samples of these preparations cleaved with *Bam*HI and *Xba*I and resolved on an agarose gel. Digestion of pE8 with these two enzymes results in a 3.75 kb fragment consisting of pCMV10 vector sequences and a 2.65 kb fragment containing the UL8 ORF. Reduction in the size of the smaller fragment corresponds to successively larger deletions extending into the 3' end of the UL8 gene in the mutated plasmids.

The endpoints of the truncated UL8 genes were determined by DNA sequencing. DNA was 3' end-labelled at the *Xba*I site and the appropriate labelled fragment was sequenced by chemical cleavage as described in section 2.2.8. An autoradiograph showing the products of sequencing reactions for the plasmids 411, 332, and 436 is presented in **Figure 11**. The sequence corresponding to the linker can be seen at the bottom of each set of tracks.

Having determined the endpoints of the deletions by DNA sequencing, the amino acid sequence at the C-terminal end of the proteins could be deduced. For convenience the plasmids were therefore renamed to indicate the number of amino acids removed (**Table 2**). **Table 2** also gives the size and C-terminal amino acid sequence (predicted from the DNA sequence) of each of the polypeptides to be expressed from these constructs.

An additional C-terminal truncation mutant (pCΔ196, colony 1114; **Figure 10**) was generated as follows. Plasmid pCΔ079 was cleaved with *Xho*I and the cohesive ends were filled in. Following ligation to 14 bp *Xba*I termination linkers the *Bam*HI-*Xba*I fragment containing the UL8 coding sequence was excised, purified and inserted into pCMV10S. The structure of pCΔ196 was confirmed by restriction enzyme mapping and is summarised in **Table 2**. This mutant was constructed for the purpose of more accurately defining a region of UL8 recognised by some anti-UL8 monoclonal antibodies (section 3.3.2), and was not used in other assays.

3.1.4 Expression of C-terminally truncated UL8 proteins

To confirm that UL8-related polypeptides could be expressed from the above plasmid constructs, each one was lipofected into BHK cells. Total cell proteins were

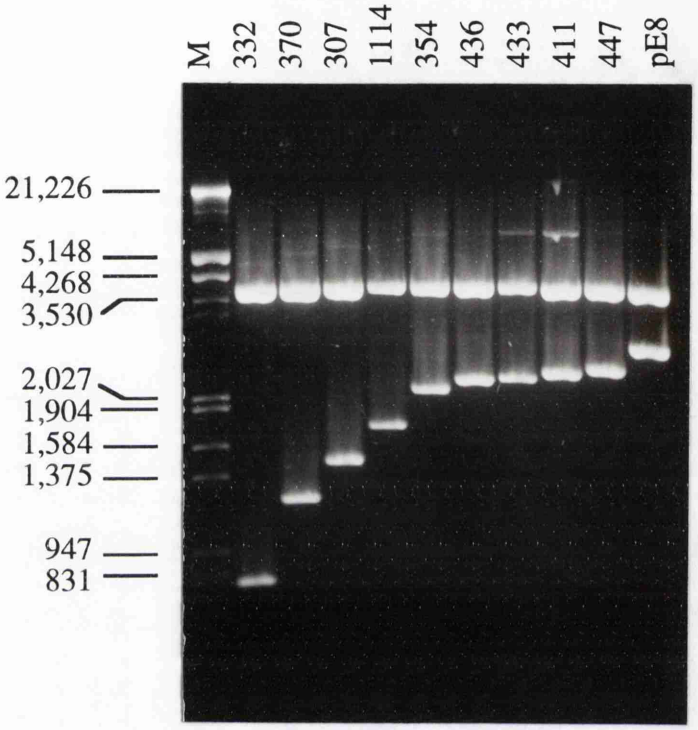


Figure 10. A set of plasmids with deletions extending into the 3' end of the UL8 ORF.

Plasmid DNA prepared from the colonies indicated was digested with *Bam*HI and *Xba*I and resolved by agarose gel electrophoresis with *Bam*HI plus *Xba*I-cleaved pE8 DNA and *Eco*RI plus *Hin*dIII-cleaved lambda DNA (M). The sizes of the lambda fragments are indicated in bp. Plasmid 1114 was derived from plasmid 354 as described in the text (page 84).

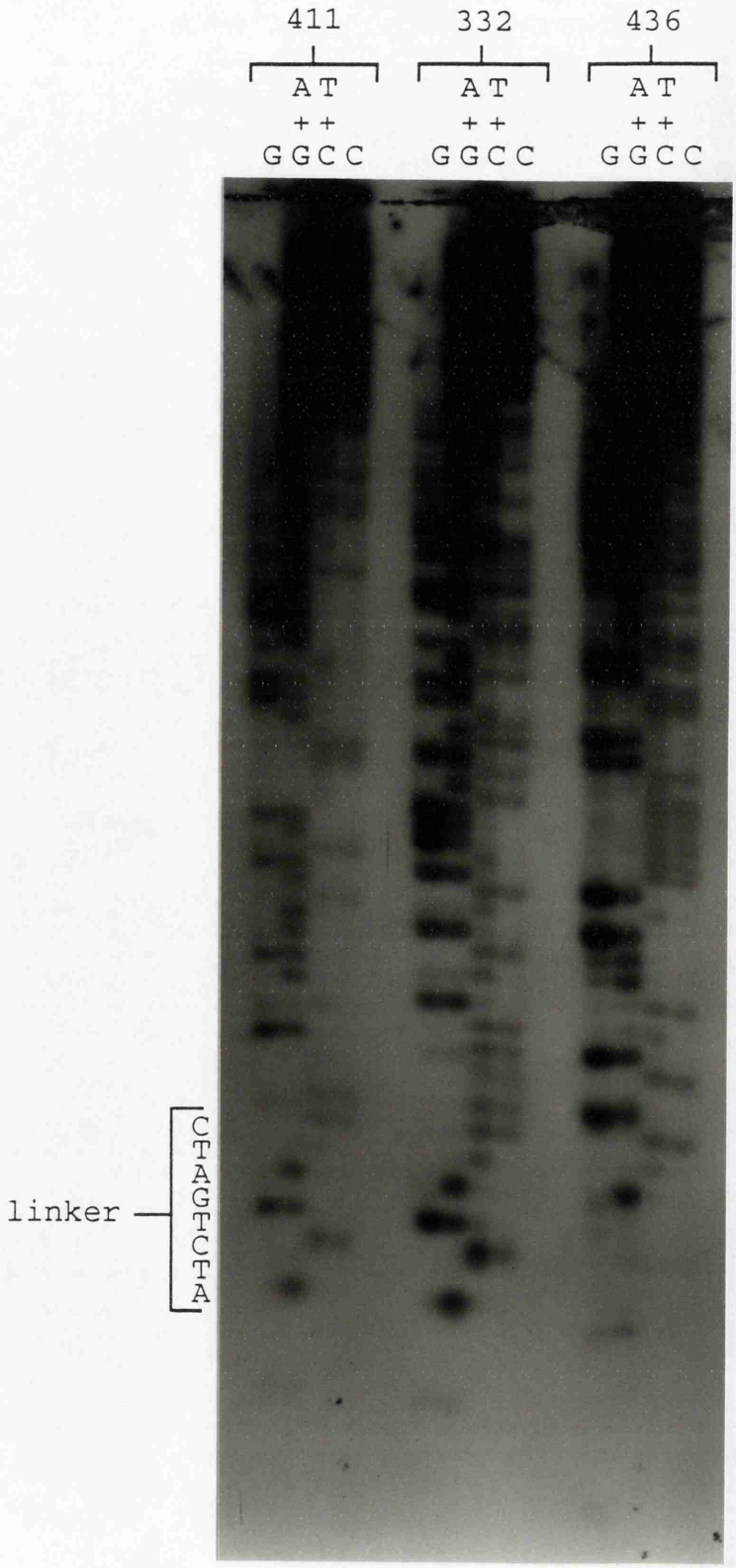
Figure 11. Determination of the endpoints of the truncated UL8 genes.

For each of the plasmids indicated, the products of the four chemical cleavage DNA sequencing reactions G, G+A, C+T and C, were electrophoresed through a denaturing 16% polyacrylamide gel. The gel was covered with clingfilm and exposed to autoradiographic film. The resulting autoradiograph is shown, with the sequence of the *Xba*I linker which marks the limit of Bal31 digestion indicated. The sequence for each plasmid, read from the bottom of the autoradiograph, is given below, with the limits of the linker (< >), the direction of the UL8 open reading frame (<-----) and the most 3' nucleotide of the UL8 coding sequence (*) indicated.

```
                    <       >*              <------
plasmid 411:        ATCTGATCCTGTAGAACAGCAGTTTCCCTATGTGT

                    <       >*              <------
plasmid 332:        ATCTGATCCCCCGTCGTCGTGGGCACGGCACTGAG

                    <       >*              <------
plasmid 436:        ATCTGATCGCGTCAGGTGCCCCCCCTCGTGTTCCC
```


411
332
436
A T
+ +
G G C C
A T
+ +
G G C C
A T
+ +
G G C C
linker
CTAGTCTA

Colony	Plasmid name (1)	Terminal nucleotide (2)	Predicted C-terminal amino acid composition of expressed protein (3)	Predicted mol. wt. of expressed protein (4)
447	pΔ3'	18174	-Ala End 750	79,925
411	pCΔ004	18242	-Ser End 746	79,446
433	pCΔ033	18328	-Arg Leu Val End 717	76,485
436	pCΔ041	18354	-Ala Ser Leu Asp End 709	75,533
354	pCΔ079	18464	-Ala End 672	71,147
114	pCΔ196	18815	-Leu Asp End 554	58,385
307	pCΔ280	19071	-Pro Ser Leu Asp End 470	49,952
370	pCΔ359	19307	-Ile End 391	41,598
332	pCΔ497	19719	-Pro Pro Ser Leu Asp End 253	27,549

Table 2. Summary of UL8 C-terminal deletion mutants.

(1) Plasmids are named according to the number of amino acids deleted from the C-terminal end of the expressed protein. pΔ3' contains a deletion in the 3' untranslated sequences, extending to within 53 bp of the UL8 ORF termination codon.

(2) Position of 3' terminal nucleotide of UL8 insert (numbering according to McGeoch *et al.*, 1988a).

(3) The last amino acid before the sequence of the expressed protein diverges from that of *wt* UL8 is numbered and additional residues encoded by the *Xba*I linker are shown where appropriate. 'End' indicates a termination codon.

(4) Molecular weights were calculated using the GeneticsComputerGroup software (GCG8 package.)

prepared, separated by SDS-PAGE and transferred by Western blotting to nitrocellulose. UL8-related polypeptides were detected using the anti-UL8 N-terminal peptide serum 064 (**Figure 12**). Detection of the product of plasmid pCΔ196 with anti-UL8 polyclonal serum 094 is shown in **Figure 23**. A protein of approximately the size predicted from DNA sequencing was identified for each of the nine plasmids.

3.1.5 Construction, characterisation and expression of UL8 N-terminal truncation mutants

The strategy for construction of plasmids containing various sizes of deletions at the 5' end of the UL8 ORF was similar to that described in section 3.1.3.

Plasmid pE8 was linearised at the unique *Eco*RI site upstream of the UL8 gene, prior to digestion with the exonuclease Bal31. The extent of the Bal31 deletions was assessed by taking a sample from each timepoint, digesting with *Sma*I, resolving the products on an agarose gel and estimating the reduction in size of the smallest (881 bp) fragment. Two pools of DNA fragments with estimated deletions of up to 200 bp and 400 bp into the UL8 ORF were chosen for cloning into pCMV10.

The 17mer (5'-**A A T T** CGCCAC **C** A T G **G** GG-3') and 13mer (3'-GCGGT**GGTACCCC**-5') oligonucleotides were phosphorylated and annealed to give a linker containing an *Nco*I site (CCATGG), an ATG codon for initiation of translation, and an *Eco*RI cohesive end. This linker was ligated to the Bal31-digested DNA fragments. The products were digested with *Eco*RI, *Bam*HI and *Pvu*II then ligated to pCMV10 DNA which had been cleaved with *Eco*RI and *Bam*HI in the presence of CIP. (It was not necessary to use the modified pCMV10 vector as the initiation codon was contained in the linker, and the termination codon was unaffected by the cloning procedures.) The DNA was used to transform competent *E. coli* DH5 bacteria and small scale plasmid preparations were made from ampicillin resistant colonies. Plasmids were screened for the presence of two bands following digestion with *Eco*RI and *Bam*HI. The presence of the linker was confirmed by cleavage with *Nco*I.

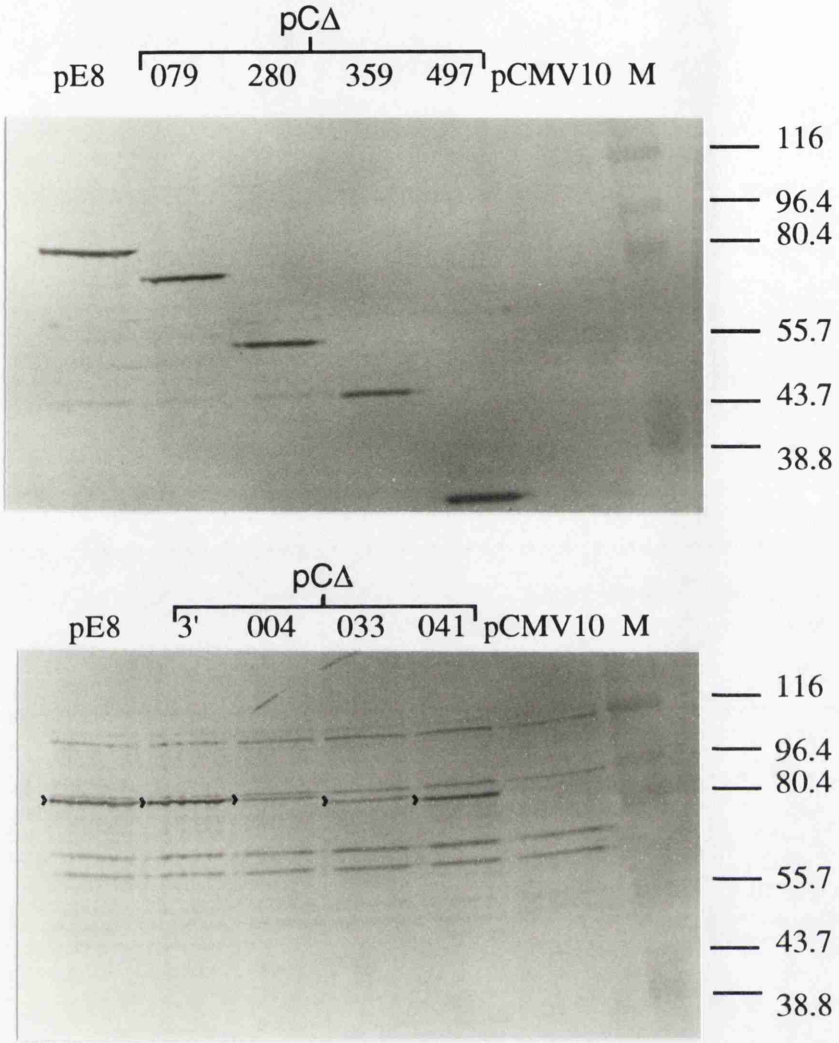


Figure 12. Expression of C-terminally truncated UL8 polypeptides in BHK cells. BHK cell monolayers were lipofected with the plasmids indicated. 30 hr post-lipofection total protein preparations were made, separated by SDS-PAGE and transferred to nitrocellulose by Western blotting. The nitrocellulose blots were incubated for 2 hr with a 1:2000 dilution of N-terminal anti-peptide serum 064 (directed against residues 2-16 of the UL8 protein). The blots were washed, incubated for 45 min with a 1:7500 dilution of anti-rabbit IgG alkaline phosphatase-conjugated antibody, washed again and incubated with colour development solution (Promega Protoblot System) until the bands were visible. The sizes of prestained molecular weight markers (M; Sigma) are indicated in kDa.

In addition, a plasmid was constructed in which the first 15 bp of the UL8 ORF were deleted. This was done by cleaving pE8 at the unique *Eco*RV site near the 5' end of the UL8 ORF, resulting in flush ends to which the *Eco*RI/*Nco*I initiation linker was ligated. This truncated UL8 fragment was then recloned into pCMV10 as described above.

The linker had been designed so that the ATG initiation codon would be in-frame with the UL8 ORF in the *Eco*RV-generated deletion mutant. However, the Bal31-generated mutants had random endpoints, and statistically only one in three of them would be expected to be in the correct frame to encode a truncated UL8 polypeptide. Therefore plasmids containing the UL8 gene fragment were lipofected into BHK cells and total cell proteins were prepared. These were analysed by SDS-PAGE followed by Western blotting. UL8-related polypeptides were identified using the polyclonal anti-UL8 serum 094.

Of the twenty Bal31-generated plasmid constructs tested, ten were found to express UL8-related polypeptides. These were from colonies 513, 515, 537, 543, 584, 5100, 5102, 5106, 5113 and 5114 (**Figure 13, lanes 1, 2, 6, 7, 10, 11, 12, 14, 15** and **16** respectively). The two *Eco*RV-generated plasmid constructs tested (from colonies 521 and 528) efficiently expressed the truncated UL8 protein (**Figure 13, lanes 3** and **5**). Large scale plasmid preparations were made of these twelve plasmids. DNA samples cleaved with *Eco*RI and *Bam*HI and resolved by agarose gel electrophoresis are shown in **Figure 14**.

Plasmids from colonies 513, 515, 584 and 5100 were selected to represent a range of deletion sizes on the basis of the size of the polypeptide products on Western blots and the size of the UL8 gene fragments determined following digestion with *Eco*RI and *Bam*HI (**Figure 13, lanes 1, 2, 10** and **11**; **Figure 14, lanes 2, 3, 6** and **7**). The deletion endpoints of these four plasmids were determined by DNA sequencing. DNA was 3' end-labelled at the *Eco*RI site and the appropriate labelled fragment was sequenced as described in section 2.2.8. The results are summarised in **Table 3,** along with the predicted sizes of the polypeptide products of these truncated genes. The

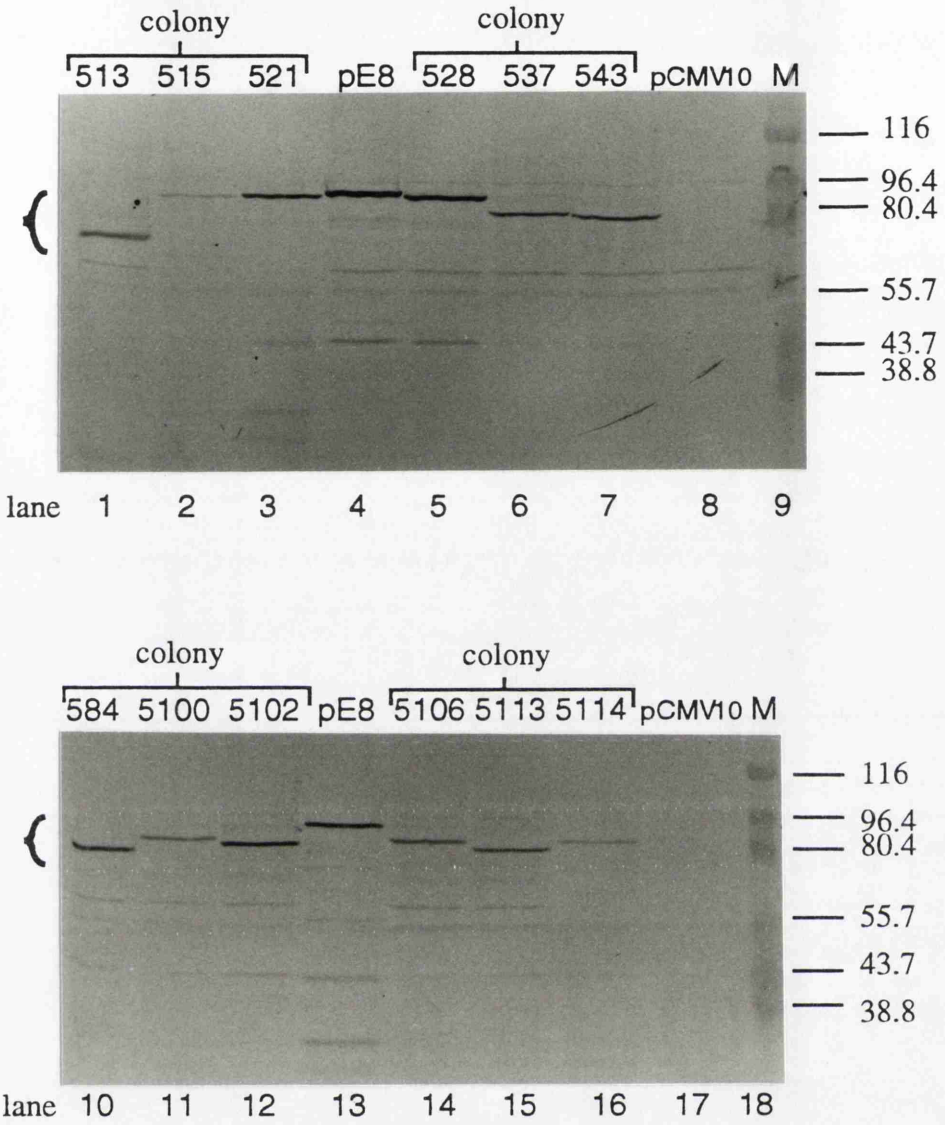


Figure 13. Expression of N-terminally truncated UL8 polypeptides in BHK cells. Small-scale plasmid preparations were purified by sequential extraction with phenol and chloroform/isoamyl alcohol followed by ethanol precipitation. Total protein preparations from BHK cells lipofected with the purified plasmids from the colonies indicated were subjected to SDS-PAGE and Western blotting. Blots were incubated with a 1:2000 dilution of anti-UL8 polyclonal serum 094. The blots were processed as described in Figure 12. The sizes of prestained molecular weight markers (M; Sigma) are indicated (kDa). [*N.B.* Only the plasmids capable of specifying UL8-related proteins are shown in this figure.]

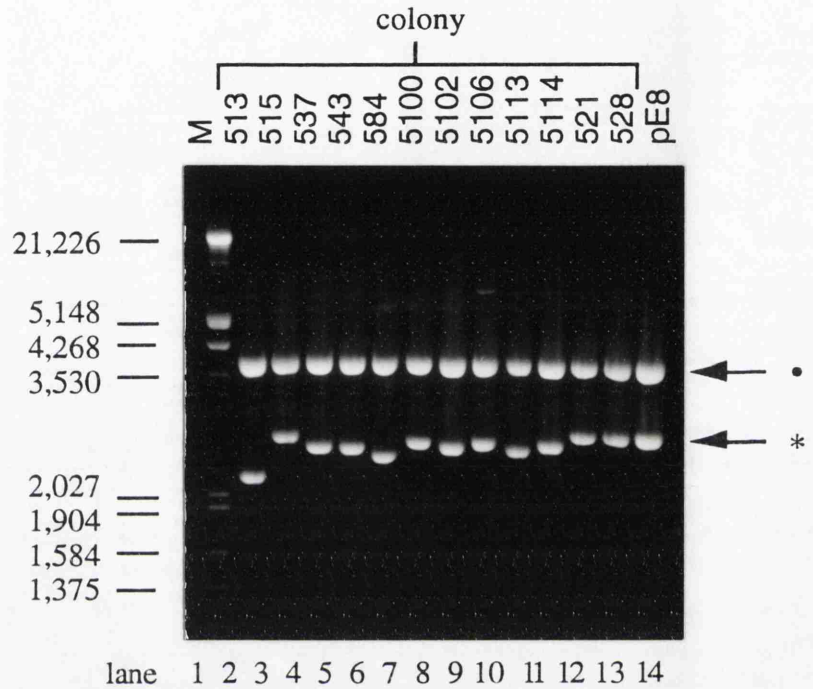


Figure 14. A set of plasmids with deletions at the 5' end of the UL8 ORF.

Plasmid DNA prepared from the colonies indicated was digested with *Eco*RI and *Bam*HI and resolved by agarose gel electrophoresis with *Eco*RI plus *Bam*HI-cleaved pE8 DNA and *Eco*RI plus *Hin*dIII-cleaved lambda DNA (M; sizes of fragments indicated in bp). The 3.74 kb fragment (•, lane 14) consists of pCMV10 vector sequences and the 2.65 kb fragment (*, lane 14) contains the UL8 ORF. The variable size of the smaller fragment in lanes 2-13 results from deletions extending various distances into the 5' end of the UL8 ORF.

Colony	Plasmid name (1)	Terminal nucleotide (2)	Predicted N-terminal amino acid sequence of expressed protein (5)	Predicted mol. wt. of expressed protein (6)
528	pNΔ005	20,463	Met Gly Ile 6	79,580
515	pNΔ023	20,409	Met Gly Pro 24	77,519
5100	pNΔ043	20,349	Met Gly Gly 44	75,156
584	pNΔ097	20,187	Met Gly Trp 98	69,642
513	pNΔ165	19,983	Met Gly Ala 166	62,453
1128	p8ΔN1(3)	(4)	Met Asp Thr 1 2 3	52,109

Table 3. Summary of N-terminally truncated UL8 proteins, and the internal deletion p8ΔN1.

(1) Plasmids are named according to the number of amino acids deleted from the N-terminal end of the expressed protein.

(2) Position of 5' terminal nucleotide of UL8 ORF (numbering according to McGeoch *et al.*, 1988a).

(3) p8ΔN1 is lacking an internal 786 bp *Not*I fragment from the UL8 ORF of pE8, so that the expressed protein lacks amino acids 78 to 339 of *wt* UL8.

(4) nucleotides 20251-19466 (inclusive) are deleted (numbering according to McGeoch *et al.*, 1988a).

(5) The first three amino acids of the expressed proteins are given, with the position of the first amino acid derived from the UL8 ORF indicated (the methionine and glycine are encoded by the *Eco*RI/*Nco*I initiation linker). p8ΔN1 does not contain the *Eco*RI/*Nco*I linker and the expressed protein is identical to *wt* UL8 for the first 77 amino acids.

(6) Molecular weights were calculated using the GCG8 software.

*Eco*RV-generated mutant, 528, was also sequenced to confirm that the deletion was as predicted.

3.1.6 Construction and characterisation of UL8 internal deletion mutant

The plasmid p8ΔN1 was constructed by excising the internal *Not*I fragment from the UL8 ORF of pE8, resulting in a deletion of 786 bp, removing amino acids 78 to 339 from the UL8 protein (**Table 3**).

Plasmid pE8 was cleaved with *Not*I which cuts twice within the UL8 insert, generating two fragments. The larger of the two fragments was purified, recircularised and used to transform *E. coli*. Ampicillin resistant colonies were selected and plasmid p8ΔN1 was obtained from one of these.

The presence of the deletion in p8ΔN1 was confirmed by restriction enzyme mapping. *Bam*HI digestion of pE8 results in a 3.76 kb fragment consisting of pCMV10 vector sequences and a 2.65 kb fragment containing UL8 coding sequences (**Figure 15, lane 1**). The equivalent UL8 coding fragment resulting from *Bam*HI digestion of p8ΔN1 is approximately 1.87 kb in size (compare **lanes 1** and **2**). Cleaveage of pE8 with both *Bam*HI and *Not*I results in the same 3.76 kb pCMV10 fragment, but the UL8 ORF is cleaved into three fragments of 247 bp, 786 bp and 1621 bp in size (**lane 3**). The 786 bp fragment is not present when p8ΔN1 DNA is double digested with *Bam*HI plus *Not*I (**lane 4**), indicating that it has been precisely deleted.

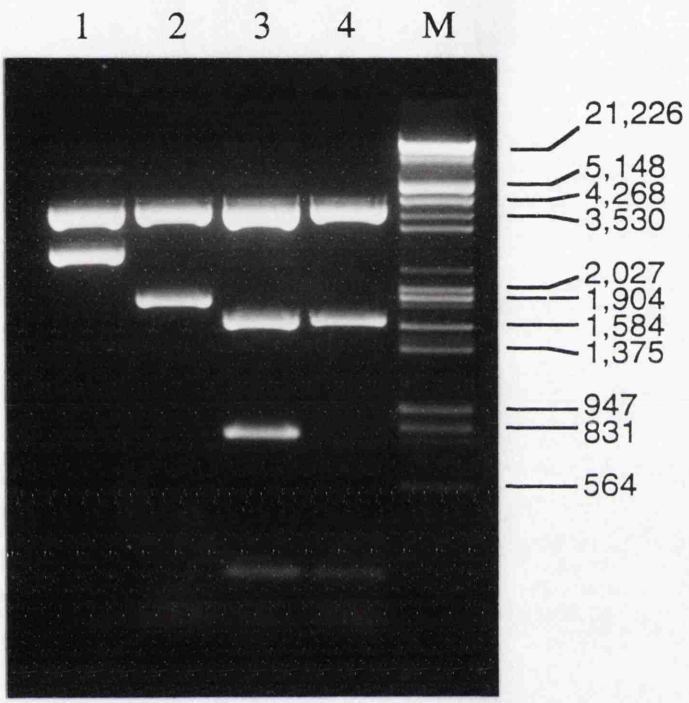


Figure 15. Restriction enzyme mapping of p8ΔN1.

Plasmids pE8 (lanes 1 and 3) and p8ΔN1 (lanes 2 and 4) were digested with *Bam*HI alone (lanes 1 and 2) or doubly digested with *Bam*HI and *Not*I (lanes 3 and 4) and resolved on an agarose gel with *Eco*RI plus *Hin*dIII-cleaved lambda DNA (M; sizes of fragments indicated in bp). The 786 bp *Not*I fragment (lane 3) is deleted in p8ΔN1 (lane 4).

Section 3.2 DEVELOPMENT OF A PLASMID-BASED ASSAY FOR THE INTRACELLULAR LOCALISATION OF THE HELICASE-PRIMASE COMPLEX

3.2.1 Introduction

Calder *et al.* (1992) investigated the cellular localisation of four of the HSV-1 proteins required for viral DNA synthesis using recombinant viruses based on the HSV-1 mutant *ts*K. The recombinant viruses *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 and *ts*K/UL52 contain additional copies of the UL5, UL8, UL9 and UL52 genes inserted under the control of the immediate early (IE) gene 3 promoter (Weir *et al.*, 1989). At the non-permissive temperature (NPT) each recombinant *ts*K virus overexpresses the products of the IE genes, including that of the inserted gene, but fails to synthesise early and late gene products, including the seven DNA replication gene products under the control of their normal promoters. Using immunofluorescent staining of BHK cells infected with various combinations of the four recombinant viruses, they demonstrated that all three components of the helicase-primase complex (UL5, UL8 and UL52) needed to be co-expressed to enable their efficient localisation to the nucleus, whereas the UL9 protein efficiently entered the nucleus when expressed alone. Single or pairwise combinations of the UL5, UL8 and UL52 proteins resulted in predominantly cytoplasmic perinuclear localisation (Calder *et al.*, 1992).

I repeated a selection of these experiments using the same recombinant viruses and anti-UL9 and -UL52 sera, along with a different anti-UL8 serum. My findings are in agreement with those of Calder and coworkers and are described below.

I proceeded to see if similar results were obtained when the same four proteins were expressed in BHK cells in the absence of the HSV-1 immediate-early (IE) proteins and virion components. This was done by expressing the UL5, UL8, UL9 and UL52 gene products from plasmid constructs tranfected into BHK cells. The establishment of this system was necessary to enable subsequent screening of UL8 mutants for ability to faciliate nuclear localisation of the helicase-primase complex without the requirement of constructing recombinant herpesviruses. These studies

were aided by the use of newly developed anti-UL8 monoclonal antibodies (Parry, 1993) which are characterised in further detail in section 3.3.

3.2.2 Cellular localisation of the UL9, UL8 and UL52 proteins in cells infected with combinations of recombinant viruses *ts*K/UL9, *ts*K/UL5, *ts*K/UL8 and *ts*K/UL52

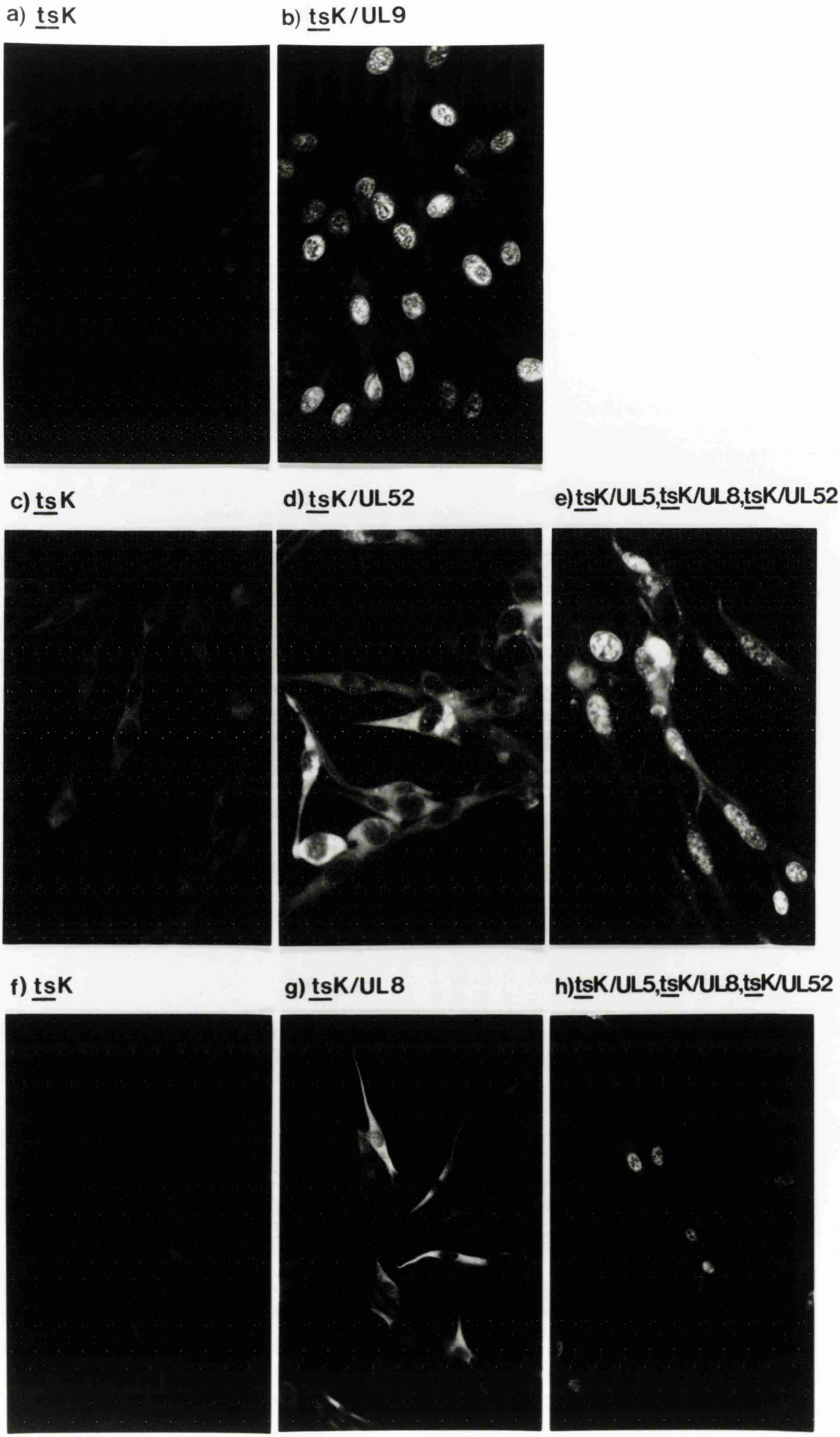
BHK cells were infected at the NPT with temperature sensitive viruses *ts*K, *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 and *ts*K/UL52 (described above and by Calder *et al.*, 1992) in the combinations indicated in **Figure 16**. 7 hr post-infection the cells were fixed and incubated with antibodies as described in Methods section 2.2.4. The primary antibodies used were anti-UL9 (**panels a** and **b**) and anti-UL52 (**panels c, d** and **e**) sera, raised in rabbits against synthetic peptides from the C-termini of the UL9 and UL52 proteins (Olivo *et al.*, 1989), and anti-UL8 serum 105 (**panels f, g** and **h**), raised in rabbits against whole purified UL8 protein (Parry, 1993).

In *ts*K/UL9-infected cells, the UL9 protein localises efficiently to the nucleus, where distinct foci of fluorescence are visible (**panel b**). Cytoplasmic staining for UL9 was not seen in any of the infected cells. In contrast, staining of *ts*K/UL52-infected cells for UL52 resulted in a diffuse cytoplasmic pattern of fluorescence, often with bright foci clustering near the nucleus (**panel d**). These foci may represent aggregates of UL52, which tends to be insoluble when expressed in the absence of UL5. When UL52 was co-expressed with UL5 and UL8 in triply infected cells, the localisation of UL52 was almost exclusively nuclear, with a speckled pattern of staining (**panel e**). These results, in agreement with those reported by Calder *et al.* (1992), indicate that UL9 is capable of efficiently entering the nucleus when expressed alone. In contrast, UL52 localises to the nucleus efficiently when co-expressed with UL5 and UL8 but not when expressed in their absence.

The use of a different anti-UL8 antibody from that used by Calder resulted in more readily visible patterns of fluorescence for UL8 expression than obtained previously. In *ts*K/UL8-infected cells, UL8 staining was cytoplasmic and generally diffuse (**panel g**). In a few cells, faint speckled nuclear fluorescence could be seen in

Figure 16. Cellular localisation of the UL8, UL9 and UL52 proteins in infected cells.

BHK cells were infected at the non-permissive temperature with the viruses *ts*K, *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 and *ts*K/UL52 as indicated. 7 hr post-infection the cells were fixed and incubated (as described in section 2.2.4) with a 1:30 dilution of C-terminal anti-peptide serum against UL9 (**panels a and b**) or against UL52 (**panels c-e**), or a 1:100 dilution of polyclonal anti-UL8 serum 105 (**panels f-h**). The cells were subsequently incubated with a 1:80 dilution of anti-rabbit IgG fluorescein-conjugated antibody, mounted under coverslips and viewed under x50 (**panels a-e**) or x25 (**panels f-h**) magnification.


a) tsK
b) tsK/UL9
c) tsK
d) tsK/UL52
e) tsK/UL5,tsK/UL8,tsK/UL52
f) tsK
g) tsK/UL8
h) tsK/UL5,tsK/UL8,tsK/UL52

addition to the strong cytoplasmic signal (**panel g**). However, it was not clear whether this represented protein actually within the nucleus or merely attached to the outside of it. Co-expression of UL5, UL8 and UL52 in triply infected cells resulted in UL8 localising almost exclusively to the nuclei with a speckled pattern of foci (**panel h**), a pattern of staining closely resembling that of UL9 in *ts*K/UL9-infected cells (compare **panels b** and **h**) and that of UL52 in triply infected cells (compare **panels e** and **h**). This again confirms the previous report that efficient nuclear localisation of UL8 is dependent on co-expression with the other two components of the helicase-primase complex. The results shown in **panel g** suggest that UL8 may be capable of interacting with or entering the nucleus to a small degree when expressed alone, although the majority remains cytoplasmic. It is not clear whether this is a genuine property of the UL8 protein, or the result of leakage of early gene expression from the *ts* virus providing small amounts of UL5 and UL52 for complex formation.

The cells in this study were fixed 7 hr post-infection and not 10 hr as described by Calder *et al.* (1992). A timecourse experiment indicated that UL9 was initially detected as a diffuse, slightly speckled pattern of nuclear fluorescence in cells 3 hr post-infection, but that this distribution became more markedly focal by 5 hr post-infection. There was no significant difference in the patterns of nuclear fluorescence seen at 5, 7 and 10 hr post-infection with *ts*K/UL9 (data not shown).

3.2.3 Cellular localisation of the UL5, UL8, UL52 and UL9 proteins in cells transfected with plasmids pE5, pE8, pE52 or pE9

In order to determine the localisations of the helicase-primase components and origin-binding protein in the absence of all other HSV-1-encoded proteins, immunofluorescence experiments were carried out on cells transfected with plasmids expressing the UL5, UL8, UL52 and UL9 gene products. The plasmids used in the immunofluorescence experiments were pE5, pE8, pE52 and pE9, consisting of the expression vector pCMV10 (Stow *et al.*, 1993) into which the UL5, UL8, UL52 and UL9 ORFs had been inserted as DNA fragments encompassing HSV-1 nucleotides 12127-15162, 17850-20492, 108967-112512 and 20666-23539 respectively of the

HSV-1 genome (McGeoch *et al.*, 1988a). These plasmids express the products of the inserted genes under the control of the HCMV major immediate early promoter.

The method for transfecting plasmid DNA into BHK cells used liposomes rather than calcium phosphate precipitation because the former procedure yielded a higher proportion of expressing cells. In addition, the sparse cell monolayers necessary for immunofluorescence tended be disrupted by the DMSO boost used in conjunction with the calcium phosphate procedure, whereas lipofection efficiency was optimal on sparse monolayers.

To optimise transfection efficiencies a DNA dose-response experiment was carried out in which equal weights of plasmids pE5, pE8 and pE52 were mixed together and various amounts of this mixture were used to lipofect BHK cells. The UL52 gene product was detected 45 hr post-lipofection by immunofluorescent staining with anti-UL52 rabbit serum and anti-rabbit IgG fluorescein-conjugated antibody. In addition, BHK cell monolayers were lipofected with various amounts of pM2 DNA and stained 45 hr post-lipofection for ß-galactosidase expression. In both cases, plates with the largest number of positive cells were identified by inspecting randomly selected fields of view, and the optimum lipofection efficiency was achieved using a total of 1-2 μg of DNA per 35mm plate. For all further immunofluorescence experiments cells were therefore lipofected with a total of 1.5μg of plasmid DNA per 35mm plate.

A preliminary timecourse experiment was conducted to determine the optimum time for fixing and staining. Four plates of BHK cell monolayers were lipofected with pE52, four with a mix of pE52 and pE5 and four more with a mixture of pE52, pE5 and pE8. At 16, 24, 39 and 48 hr post-lipofection, three plates of cells (one from each plasmid combination) were fixed and incubated with anti-UL52 rabbit serum and anti-rabbit IgG fluorescein-conjugated antibody. At 16 hr post-lipofection bright specific fluorescence could be seen in the cells, although some cells fluoresced quite weakly compared to others. Similar patterns of fluorescence were seen in cells fixed 24 hr after lipofection, with fewer weakly fluorescing cells. At 39 hr post-lipofection the brightness of the fluorescence was beginning to obscure details of the

pattern. By 48 hr after lipofection the signal was so strong that the whole cell appeared to fluoresce. For all subsequent experiments the cells were fixed 20-24 hr after lipofection.

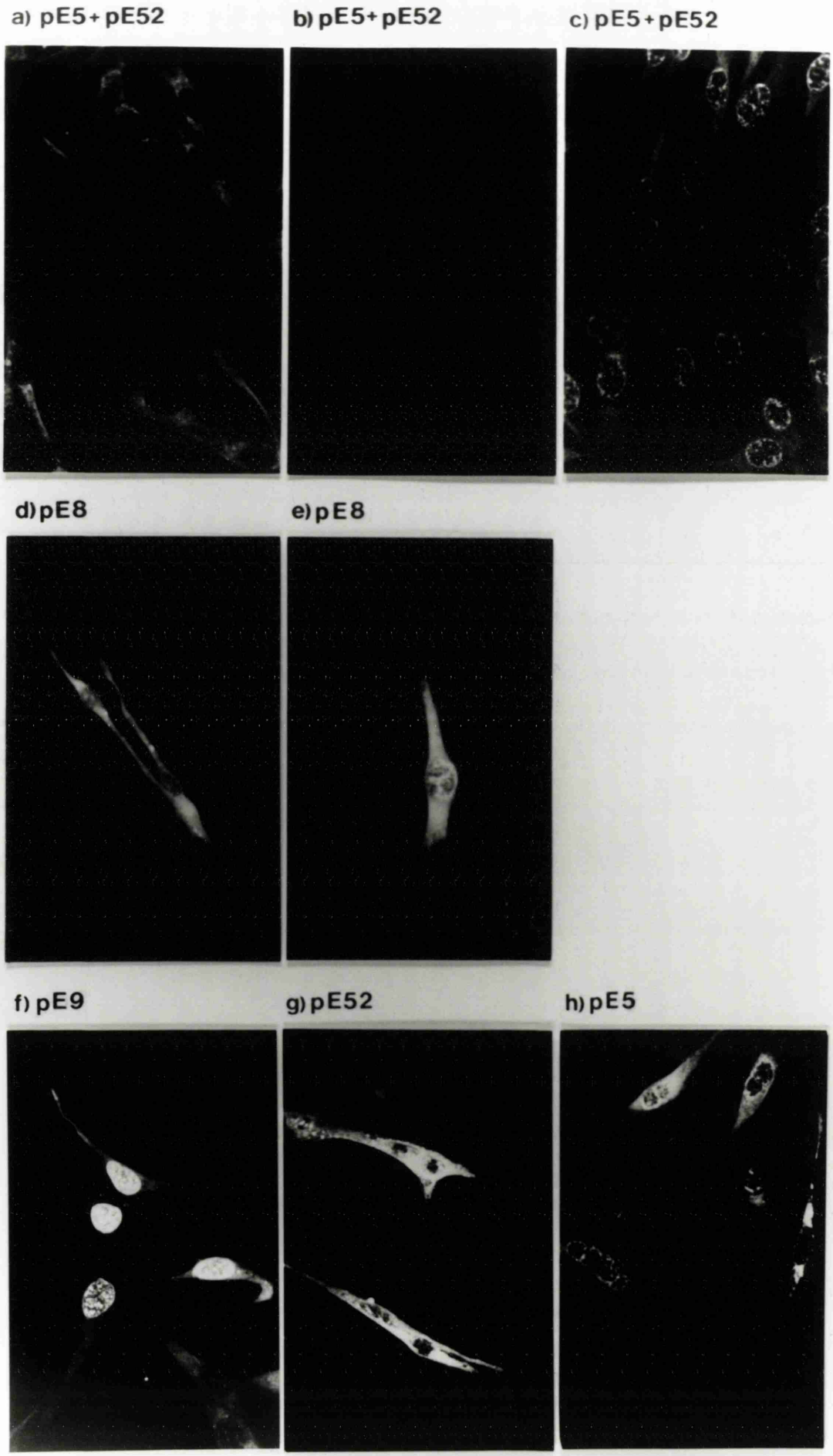
The anti-UL5, anti-UL9 and anti-UL52 sera raised against synthetic C-terminal peptides had previously been shown to specifically recognise the corresponding gene products (Olivo *et al.*, 1989; Calder *et al.*, 1992). Similar controls to ensure that the anti-UL8 antibodies used in these studies did not recognise UL5, UL52 or cellular proteins were performed by incubating the antibodies with BHK cells lipofected with pE5 and pE52. Anti-UL8 monoclonal antibody (MAb) 0811 showed a complete absence of non-specific staining (**Figure 17b**) whereas staining with MAb 0809 resulted in distinct perinuclear foci (**Figure 17c**). For this reason MAb 0811 was used for detection of UL8 in subsequent immunofluorescence experiments. The significance of the MAb 0809 staining pattern is considered further in Section 3.3.6. The polyclonal anti-UL8 serum 105, which did not cross-react with proteins in *ts*K infected cells (**Figure 16f**), also gave a relatively low background level of cytoplasmic staining in cells lipofected with pE5 and pE52 (**Figure 17a**). Taken together with the positive signal obtained with cells co-infected with *ts*K/UL5, *ts*K/UL8 and *ts*K/UL52 (**Figure 16h**), this confirms its specificity for the UL8 protein.

Figure 17 also shows that BHK cells lipofected individually with plasmids pE5, pE8, pE9 and pE52 express proteins which are detectable by immunofluorescence using the corresponding antisera. **Panels 17d** and **17e** show BHK cells lipofected with pE8 and stained with MAb 0811. In approximately 50% of fluorescing cells UL8 was located almost exclusively to the cytoplasm (**Figure 17d**). A distinctly speckled pattern of nuclear fluorescence, usually accompanied by cytoplasmic fluorescence, was observed in approximately 25% of fluorescing cells (**Figure 17e**). For the remaining 25% of cells the fluorescence was too strong to determine the intracellular location of UL8.

In agreement with previous findings using the *ts*K/UL9 recombinant (Calder *et al.*, 1992) UL9, when expressed alone, was capable of localising efficiently to the nucleus in 100% of fluorescing cells, where it apppeared to be concentrated in foci

Figure 17. Cellular localisation of the UL5, UL8, UL52 and UL9 proteins in transfected cells.

BHK cells were lipofected with plasmids pE5, pE8, pE9 and pE52 as indicated. 24 hr post-lipofection the cells were fixed and incubated with the following primary antibodies: a 1:100 dilution of polyclonal anti-UL8 serum 105 (**panel a**); 1:200 dilutions of anti-UL8 monoclonal antibodies 0811 (**panels b, d** and **e**) and 0809 (**panel c**); and 1:40, 1:100 and 1:50 dilutions respectively of C-terminal anti-peptide sera to UL9 (**panel f**), UL52 (**panel g**) and UL5 (**panel h**). The cells were subsequently incubated with 1:80 dilutions of anti-rabbit IgG (**panels a** and **f-h**) or anti-mouse IgG (**panels b-e**) fluorescein-conjugated antibodies, mounted and viewed under x50 magnification.


a) pE5+pE52
b) pE5+pE52
c) pE5+pE52
d) pE8
e) pE8
f) pE9
g) pE52
h) pE5

(**Figure 17f**). In approximately 20% of these cells additional weak cytoplasmic fluorescence was seen, usually accompanying very strong fluorescence in the nucleus.

In contrast, UL52 was found exclusively in the cytoplasm of lipofected cells, exhibiting either diffuse or speckled cytoplasmic fluorescence (**Figure 17g**). Speckled and diffuse cytoplasmic fluorescence were also the predominant patterns observed in cells lipofected with pE5 (**Figure 17h**). Although perinuclear or possibly nuclear foci of fluorescence could be seen in the occasional cell (**Figure 17h**), this pattern was rare and its significance is not clear.

These results using plasmid expression vectors thus extend the findings from recombinant *ts*K infections (Calder *et al.*, 1992), and demonstrate that in the absence of other viral proteins UL9 alone localises efficently to the nucleus of cells whereas the components of the helicase-primase complex, when expressed individually, do not. The nuclear localisation of UL8 in a minority of cells also occurs in the absence of all other HSV-1 proteins.

3.2.4 Cellular localisation of the UL5, UL8 and UL52 proteins when co-expressed in transfected cells

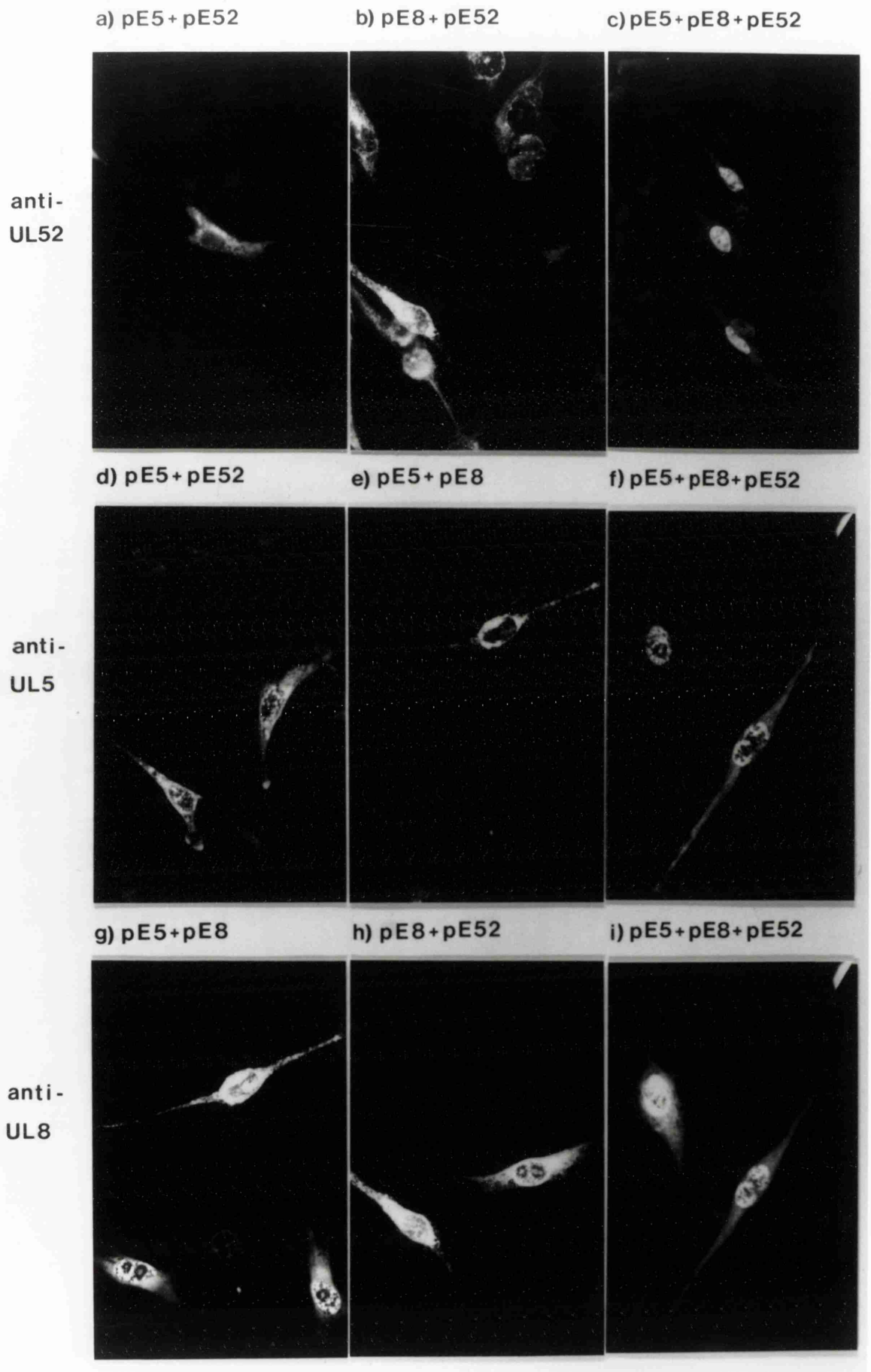
The effect of co-expressing two and three subunits of the helicase-primase complex in BHK cells was investigated by lipofection with combinations of plasmids pE5, pE8 and pE52. The cells shown in **Figure 18** were stained with anti-peptide sera to UL52 (**a-c**) or UL5 (**d-f**) or with anti-UL8 MAb 0811 (**g-i**). Cells cotransfected with pE8 and either UL5 or UL52 or both, were incubated with a second antibody (anti-UL8 for **panels b**, **c**, **e** and **f**; anti-UL5 for **panels g** and **i**; anti-UL52 for **panel h**) to determine whether individual cells were co-expressing both proteins. For this reason the anti-UL52 and anti-UL5 antibodies were detected using a fluorescein-conjugated anti-rabbit IgG serum, and the anti-UL8 MAb was detected using a rhodamine-conjugated anti-mouse IgG serum.

UL52 protein remained in the cytoplasm when co-expressed with UL5 (**panel a**) or UL8 (**panel b**), but was localised highly efficiently to the nuclei of cells cotransfected with pE52, pE8 and pE5 (**panel c**).

Figure 18. Cellular localisation of the UL5, UL8 and UL52 proteins when co-expressed.

BHK cells were lipofected with plasmids pE5, pE8 and pE52 in the combinations indicated. 22 hr post-lipofection the cells were fixed and incubated with a 1:100 dilution of anti-peptide serum to UL52 (**panels a-c** and **h**), a 1:50 dilution of anti-peptide serum to UL5 (**panels d-f, g** and **i**) or a 1:200 dilution of anti-UL8 monoclonal antibody 0811 (**panels b, c, e, f** and **g-i**). Following incubation with fluorescein-conjugated anti-rabbit IgG antibody (all panels) and rhodamine-conjugated anti-mouse IgG antibody (all panels except **a** and **d**), the cells were mounted under coverslips and viewed under x50 magnification. The following pairs of panels show the same cells viewed under different filters: **b** and **h**; **e** and **g**; **f** and **i**.

Note The cells shown in **panels g** and **h** were selected to represent the full complement of fluorescence patterns observed, such that each panel does not necessarily reflect the true frequency with which any particular pattern was observed.


a) pE5+pE52
b) pE8+pE52
c) pE5+pE8+pE52
anti-
UL52
d) pE5+pE52
e) pE5+pE8
f) pE5+pE8+pE52
anti-
UL5
g) pE5+pE8
h) pE8+pE52
i) pE5+pE8+pE52
anti-
UL8

UL5, when co-expressed with UL52 (**panel d**) or UL8 (**panel e**), exhibited a similar pattern to that seen in cells transfected with pE5 alone, namely cytoplasmic fluorescence with some additional perinuclear or nuclear foci. Again it was not clear whether the latter represented UL5 protein inside the nucleus or on the surface of the nucleus. Co-transfection of cells with pE5, pE8 and pE52, however, resulted in efficient nuclear localisation of UL5 in the vast majority of cells (**panel f**).

Co-expression of UL8 with UL5 resulted in predominantly cytoplasmic UL8 in the transfected cells (uppermost cell in **panel g**, with **panel e** showing the same cell expressing UL5). A minority of cells exhibited predominantly nuclear fluorescence for UL8, but in every case these cells failed to show any expression of UL5 (compare the bottom two cells in **panel g** with **panel e**). Likewise, cells co-expressing UL8 and UL52 exhibited a cytoplasmic pattern of fluorescence for UL8 (**panel h**, left-hand cell, with **panel b** showing the same cell expresses UL52). Again, the occasional cell showing nuclear localisation of UL8 (**panel h**, right-hand cell) was not expressing UL52 (**panel b**). In cells cotransfected with pE5, pE8 and pE52, UL8 was efficiently localised to the nucleus, such that the majority of fluorescing cells exhibited nuclear staining (**panel i**).

These results, in agreement with the previous findings obtained using recombinant viruses (Calder *et al.*, 1992), show that UL5 and UL52 do not localise efficiently to the nucleus unless co-expressed with both of the other components of the helicase-primase complex, and further demonstrate that no other HSV-1-encoded proteins are required for this nuclear localisation.

A proportion of the UL8 protein, when expressed alone, appeared to be capable of nuclear localisation in some cells, where it exhibited a punctate pattern of fluorescence. When expressed with both UL5 and UL52, UL8 was efficiently localised to the nucleus in the majority of cells. Co-expression with UL5 or with UL52 resulted in cytoplasmic localisation of UL8, implying that formation of complexes between UL8 and UL5, or UL8 and UL52, is not sufficient to enable transport of UL5 or UL52 into the nucleus and may actually inhibit UL8 from accumulating in the nucleus. To investigate further the effect that interaction of UL8 with other subunits of

the complex has on the localisation of UL8, the following double-staining experiments were performed.

3.2.5 Colocalisation of UL8 with UL5 or UL52 in transfected cells

BHK cells cotransfected with pE8 and pE5, pE8 and pE52, or with pE5, pE8 and pE52 were fixed and double-stained with anti-UL8 MAb 0811 and either anti-UL5 or anti-UL52 C-terminal anti-peptide serum. The cells were subsequently incubated with anti-mouse IgG rhodamine-conjugated and anti-rabbit IgG fluorescein-conjugated antibodies, such that red fluorescence indicated UL8 protein and green fluorescence indicated UL5 or UL52 protein, depending on the primary antibody used. Some typical results are shown in **Figure 19**.

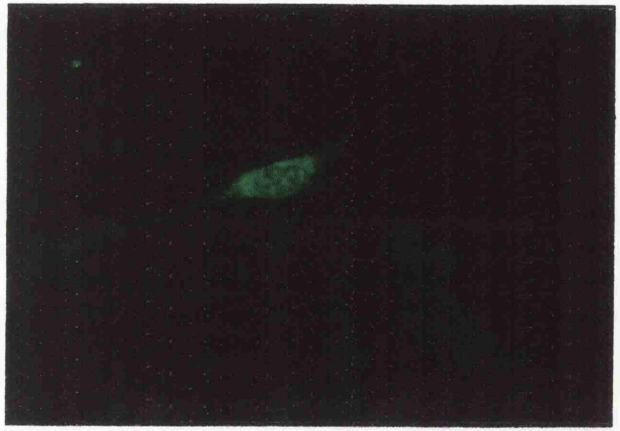
Panels a, **b** and **c** show cells lipofected with pE8 and pE5. A single cell expressing UL5 can be seen in **panel a**. Cytoplasmic UL5 is clustered near the nucleus, with some additional fluorescence representing UL5 either over the surface of, or within, the nucleus. This cell also expresses UL8 (**panel b**) and its distribution closely resembles that of UL5 (compare **panels a** and **b**). Other cells expressing UL8 in a punctate nuclear distribution with fainter diffuse cytoplasmic staining (**panel b**) fail to show any expression of UL5 (**panel a**). **Panel c** is a double exposure, combining **panels a** and **b**. The strong yellow fluorescence indicates colocalisation of the two proteins. These results clearly indicate that UL8 protein is capable of localising to the nucleus in the absence of all other HSV-1 encoded proteins. The frequency of nuclear localisation in UL8-expressing cells appeared to be higher than observed in section 3.2.3, but exclusively cytoplasmic UL8 was also observed, suggesting that its distribution may be affected by the state of the cell and/or the level of UL8 expression. However, co-expression of UL5 with UL8 was not sufficient for efficient nuclear localisation of UL5, although the two proteins do appear to colocalise.

Panels d, **e** and **f** show cells lipofected with pE5, pE8 and pE52 and incubated with anti-UL5 and anti-UL8 antibodies. In one cell UL5 is localised almost exclusively to the nucleus as punctate foci, whereas in another cell the fluorescence is

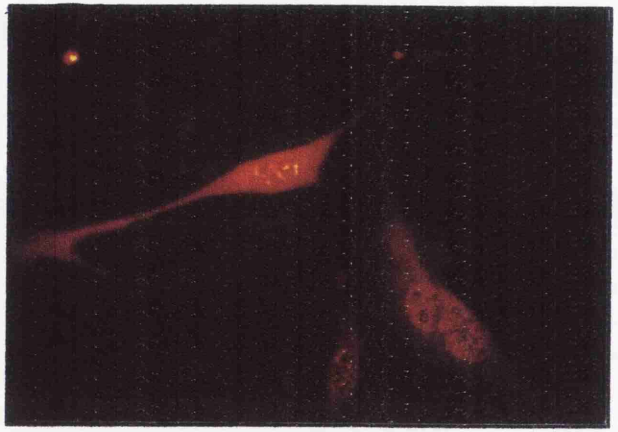
Figure 19. Colocalisation of UL8 with UL5 and UL52 in transfected cells.

BHK cells were lipofected with plasmids pE5, pE8 and pE52 in the combinations indicated. 24 hr post-lipofection the cells were fixed and incubated with the following primary antibodies: a 1:200 dilution of anti-UL8 monoclonal antibody 0811 followed by a 1:70 dilution of anti-peptide serum to UL5 (**panels a-f**); a 1:200 dilution of anti-UL8 monoclonal antibody 0811 followed by a 1:100 dilution of anti-peptide serum to UL52 (**panels g-l**). The cells were subsequently incubated with 1:80 dilutions of fluorescein-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG antibodies, and were mounted. Cells were viewed under x50 magnification using appropriate filters to detect green fluorescence, representing UL5 (**panels a** and **d**) or UL52 (**panels g** and **j**) protein, and red fluorescence representing UL8 protein (**panels b, e, h** and **k**). **Panels c, f, i** and **l** are double exposures of the previous two panels, showing colocalisation of the two proteins in yellow.

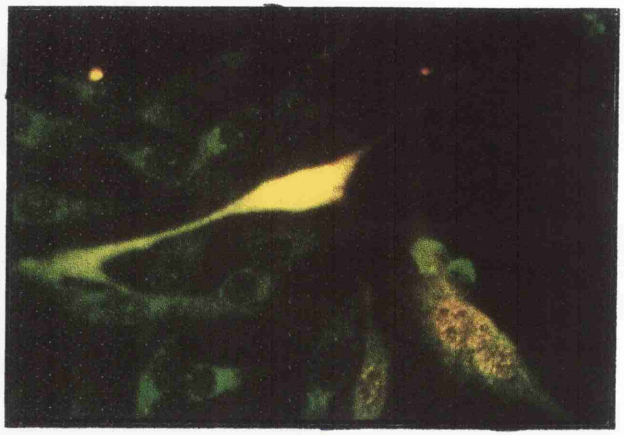
a) pE5 + pE8



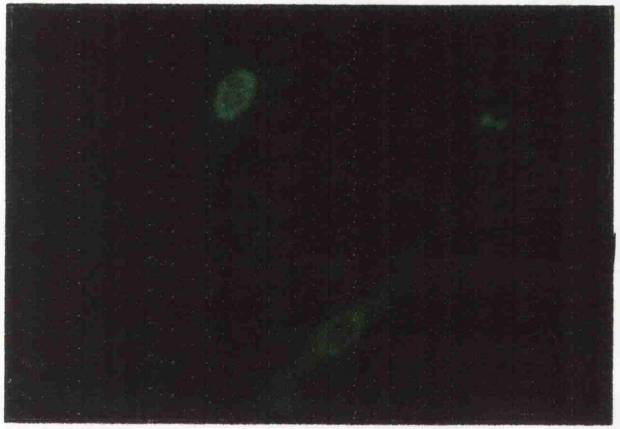
b) pE5 + pE8



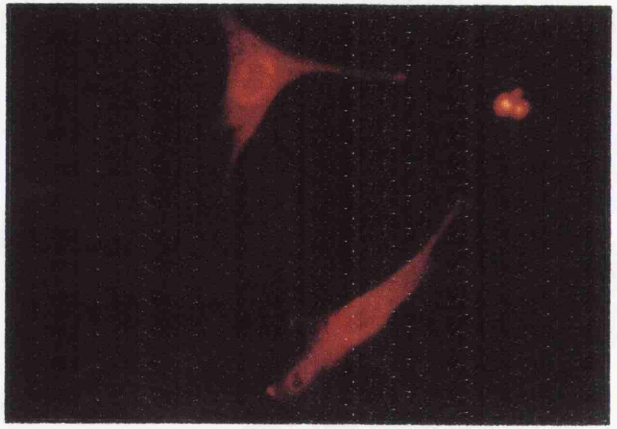
c) pE5 + pE8



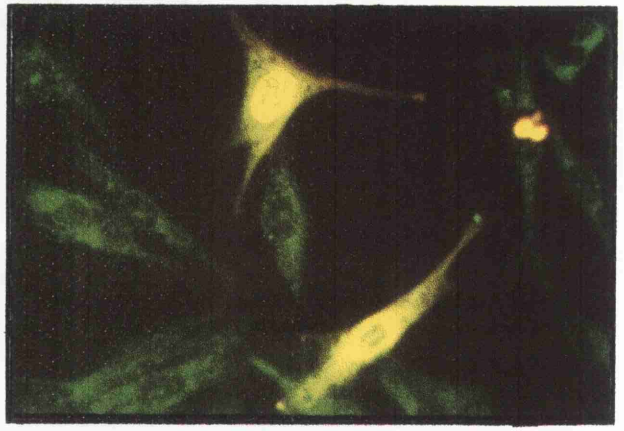
d) pE5 + pE8 + pE52



e) pE5 + pE8 + pE52

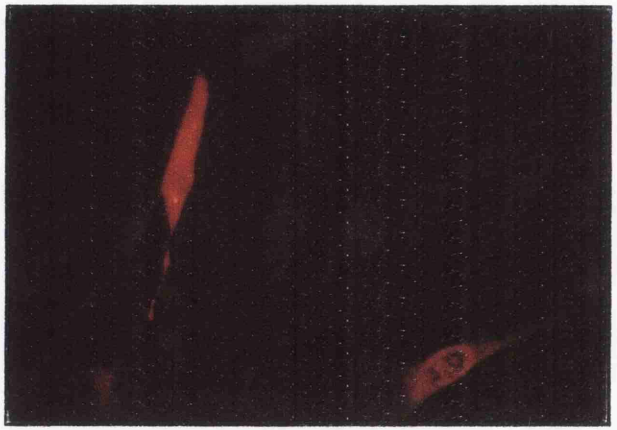


f) pE5 + pE8 + pE52

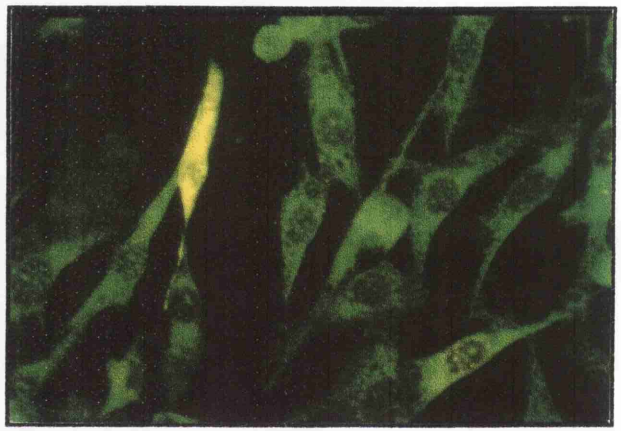


g) pE8 + pE52

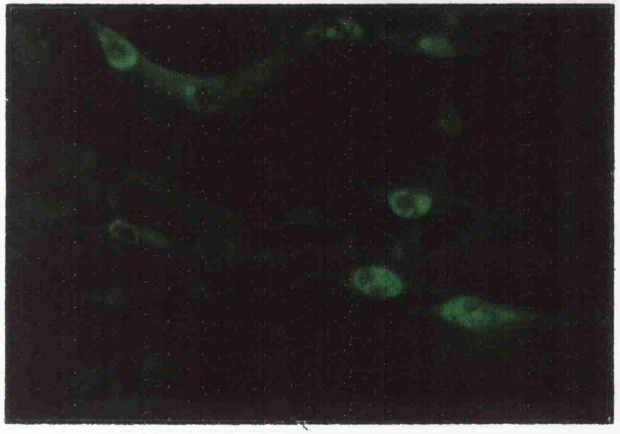
h) pE8 + pE52



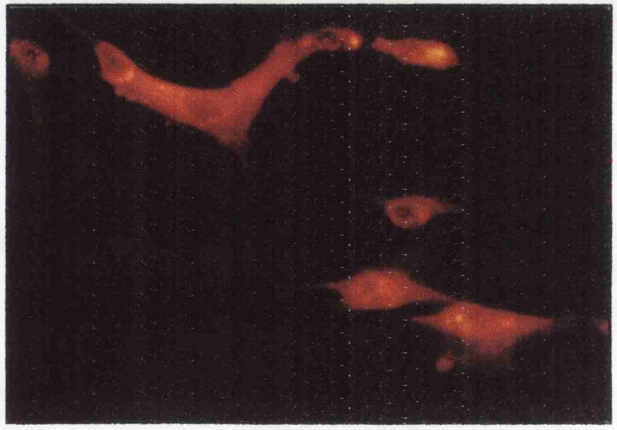
i) pE8 + pE52



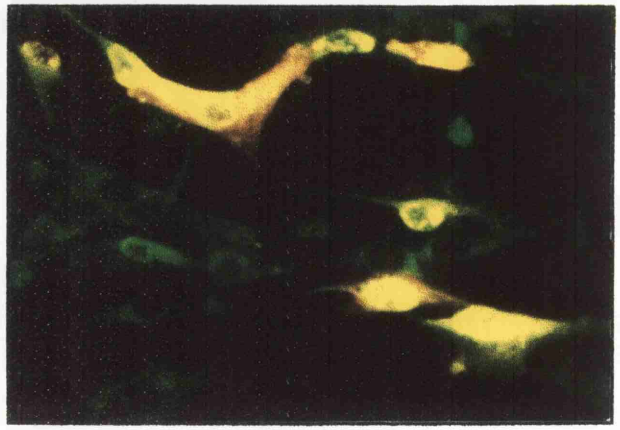
j) pE5 + pE8 + pE52



k) pE5 + pE8 + pE52



l) pE5 + pE8 + pE52



weaker but UL5 still appears to be predominantly in nuclear foci (**panel d**). Both of these cells express UL8 (**panel e**) and whilst foci of UL8 colocalised with those of UL5, there seemed to be a much higher level of diffuse cytoplasmic fluorescence for UL8 than UL5. Although the presence of UL52 protein in these cells could not be shown directly, presumably the difference in UL5 distribution between **panel a** and **panel d** is the result of UL52 being expressed in the latter but not the former. Thus it can be inferred that co-expression of UL5 with UL8 and UL52 is sufficient for efficient nuclear localisation of UL5. The less efficient nuclear localisation of UL5 seen in one cell in **panel d** may be due to lower expression of UL52 than UL5 and UL8. Certainly the relatively strong cytoplasmic fluorescence for UL8 compared to UL5 suggests that these proteins are not expressed at equivalent levels.

Panels g, h and **i** show cells lipofected with pE8 and pE52. The results were similar to those obtained with pE8 and pE5. UL52 was located exclusively in cytoplasmic foci (**panel g**) and UL8 colocalised to these foci, although UL8 protein was also found elsewhere in the cytoplasm (**panel h**). Again UL8 was located in punctate foci in the nucleus of cells not expressing UL52 (**panels g, h** and **i**). Co-expression of UL52 with UL8 is therefore not sufficient for nuclear localisation of UL52.

Panels j, k and **l** show cells lipofected with pE5, pE8 and pE52 and incubated with anti-UL52 and anti-UL8 antibodies. Several of the cells in **panel j** show efficient nuclear localisation of the UL52 protein. All of these cells also express UL8, although there appeared to be an excess of UL8 protein in these cells such that UL8 was detectable throughout the cytoplasm in addition to colocalising with UL52 in the nucleus (**panels k** and **l**). Again it is assumed that these cells are expressing UL5, since nuclear localisation of UL52 was not seen in cells lipofected with just pE52 and pE8. Thus co-expression of UL52 with UL5 and UL8 is sufficient for nuclear localisation of UL52. Two further cells are expressing UL8 in the absence of UL52 (compare **panels k** and **j**), one showing predominantly punctate nuclear UL8 (**panel k**, top left-hand corner) and the other exclusively cytoplasmic UL8 (**panel k**, large cell just left of centre). The difference between these two patterns may be due to one cell

expressing UL5 and the other not. From the double lipofections it might be predicted that there is no UL5 protein in the cell containing nuclear UL8 (top left-hand corner), but that UL5 is being expressed in the large cell containing cytoplasmic UL8.

The very bright fluorescence of UL8 protein, frequently observed in cells lipofected with pE5 and pE8, pE52 and pE8, or pE5, pE8 and pE52, suggests that UL5 and UL52 may be poorly expressed relative to UL8, making it difficult to follow the effects of UL5 and UL52 on UL8 localisation. The three plasmids used for lipofection differ in size by less than 1 kb and therefore, although lipofection mixes were made up using equivalent weights of DNA, are present in approximately equimolar amounts. It is probable that the different levels of expression are due to differences in stability of the mRNAs or in turnover rates for the three proteins, since all three genes are inserted under the control of the same promoter.

3.2.6 Discussion

Using temperature-sensitive HSV-1 recombinants in immunofluorescence assays, Calder *et al.* (1992) demonstrated that all three components of the helicase-primase complex (UL5, UL8 and UL52) must be co-expressed in order for their efficient localisation to the nucleus, whereas the UL9 protein efficiently enters the nucleus when expressed alone. They suggested a potential role for the UL8 protein may be in facilitating nuclear uptake or retention of the helicase-primase complex.

It remained possible, however, that other viral IE proteins which are expressed in *ts*K-infected cells at the NPT, or components of the input virions, could be involved in the localisation of viral DNA replication proteins. To exclude these possibilities, the localisation of the UL5, UL8, UL9 and UL52 proteins when expressed from plasmids transfected into BHK cells was investigated.

The results demonstrate that UL9 localises efficently to the nucleus of cells in the absence of all other viral proteins, and that co-expression of the three components of the helicase-primase complex is similarly sufficient for their nuclear localisation. Whereas UL52 and probably UL5, when expressed individually or in pairwise combinations with other components of the complex, do not enter the nucleus, UL8

was frequently observed in punctate nuclear foci in the absence of all other viral proteins. Whether this staining pattern represents protein that has been taken up by the nucleus, as opposed to remaining bound to the nuclear surface, was not easy to determine. The use of confocal microscopy would overcome this problem, since sections through a cell can be viewed individually, permitting a more precise location of the fluorescent signal. The reason why this nuclear localisation of UL8 was not observed with the recombinant viruses may be connected to the high level expression of UL8 from plasmid pE8.

When either UL5 or UL52 was co-expressed with UL8, the association of UL8 with the nucleus was decreased. Colocalisation of UL8 with UL5 or UL52 was observed in the cytoplasm, in agreement with the previous report that UL8 can interact separately with each of these proteins (McLean *et al.*, 1994).

The intranuclear pattern of UL9 and the helicase-primase complex is relevant to the formation and function of prereplicative sites. UL5, UL8 and UL52 colocalise with mDBP (UL29) to replication compartments in infected cells and to prereplicative sites in infected cells grown in the presence of PAA (Lukonis & Weller, 1996). Prereplicative sites form in the absence of functional viral polymerase holoenzyme and are characterised by numerous punctate foci of mDBP. Cotransfection of cells with the genes encoding mDBP, UL5, UL8 and UL52 results in mDBP localising to punctate structures resembling prereplicative sites in 40% of the cells, whereas mDBP alone or in the absence of any one component of the helicase-primase exhibits a diffuse nuclear distribution (Liptak *et al.*, 1996). These data suggest that mDBP and the trimeric helicase-primase complex are taken up independently into the nucleus but that mDBP may interact with the complex. It will be interesting to determine whether UL9 and the helicase-primase complex independently localise to the same nuclear sites and the relationship of these sites to pre-replicative sites as defined by the presence of mDBP. Such information, and the identification of host proteins which define such sites, should contribute to a further understanding of the sequence of events leading up to the initiation of HSV-1 DNA synthesis.

* and had been partially characterised in Western blotting and immunoprecipitation experiments (Parry, 1993).

Section 3.3 CHARACTERISATION OF anti-UL8 MONOCLONAL ANTIBODIES

3.3.1 Introduction

A set of twenty mouse monoclonal antibodies (MAb 0801 to MAb 0820) had previously been raised by Dr M. Parry against purified UL8 protein (Parry *et al.*, 1993; Parry, 1993; McLean *et al.*, 1994). These MAbs had been screened for reactivity with the UL8 protein by ELISA assay (Parry, 1993). In order to use these MAbs to analyse the UL8 mutants it was essential to first know whether they recognised *wt* UL8 protein when used in Western blotting, immunofluorescence and immunoprecipitation experiments, and the approximate locations of epitopes.

3.3.2 Ability of monoclonal antibodies to recognise UL8 protein by Western blotting

All twenty MAbs were screened as ascitic fluids for the ability to recognise denatured *wt* UL8 protein bound to nitrocellulose. A preparation of total protein from BHK cells lipofected with pE8 was resolved by SDS-PAGE using gels with a single well running the width of the gel. Strips of nitrocellulose were carefully aligned over the gels and the proteins were transferred by electroblotting. One of the nitrocellulose strips was incubated with the polyclonal anti-UL8 serum 094, the rest were each incubated with a single MAb. MAbs 0809, 0811, 0812, 0814, 0817 and 0818 (**Figure 20**) and 0819 (not shown) showed specific recognition of a protein which co-migrated with the protein recognised by 094 antibody and which was of the expected size for UL8.

Several antibodies (*e.g.* 0810, 0815 and 0816, **Figure 20**) recognised a protein migrating slightly faster than UL8. A protein of similar mobility was also detected with MAbs 0802, 0803, 0806, 0807 and 0808 (**Figure 21**). In this case the gel was blotted using a single sheet of nitrocellulose, which was marked before being cut into strips to avoid any possible problems with aligning the strips later.

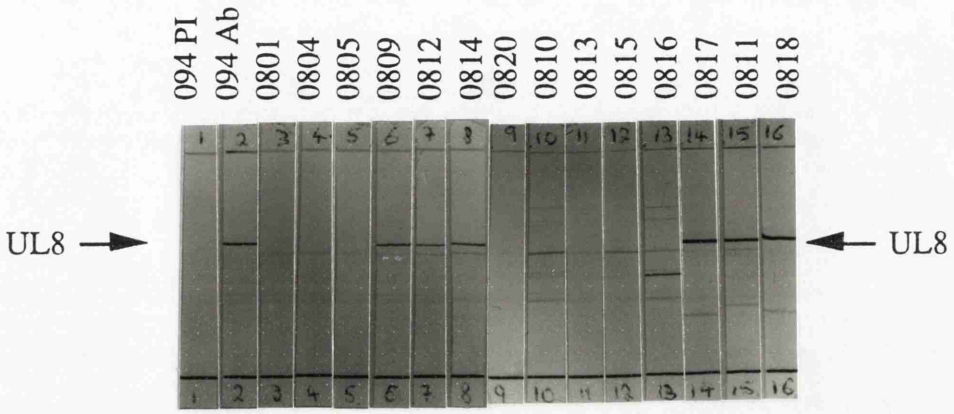


Figure 20. Screening monoclonal antibodies (MAbs) for the ability to recognise *wt* UL8 by Western blotting.

A preparation of total protein from BHK cells lipofected with pE8 was resolved by SDS-PAGE and electroblotted on to strips of nitrocellulose. Strips were incubated with 1:2000 dilutions of pre-immune serum 094 (094 PI; strip 1), polyclonal anti-UL8 serum 094 (094 Ab; strip 2) or anti-UL8 MAbs as indicated (strips 3-16). The strips were washed and incubated with 1:7500 dilutions of anti-rabbit IgG (strips 1 and 2) or anti-mouse IgG (strips 3-16) alkaline phosphatase-conjugated antibody. Following further washes the strips were incubated with colour development solution (Promega Protoblot System) until bands were visible. The position of UL8 protein is indicated.

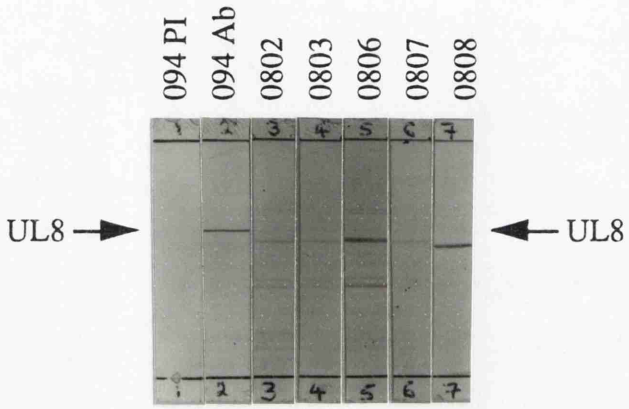


Figure 21. Further screening of monoclonal antibodies by Western blotting.

A preparation of total protein from BHK cells lipofected with pE8 was resolved by SDS-PAGE and electroblotted onto a single sheet of nitrocellulose. This sheet was cut into strips which were incubated with 1:2000 dilutions of pre-immune serum 094 (094 PI; strip 1), polyclonal anti-UL8 serum 094 (094 Ab; strip 2) or anti-UL8 MAbs as indicated (strips 3-7). The strips were processed as described in Figure 20. The position of UL8 protein is indicated.

3.3.3 Locating epitopes recognised by anti-UL8 monoclonal antibodies

The seven MAbs which efficiently recognised denatured *wt* UL8 were screened against a panel of N- and C-terminally truncated UL8 proteins to map the approximate locations of the linear epitopes. Total protein preparations from BHK cells lipofected with plasmids expressing the truncated proteins were subjected to SDS-PAGE and Western blotting and were incubated with 094 (to confirm expression of the UL8-related proteins) or with one of the seven MAbs. The antibodies could be divided into three groups according to the pattern of bands following colour development, indicating that the MAbs were recognising epitopes in at least three different regions of UL8. One blot from each category is shown in **Figure 22**, along with the blot incubated with 094 antibody which shows the relative positions of the bands representing *wt* and mutated UL8 proteins (**Figure 22a**). For each blot, **lane 9** was loaded with a total protein sample from BHK cells lipofected with the expression vector pCMV10, to control for cross-reactivity of the antibody with cellular proteins.

MAbs 0811 and 0812 recognised all of the mutant UL8 polypeptides tested (**Figure 22b**), indicating that deletion of up to 497 amino acids from the C-terminal end and up to 165 amino acids from the N-terminal end of UL8 does not remove the epitope or epitopes recognised by these two MAbs. The epitopes recognised by MAbs 0811 and 0812 must therefore lie in a region of UL8 from amino acids 166 to 253, designated Region 1.

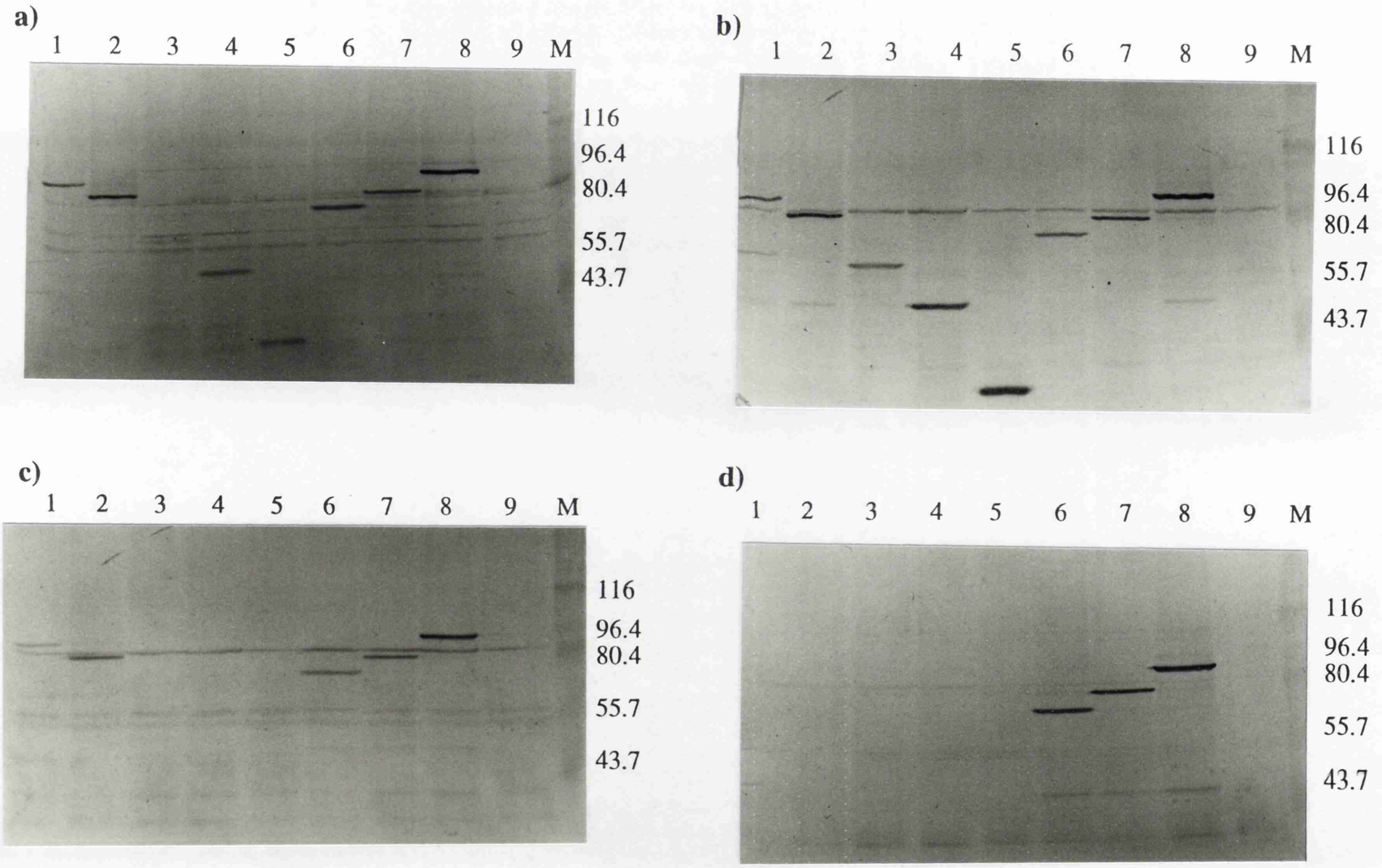
MAbs 0809 and 0814 recognised UL8 mutant polypeptides with up to 79 amino acids deleted from the C-terminus, but did not recognise mutants with C-terminal deletions of 280 amino acids or larger (**Figure 22c**). Both antibodies recognised the two N-terminally truncated polypeptides. From this it can be concluded that these two antibodies recognise epitopes lying in a region between amino acids 470 and 671 of UL8, designated Region 2.

MAbs 0817, 0818 and 0819 all recognised the two N-terminally truncated polypeptides, but did not recognise any of the mutants with C-terminal deletions (**Figure 22d**). This implies that the very C-terminal 33 amino acids of UL8 include the

Figure 22. Monoclonal antibodies that recognise UL8 protein by Western blotting can be divided into three categories according to their ability to recognise a set of truncated UL8 polypeptides.

Four SDS-polyacrylamide gels were loaded in an identical order with total protein preparations from BHK cells lipofected with the plasmids indicated below. Following electrophoresis and electroblotting of the proteins on to nitrocellulose, the blots were incubated with 1:2000 dilutions of polyclonal anti-UL8 serum 094 (**a**), MAb 0811 (**b**), MAb 0814 (**c**) and MAb 0817 (**d**). Blots were washed, incubated with 1:7500 dilutions of anti-rabbit IgG (**a**) or anti-mouse IgG (**b-d**) alkaline phosphatase-conjugated antibody and colour developed (Promega Protoblot System). The sizes of prestained molecular weight markers (M; Sigma) are indicated in kDa.

Lane	Plasmid
1	pCΔ033
2	pCΔ079
3	pCΔ280
4	pCΔ359
5	pCΔ497
6	pNΔ165
7	pNΔ097
8	pE8
9	pCMV10


a)
1 2 3 4 5 6 7 8 9 M
116
96.4
80.4
55.7
43.7
b)
1 2 3 4 5 6 7 8 9 M
116
96.4
80.4
55.7
43.7
c)
1 2 3 4 5 6 7 8 9 M
116
96.4
80.4
55.7
43.7
d)
1 2 3 4 5 6 7 8 9 M
116
96.4
80.4
55.7
43.7

epitope or epitopes to which these three antibodies bind. This region from amino acids 718 to 750 of the UL8 protein was designated Region 3.

In order to narrow down Region 2, the mutant pCΔ196 was constructed (Section 3.1.2). A total protein preparation from BHK cells lipofected with this plasmid was resolved by SDS-PAGE along with total protein preparations from cells lipofected with pE8, pCΔ079 and pCΔ280. The proteins were Western blotted and incubated with 094 antibody, MAb 0809 or MAb 0814 (**Figure 23**). Both MAbs recognised the mutant CΔ196 protein, indicating that the epitope or epitopes recognised by these two MAbs lie in the region of UL8 between amino acids 470 and 554.

The diagram in **Figure 24** shows a summary of the epitope-containing regions of UL8 and the MAbs which bind to them.

3.3.4 Two different patterns of bands on Western blots following a timecourse of UL8 gene expression

Whilst determining the parameters for Western blot analysis of wild-type and mutant UL8 proteins, an interesting cross-reactivity of at least one of the MAbs was uncovered.

A timecourse for UL8 gene expression was carried out by making preparations of total proteins from BHK cells at various times after lipofection with pE8. The protein samples were then resolved by SDS-PAGE, Western blotted on to nitrocellulose and detected using the MAbs 0812 and 0817. Using the MAb 0817, a protein of the expected size was detected 16hr after lipofection, and gradually accumulated up to 32hr post-lipofection (**Figure 25a**).

When a Western blot prepared using the same protein preparations was stained using MAb 0812, a different pattern of bands could be seen. A faint band of the expected size for UL8 (apparent mol wt approximately 85,000) was visible at 28hr post-lipofection, increasing slightly up to 48 hr post-lipofection (**Figure 25b**). However a more strongly stained polypeptide (protein X), smaller than UL8 with an apparent mol wt of approximately 78,500, appears and disappears in a cyclical manner.

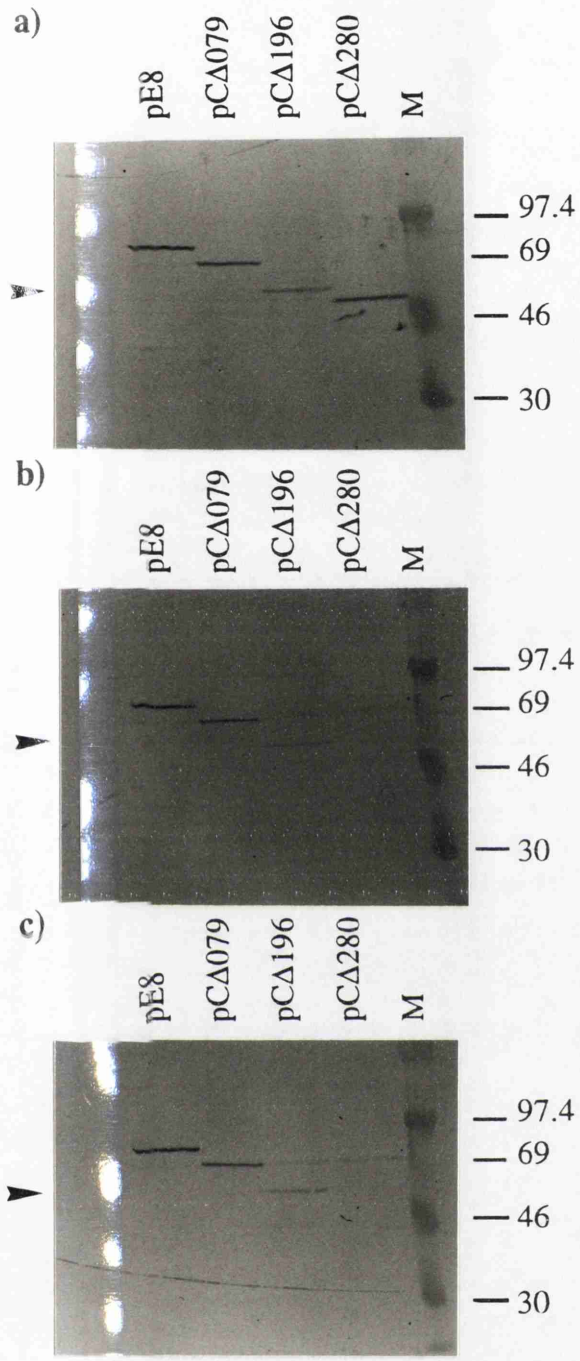


Figure 23. The region of UL8 containing epitopes recognised by MAbs 0809 and 0814 can be narrowed down to amino acids 470-554.

Total protein preparations from BHK cells lipofected with the plasmids indicated were subjected to SDS-PAGE and Western blotting in triplicate. Blots were incubated with 1:2000 dilutions of polyclonal anti-UL8 serum 094 (**a**), MAb 0809 (**b**) or MAb 0814 (**c**) and processed as described in Figure 22. The position of protein CΔ196 is indicated and the sizes of the prestained molecular weight markers (M; Amersham) are given in kDa.

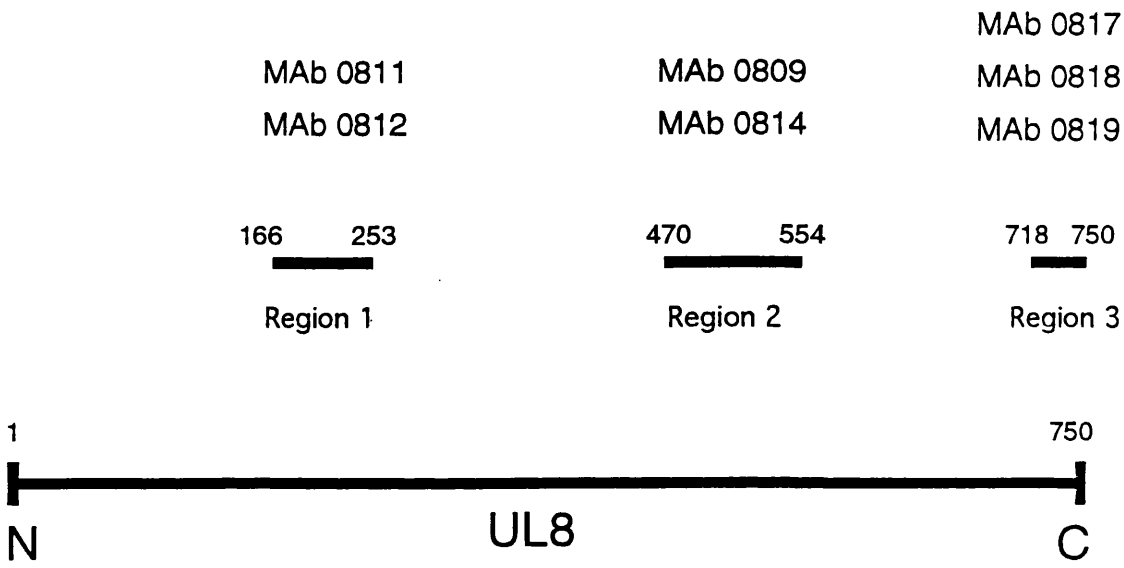


Figure 24. Summary of the epitope-containing regions of UL8 and the monoclonal antibodies which bind to them.

The UL8 protein is represented as a single bar with its first and last amino acids numbered and the N- and C-termini indicated. Above it are marked the three epitope-containing regions along with the amino acid positions which constitute the limits of each region. Above each region are listed the MAbs which bind to that region.

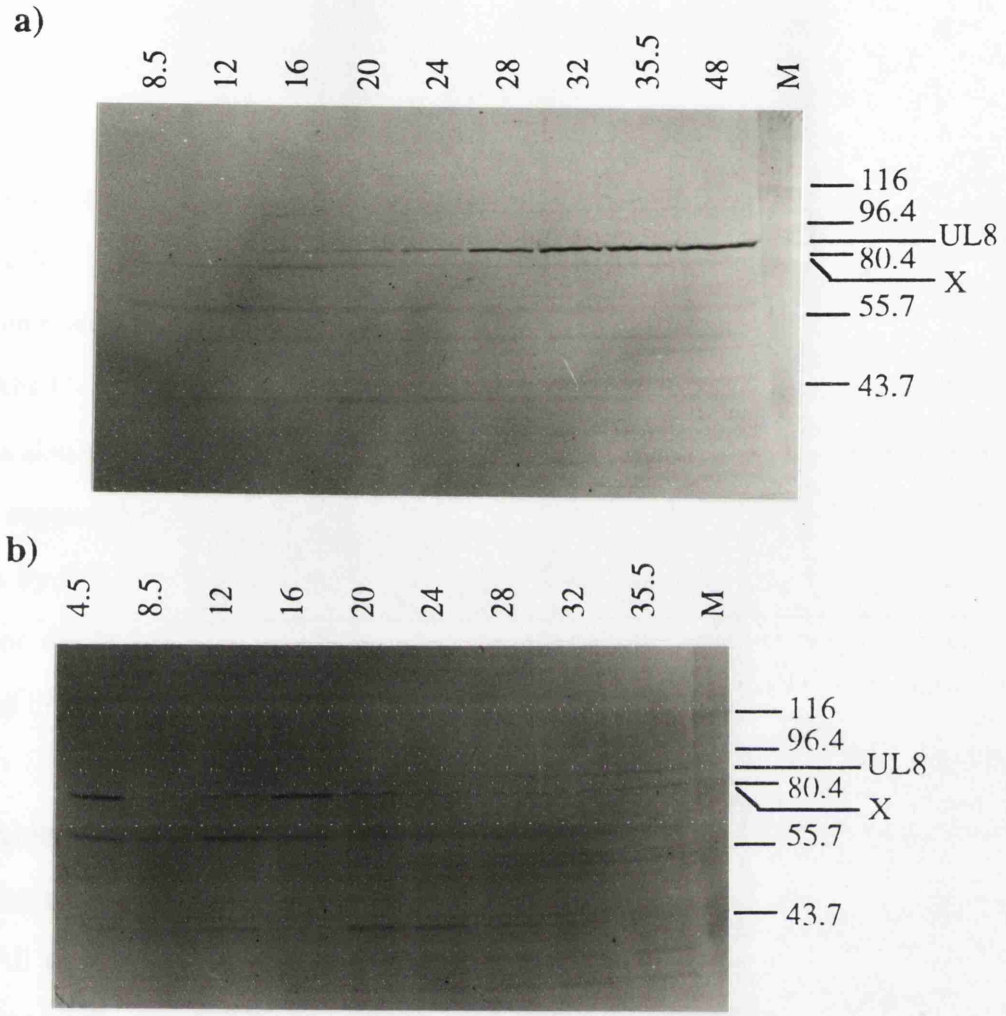


Figure 25. Two monoclonal antibodies show different patterns of bands on Western blots following a timecourse of UL8 gene expression.

Cells were lipofected with pE8 and total protein preparations were made at the times indicated (in hrs) after lipofection. These were resolved by SDS-PAGE, Western blotted and incubated with 1:2000 dilutions of MAb 0817 (**a**) or 0812 (**b**). Lane M on both blots contains prestained molecular weight markers (sizes in kDa as indicated). The positions of UL8 protein and the BHK cell protein X are marked.

This cyclical pattern was seen reproducibly, with maxima at 4, 16, 32 and 48hr post-lipofection, although the relative amounts of protein X at each maximum varied between experiments.

A strongly stained band of similar size can also be seen in the protein samples stained with the other Region 1 antibody, MAb 0811 (**Figure 22b**) and with the Region 2 MAb 0814 (**Figure 22c**). A band of similar mobility to protein X also appears on many of the strips shown in **Figures 20** and **21**, especially those incubated with MAbs 0806 and 0810. Protein X is unlikely to be a proteolytic product of UL8 since it is also seen in pCMV10-transfected cells (**Figures 22b** and **22c**, **lane 9**). These findings suggest that the cells used for lipofection may to some extent be synchronised (perhaps by the process of trypsinising and reseeding prior to lipofection) and that several of the MAbs cross-react quite strongly with a BHK cell protein which is expressed in a cell cycle dependent manner. This observation was not followed up.

3.3.5 Ability of monoclonal antibodies to recognise UL8 protein by immunofluorescence

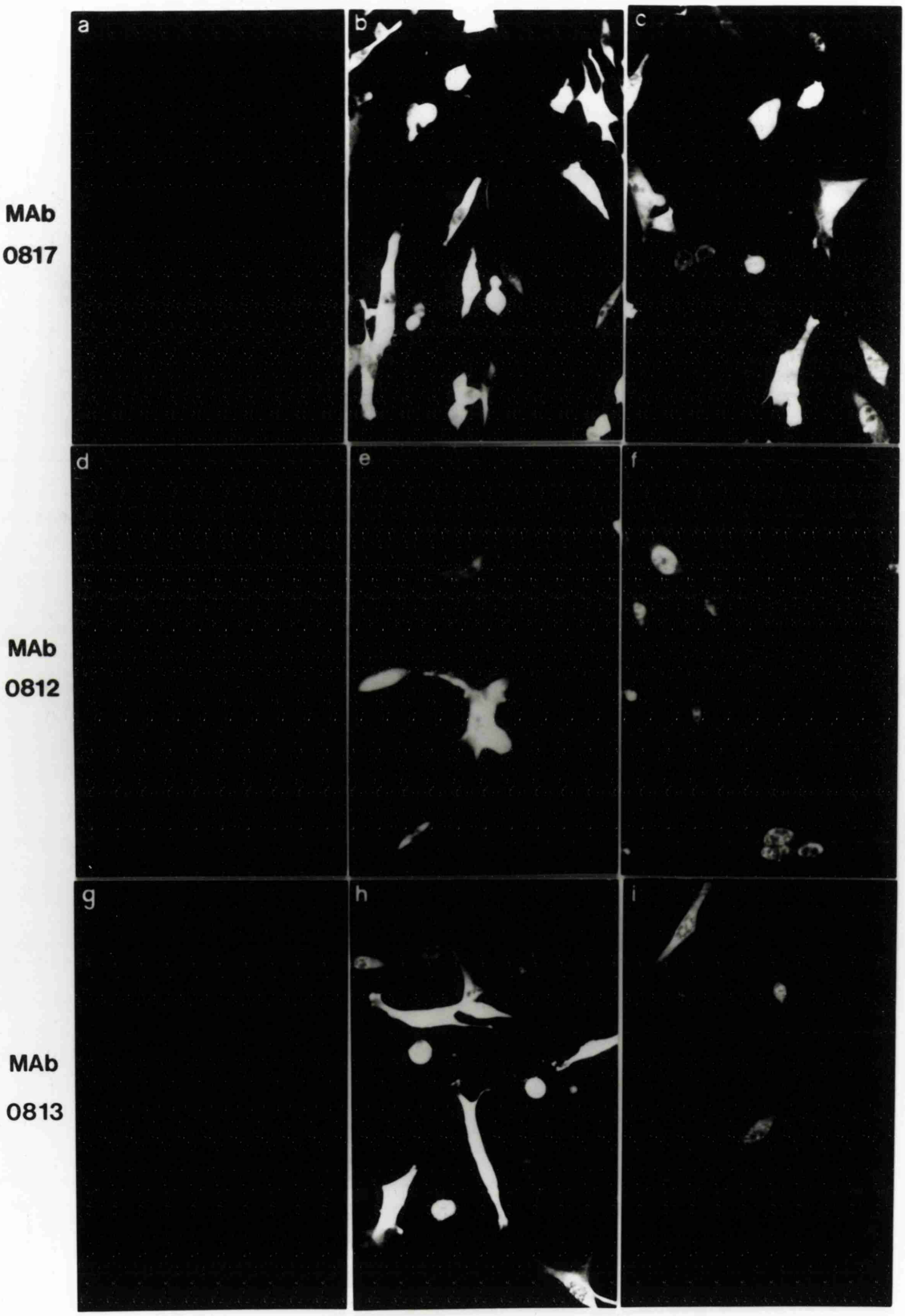
All twenty MAbs were screened for their ability to recognise UL8 protein within fixed cells. For each MAb, three plates of BHK cells were lipofected, one with pCMV10, one with pE8 and one with a mixture of pE5, pE8 and pE52. 24 hr later the cells in all three plates were fixed and incubated with a single MAb and an anti-mouse IgG fluorescein-conjugated antibody.

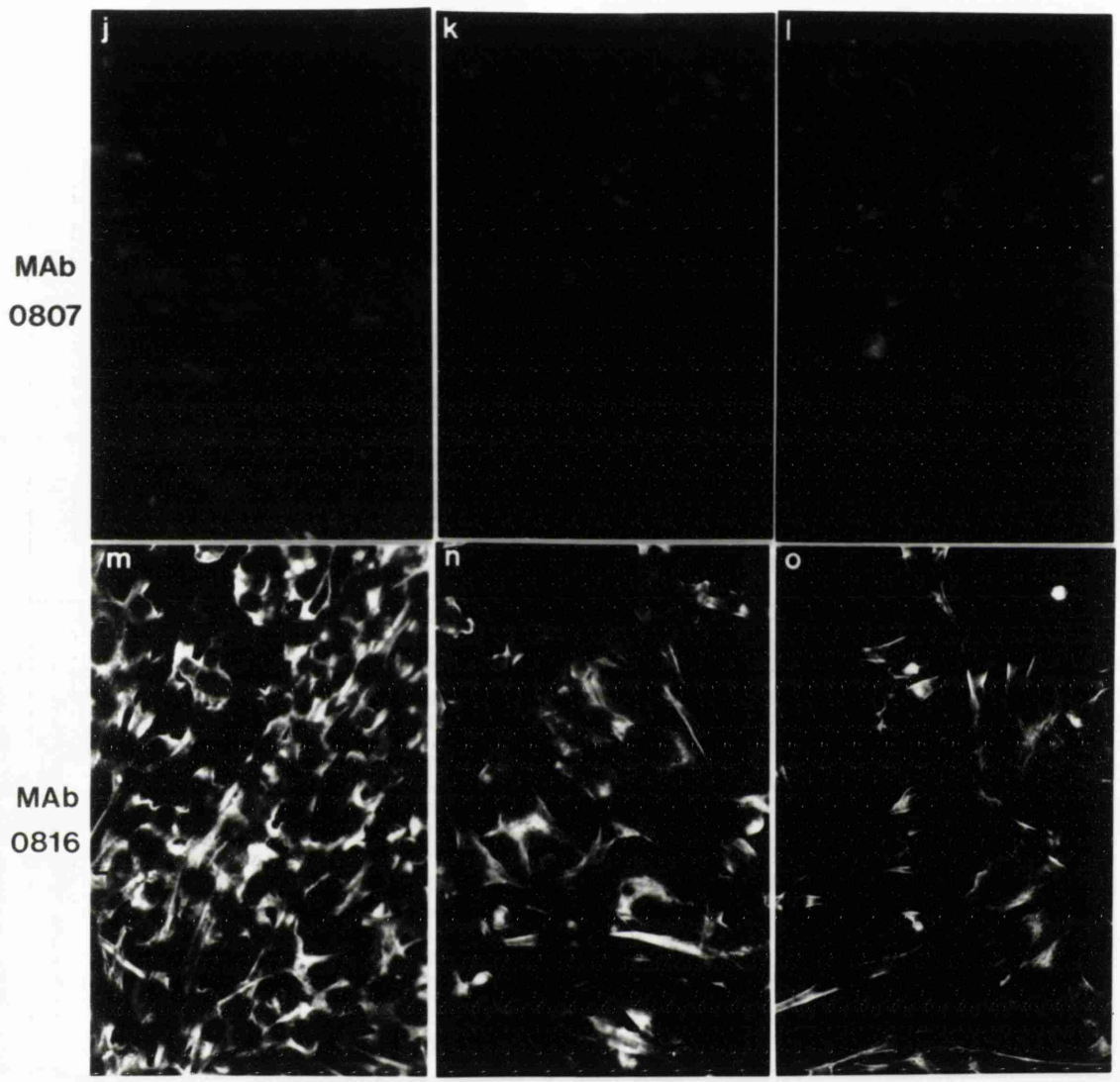
MAbs 0805, 0811, 0817, 0818 and 0819, which showed a very low background of fluorescence in pCMV10-lipofected cells and strong specific recognition of UL8 both in cells lipofected with pE8 alone and those lipofected with pE5, pE8 and pE52, were scored as '++' (cells incubated with MAb 0817 are shown in **Figure 26a-c**). MAbs 0804, 0812 and 0815, showing a similar pattern of recognition but with a weaker fluorescent signal, were scored as '+' (**Figure 26d-f**: cells incubated with MAb 0812).

MAbs 0813 (**Figure 26g-i**), 801 and 0814 exhibited specific recognition of UL8 when expressed without UL5 and UL52 (**g** and **h**) but the fluorescent signal was

Figure 26. Monoclonal antibody recognition of UL8 protein by immunofluorescence.

BHK cells lipofected with pCMV10 (**panels a, d, g, j** and **m**), pE8 (**panels b, e, h, k** and **n**) or with pE5, pE8 and pE52 (**panels c, f, i, l** and **o**) were fixed 24 hr post-lipofection and incubated with a 1:200 dilution of MAb 0817 (**a-c**), 0812 (**d-f**), 0813 (**g-i**), 0807 (**j-l**) or 0816 (**m-o**). The cells were washed, incubated with a 1:80 dilution of anti-mouse IgG fluorescein-conjugated antibody, washed again and mounted under coverslips. Cells were viewed under x25 magnification.


pCMV10
pE8
pE5+pE8+pE52
MAb 0817
MAb 0812
MAb 0813
a
b
c
d
e
f
g
h
i


pCMV10
pE8
pE5+pE8+pE52
j
k
l
MAb
0807
m
n
o
MAb
0816

greatly reduced when all three genes were lipofected together (**Figure 26i**). These MAbs were scored as being able to recognise UL8 alone, but not in the presence of UL5 and UL52.

When there was little detectable fluorescence in any of the three plates the MAbs were scored as '-' (**Figure 26j-l**: cells incubated with MAb 0807). MAbs 0806, 0810 and 0816 showed no specific recognition of UL8 protein (*i.e.* no difference between the three plates of cells) but there was a very clear pattern of fluorescence in the cells which resembled cytoskeletal fibres, particularly around the periphery of the cells (**Figure 26m-o**: MAb 0816). As all three of these MAbs had also exhibited a relatively strong affinity for host proteins in Western blots, these three MAbs were scored as being positive for proteins expressed by BHK cells (BHK +).

Table 4 summarises the reactivity of these anti-UL8 MAbs in Westen blotting and immunofluorescence experiments, and also in immunoprecipitation experiments as determined by Dr G. McLean.

3.3.6 Discussion

The availability of a panel of anti-UL8 monoclonal antibodies provides a potentially powerful tool for analysing both wild-type and mutated UL8 proteins. In general the abilities of the MAbs to detect *wt* UL8 protein by immunofluorescence (IF) and immunoprecipitation (IP) were similar - only MAb 0820 detected UL8 in one assay (IP) but not the other (IF). Three MAbs (0804, 0805 and 0815) which were positive by both IF and IP however failed to recognise UL8 on a Western blot. These results are consistent with the UL8 protein maintaining a relatively native conformation in the IF and IP assays but being more extensively denatured by Western blotting. MAbs 0804, 0805 and 0815 are therefore likely to recognise conformational epitopes. In contrast, MAb 0809 recognises denatured but not native UL8 protein. Such antibodies might be useful for identifying whether mutated UL8 proteins can adopt a correctly folded conformation.

MAbs 0801, 0813 and 0814 may also prove useful since they recognise uncomplexed but not complexed UL8. Further definition of their epitopes may allow

Table 4. Summary of the reactivities of twenty monoclonal antibodies raised against purified UL8 protein.

(a) MAbs that recognise *wt* UL8 protein by Western blotting are marked +, those that do not are marked -. The number in brackets refers to the region that epitopes have been mapped to: **(1)** amino acids 166-253; **(2)** amino acids 470-554; **(3)** amino acids 718-750. A host band (protein X) of greater mobility than UL8, with an apparent molecular weight of approximately 80kDa, was strongly **(X)** or weakly **(x)** stained by the MAbs indicated. **(*)** indicates MAbs which strongly stain bands of greater mobility than protein X.

(b) MAbs not capable of recognising UL8 protein by immunofluorescence are marked -, those that show specific staining of UL8 protein by immunofluorescence are graded as giving strong (++) or weaker (+) staining. **(UL8)** indicates that the MAb binds to UL8 alone but shows reduced staining upon coexpression of UL8 with UL5 and UL52. **(BHK+)** indicates that the MAb crossreacts with cellular proteins to give a distinctive pattern of fluorescence.

(c) MAbs are scored as strongly (++), weakly (+), or incapable of (-) precipitating UL8 protein from extracts of insect cells infected with AcUL8 (see section 3.5). Some of the MAbs were not tested **(nt)**. Immunoprecipitation data kindly provided by Dr G. McLean.

Antibody	Western blot [a]			Immunofluorescence [b]	Immunoprecipitation [c]
MAb 0801	-		(x)	+ (UL8)	++
MAb 0802	-		(x)	-	-
MAb 0803	-		(x)	-	-
MAb 0804	-		(x)	+	++
MAb 0805	-		(x)	++	++
MAb 0806	-		(X)	- (BHK)	-
MAb 0807	-		(x)	-	-
MAb 0808	-		(*)	-	nt
MAb 0809	+	(2)	(X)	-	nt
MAb 0810	-		(X)	- (BHK)	nt
MAb 0811	+	(1)	(X)	++	++
MAb 0812	+	(1)	(X)	+	nt
MAb 0813	-		(x)	++ (UL8)	+
MAb 0814	+	(2)	(X)	+ (UL8)	nt
MAb 0815	-		(X)	+	nt
MAb 0816	-		(x/*)	- (BHK)	nt
MAb 0817	+	(3)	(x)	++	++
MAb 0818	+	(3)		++	++
MAb 0819	+	(3)	(x)	++	++
MAb 0820	-			-	++

identification of surface regions of UL8 involved in intersubunit interactions. It would be interesting to express UL8 with UL5 or UL52 alone to see whether one protein is capable of blocking the antibody binding site.

Epitopes recognised by MAbs 0817, 0818 and 0819 were mapped by Western blotting to the C-terminal 33 amino acids of UL8. The ability of these three MAbs to bind to UL8 in all three assays suggests that the very C-terminus of UL8 may be relatively exposed in the tertiary structure of UL8, rather than buried within the molecule. Preliminary results have indicated that this region interacts with the UL30 subunit of the HSV-1 polymerase and that MAbs 0817, 0818 and 0819 can block the interaction (H. Marsden, personal communication). It would therefore be interesting to determine whether these MAbs remain capable of detecting UL8 in immunofluorescence assays in HSV-1 infected cells coexpressing UL8 and UL30.

It is perhaps surprising that several MAbs, recognising different epitopes of UL8 or even failing to recognise UL8 at all by Western blot analysis, appear to recognise the same cellular protein with an apparent molecular weight slightly less than that of UL8 (designated protein X). Since this was not observed with all MAbs it is unlikely to represent an interaction of the secondary anti-mouse IgG antibody with a protein on the blot. The reason for this reactivity with a hamster cell protein is not clear, particularly since the immunogen used for MAb generation was purified protein from insect cells infected with a recombinant baculovirus. A possible explanation is that the reactivities are due to other antibodies produced by the mice used to generate the ascitic fluids, unconnected with the injected hybridoma cells (between 2 and 10% of the antibodies from ascitic fluid will be from the mouse's current antibody repertoire and not from the hybridoma: Harlow & Lane, 1988).

The staining patterns obtained with MAb 0809 present an interesting paradox. In cells lipofected with pE5 and pE52, staining with MAb 0809 results in predominantly perinuclear, punctate fluorescence (**Figure 17c**). However, in a separate set of experiments, cells lipofected with either pCMV10, pE8, or with pE5, pE8 and pE52, and stained with MAb 0809, exhibited virtually no detectable fluorescence at all (data not shown, see **figure 26 j-l** for a comparable pattern resulting from staining with

MAb 0807). Further work is obviously required to determine whether there is any specific recognition of UL5 and/or UL52 by MAb 0809. If it turns out that the MAb can recognise one or other of these proteins or a complex of both, this could possibly be explained by postulating that the MAb 0809 ascitic fluid also contains anti-idiotype antibodies reactive with a region of UL8 involved in interacting with UL5 and/or UL52. The combining surface of such anti-idiotype antibodies would resemble a region of the surface of the UL8 protein involved in the interaction. Consistent with this, MAb 0809 recognises an epitope between amino acids 470-554 of UL8, whilst a second MAb (0814), which recognises an epitope in the same region, detects UL8 when expressed alone but not when it is coexpressed with UL5 and UL52. It should be noted, however, that if such anti-idiotype antibodies are present, then there should be some recognition of the native protein by IF/IP assays. A precedent for this theory comes from Marsden *et al.* (1994), who used UL42-reactive anti-idiotype antibodies present in the serum of rabbits immunised with peptides from UL30 to demonstrate complementarity of the structure of the UL30 C-terminus to a region of UL42.

Section 3.4 ANALYSIS OF UL8 DELETION MUTANTS

3.4.1 Ability of UL8 deletion mutants to support HSV-1 DNA replication

A transient transfection assay for HSV-1 origin-dependent DNA replication, in which the seven HSV-1 genes are expressed under the control of the efficient HCMV major IE promoter (Heilbronn & Zur Hausen, 1989; Stow *et al.*, 1993), was used to test the mutant UL8 proteins for replicative ability. Plasmids carrying *wt* copies of the other six HSV-1 replication genes (pE5, pE9, pE29, pE30, pE42 and pE52), and a similar plasmid containing either wild-type UL8 (pE8) or a mutated UL8 gene, were co-transfected into BHK cells together with pS1 (an HSV-1 ori_S-containing plasmid). DNA was extracted 30 hr post-transfection, cleaved with *Eco*RI and *Dpn*I and subjected to agarose gel electrophoresis and Southern blotting. Replication of plasmid pS1 was detected by hybridisation to a labelled probe (plasmid pTZ19U) which hybridises specifically to vector DNA sequences. Replicated pS1 is resistant to digestion by *Dpn*I and so can be distinguished from *Dpn*I-sensitive unreplicated pS1 molecules. *Eco*RI is used to reduce the replicated concatemeric DNA molecules to unit length species. In all transient replication assays, ethidium bromide-stained agarose gels were examined by UV light to check that recovery of DNA from all samples was equivalent.

In agreement with previous results, efficient replication of the origin-containing plasmid occurred when all seven *wt* DNA replication proteins were present (**Figure 27, lanes 2** and **7**), but not when UL8 was omitted (**lanes 1** and **14**). All but two of the UL8 deletion mutants failed to support replication of pS1 in the presence of the other six replication proteins (*e.g.* **lanes 4, 5, 8, 9, 12** and **13**). Of the two mutants that were able to support replication, pNΔ005 exhibited replicative ability similar to the *wt* protein (compare **lanes 10** and **11** to **lane 7**) but replication by pCΔ004 was reduced approximately 4 to 5-fold (**lane 3**).

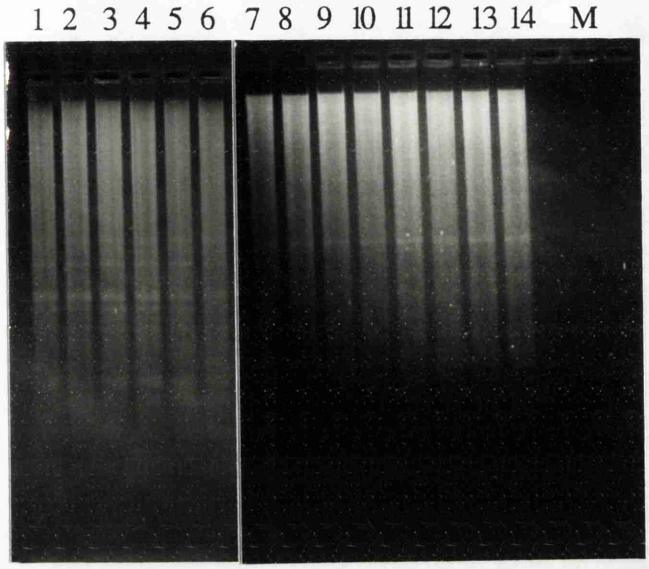
To quantify the levels of replication more accurately, the experiment was repeated and the Southern blot exposed to a PhosphorImager screen. The data were processed using the ImageQuant package (Molecular Dynamics) and are summarised

Figure 27. Transient assay of HSV-1 origin-dependent DNA replication.

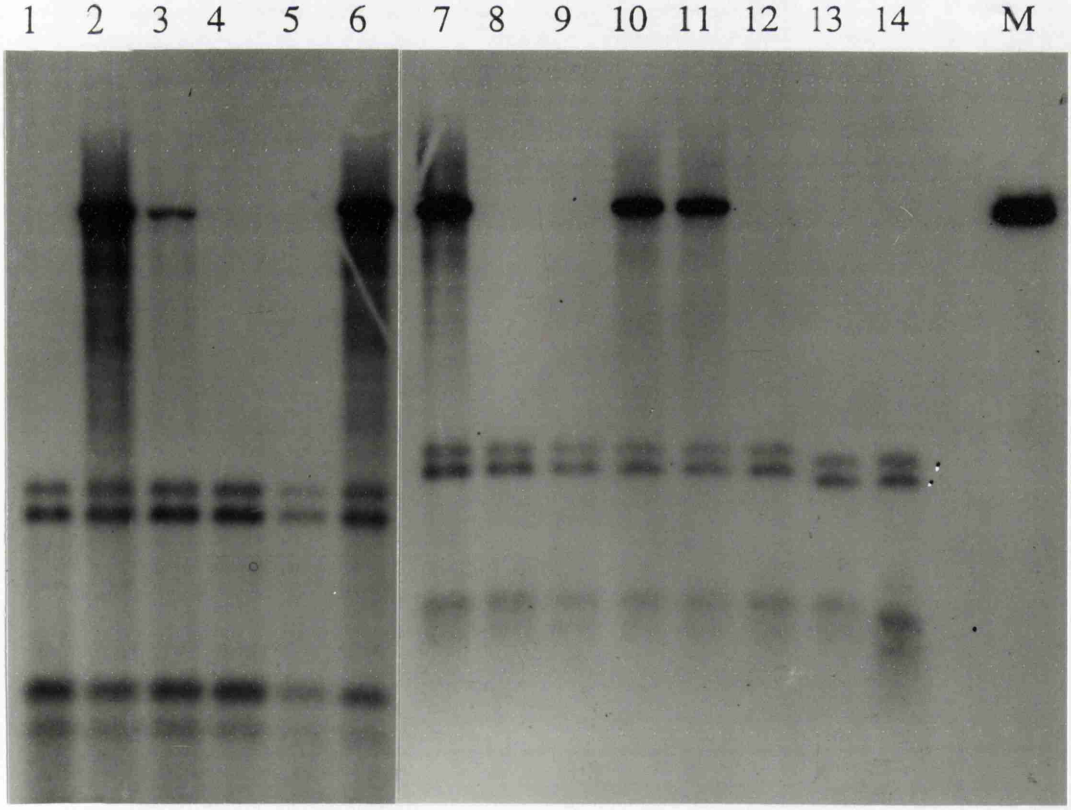
Cells were cotransfected with the plasmids indicated below, together with plasmids expressing the other six HSV-1 DNA replication proteins (pE5, pE9, pE29, pE30, pE42 and pE52) and the origin-containing plasmid pS1. 30 hr post-transfection DNA was extracted and cleaved with *Eco*RI and *Dpn*I, subjected to agarose gel electrophoresis (**a**: ethidium bromide stained gels) and Southern blotting, and hybridised to ^{32}P-labelled pTZ19U (**b**: autoradiographs). The marker (lane M) indicates the position of linearised pS1.

Lane	Plasmid	Lane	Plasmid
1	pCMV10	8	pNΔ165
2	pE8	9	pNΔ023
3	pCΔ004	10	pNΔ005
4	pCΔ033	11	pNΔ005
5	pCΔ041	12	pNΔ043
6	pCΔ3'	13	pNΔ097
7	pE8	14	pCMV10

a)



b)



Plasmids	Replicated pS1 DNA
6 wt + pCMV10	4.4 *
6 wt + pE8	100.0
6 wt + pNΔ005	88.8
6 wt + pNΔ023	0.9
6 wt + pNΔ033	0.3
6 wt + pNΔ004	22.8

Table 5. Quantification of transient assays of HSV-1 origin-dependent DNA replication.

The transient replication assay was performed as described in Figure 27 with pS1 (containing ori_S), the six plasmids pE5, pE9, pE29, pE30, pE42 and pE52 (6 wt) and additional plasmids as indicated. The resulting Southern blot was exposed to a PhosphorImager screen and the data processed using ImageQuant (Molecular Dynamics). The amount of replicated pS1 DNA is shown as a percentage of that present in the 6 wt + pE8 sample.

* The figure of 4.4% for pS1 DNA replicated in the presence of six of the replication genes (6 wt + pCMV10) was artificially high due to overlap of the signal from the pS1 marker DNA, which was loaded in the neighbouring track on the gel.

in **Table 5**. The amount of replicated pS1 DNA is given as a percentage of that synthesised in the presence of *wt* UL8 and the other six replication proteins (6 wt + pE8). When pNΔ005 was substituted for pE8, the amount of pS1 DNA replicated was 88.8% of the wild-type level, indicating that pNΔ005 is relatively unimpaired in UL8 replicative functions. In the presence of pCΔ004 the amount of replicated pS1 DNA was reduced to 22.8% of the wild-type level. Deletion of four amino acids from the C-terminus of the UL8 protein therefore interferes with but does not abolish its ability to function in DNA replication.

Since both pCΔ033 and pNΔ023 were unable to support pS1 synthesis (less than 1% of the wild-type level), these experiments indicate that sequences essential for UL8 replicative function are present within 33 amino acids of the C-terminus and 23 amino acids of the N-terminus of the protein.

3.4.2 Ability of UL8 deletion mutants to act as dominant inhibitors of replication

For some proteins, co-expression of a mutant form interferes with the functions of the wild-type form (for an overview of the mechanisms by which mutant proteins can exert dominant negative effects, see Herskowitz, 1987). In the case of DNA replication proteins this may result in a reduction in or complete absence of DNA synthesis (*e.g.* HSV-1 UL9: Stow *et al.*, 1993).

Selected plasmids encoding mutant UL8 proteins were therefore assayed for ability to interfere with HSV-1 origin-dependent DNA synthesis in transfected BHK cells. Plasmids containing *wt* copies of all 7 HSV-1 DNA replication genes, including UL8, were co-transfected into BHK cells along with one of the UL8 mutants and the origin-containing pS1. At 30 hr post-transfection the DNA was extracted and analysed by Southern blotting as described in Section 3.4.1. The blot was exposed to a PhosphorImager screen and the data processed using the ImageQuant package (Molecular Dynamics).

An autoradiograph of the blot from a single experiment is shown in **Figure 28** and the results from four separate experiments are summarised in **Table 6**. Although

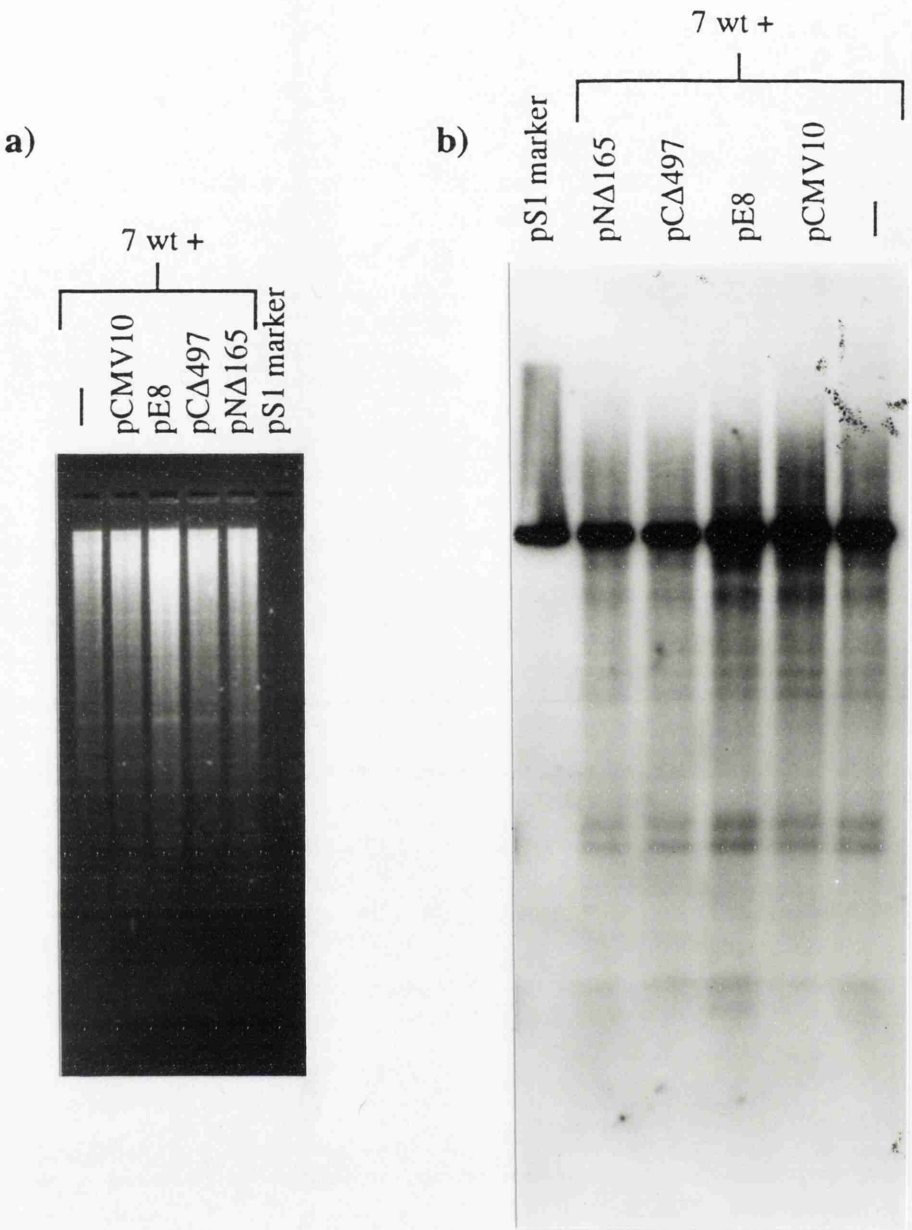


Figure 28. Inhibition of HSV-1 origin-dependent DNA replication.

Cells were cotransfected with pS1 (containing ori_S), seven plasmids carrying *wt* HSV-1 DNA replication genes (7 wt: pE5, pE8, pE9, pE29, pE30, pE42, pE52) and additional plasmids as indicated. The DNA was prepared and analysed as described in Figure 27. **a)** ethidium bromide-stained gel; **b)** autoradiograph of the corresponding Southern blot. Quantification of these data are presented as experiment no. 1 in Table 6.

expt no. / plasmids	1	2	3	4
7 wt	100	100	100	100
7 wt + pCMV10	97	92	107	135
7 wt + pE8	112	109	140	60*
7 wt + pNΔ005	nd	nd	73	177
7 wt + pNΔ023	nd	nd	26*	101
7 wt + pNΔ165	55	44	nd	nd
7 wt + pCΔ497	44	32	nd	nd
7 wt + pCΔ033	nd	nd	87	nd
7 wt + pCΔ004	nd	nd	153	131
7 wt + p8ΔN1	nd	nd	149	121
7 wt + p9CT	nd	nd	nd	15

Table 6. Inhibition of HSV-1 origin-dependent DNA replication.

The table shows data from a series of experiments. Transient replication assays were carried out as described in Figure 27. BHK cells were transfected with pS1 (origin-containing), seven plasmids each containing one of the *wt* HSV-1 DNA replication genes (7 wt) and additional plasmids as indicated. The figures in each column represent the amount of replicated pS1 DNA, expressed as a percentage of the amount in the 7 wt sample. **nd** signifies not done. **p9CT** expresses the C-terminal domain of the UL9 protein and has previously been shown to act as a potent dominant inhibitor of replication in this assay (Stow *et al.*, 1993). * signifies there was a loss of DNA in this sample as judged by examination of the ethidium bromide-stained gel by UV light.

there was some variability between experiments, it is clear from **Table 6** that none of the UL8 mutants tested exhibited a strong dominant inhibitory phenotype. For example, the product of the most inhibitory plasmid, pCΔ497, was found to reduce the amount of replicated pS1 DNA to an average 38% of the amount synthesised in the presence of the seven *wt* replication proteins (a 3-fold reduction). In comparison, Stow *et al.* (1993) reported a 30-fold reduction exerted by the UL9 C-terminal domain, and a 7-fold reduction by the UL9CT construct was observed in experiment no. 4 (**Table 6**).

3.4.3 Ability of UL8 deletion mutants to affect the intracellular localisation of UL5 and UL52

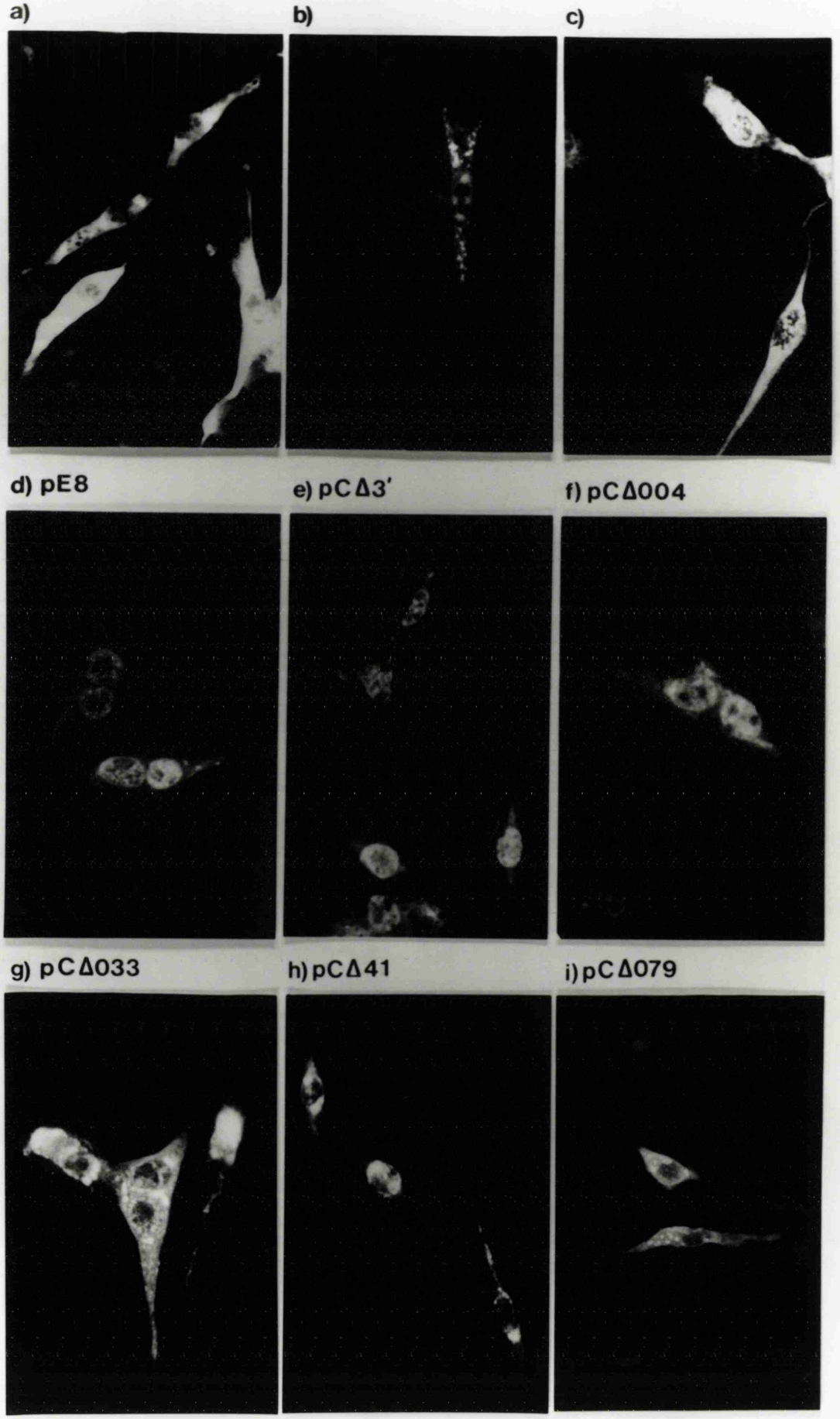
It has been established, using HSV-1 recombinants, that co-expression of UL5, UL8 and UL52 is necessary for efficient localisation of all three proteins to the nucleus (Calder *et al.*, 1992). Moreover, cotransfection experiments demonstrate that when expressed in the absence of all other viral proteins, UL5 and UL52 remain cytoplasmic, but that co-expression with the other two components of the helicase-primase complex is sufficient for nuclear uptake of all three proteins (Section 3.2). Since UL5 and UL52 are capable of forming a stable subassembly with helicase and primase activities (Calder & Stow, 1990; Dodson & Lehman, 1991), this suggests that UL8 may have an important role to play in facilitating nuclear localisation of the helicase-primase complex. The UL8 deletion mutants were therefore tested for their ability to replace *wt* UL8 protein in facilitating the nuclear localisation of UL5 and UL52.

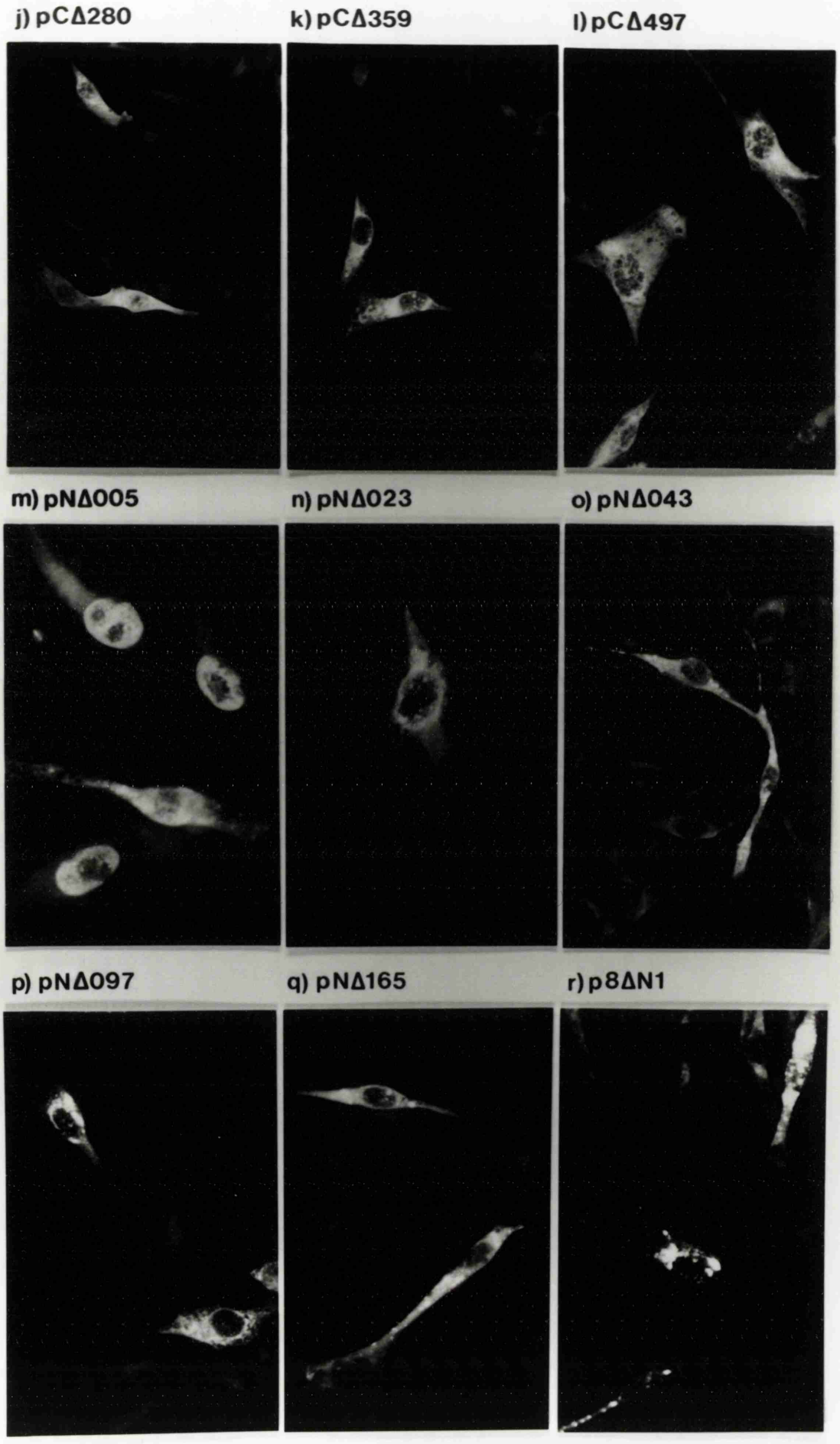
BHK cells were cotransfected (using the liposome method) with plasmids pE5, pE52 and either pE8 or one of the plasmids containing a mutated UL8 gene. 24 hr post-lipofection the cells were fixed and incubated with C-terminal anti-peptide rabbit serum against UL5 or UL52 (Olivo *et al.*, 1989). The cells were then washed and stained with anti-rabbit IgG fluorescein-conjugated antibody.

The first four panels of **Figure 29** show the localisation of UL52 protein when expressed alone (**panel a**) and in conjuction with other components of the helicase-primase complex (**panels b-d**). UL52 showed a predominantly cytoplasmic pattern of

Figure 29. Effect of mutant UL8 proteins on the intracellular localisation of UL52.

Cells were lipofected with pE52 (**panel a**), pE8 and pE52 (**panel b**), pE5 and pE52 (**panel c**) or co-lipofected with pE5, pE52 and a third plasmid as indicated (**panels d-r**). 24 hr post-lipofection the cells were fixed, incubated with a 1:100 dilution of C-terminal anti-peptide rabbit serum to UL52, stained with a 1:80 dilution of anti-rabbit IgG fluorescein-conjugated antibody and mounted under coverslips. Cells were viewed under x50 magnification.


a)
b)
c)
d) pE8
e) pCΔ3'
f) pCΔ004
g) pCΔ033
h) pCΔ41
i) pCΔ079


j) pCΔ280
k) pCΔ359
l) pCΔ497
m) pNΔ005
n) pNΔ023
o) pNΔ043
p) pNΔ097
q) pNΔ165
r) p8ΔN1

fluorescence when expressed alone (**panel a**), and in the presence of UL8 (**panel b**) or UL5 (**panel c**). In the presence of both UL5 and UL8, UL52 localised efficiently to the nucleus (**panel d**).

Cotransfection of pE5 and pE52 with pCΔ3', which expresses wild-type UL8 protein, resulted in a similar pattern of nuclear localisation for UL52 as observed with pE8 (**panel e**). Likewise, when CΔ004 was coexpressed with UL5 and UL52, a nuclear pattern of fluorescence was seen for UL52 (**panel f**). However, cotransfection of pCΔ033, pE5 and pE52 resulted in cells showing cytoplasmic fluorescence only (**panel g**), indicating that CΔ033 is unable to replace *wt* UL8 in facilitating the efficient nuclear localisation of UL52. Similarly, no nuclear fluorescence was detected in cells coexpressing UL5, UL52 and any of the other five C-terminally truncated mutants (**panels h-l**).

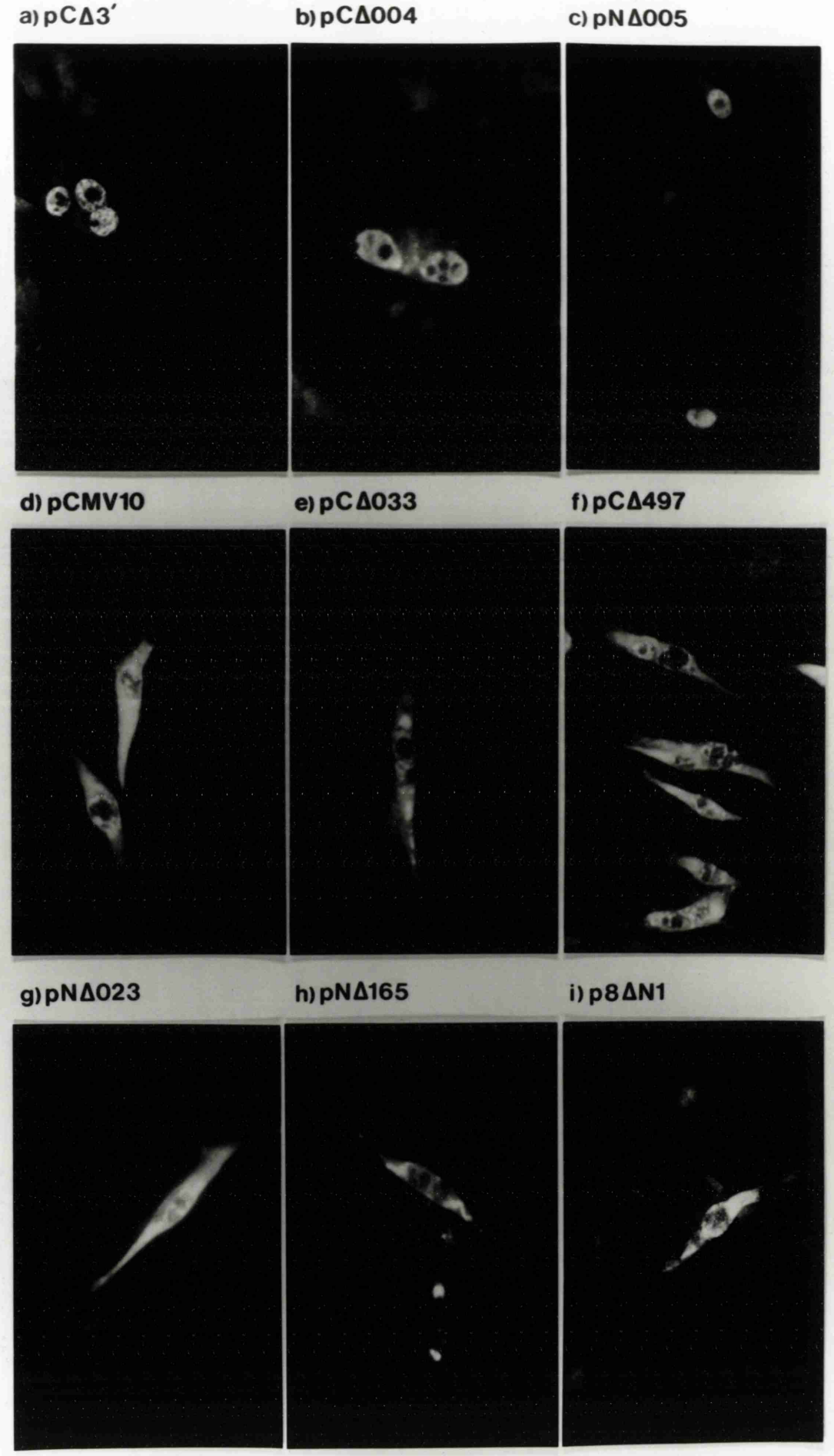
When N-terminal mutants were assayed, only cells cotransfected with pE5, pE52 and pNΔ005 exhibited nuclear fluorescence indicative of UL52 localising efficiently to the nucleus (**panel m**). Note that one of the cells in **panel m** shows a cytoplasmic pattern of fluorescence. It is likely that this cell did not take up all three plasmids during lipofection, since double labelling studies described in Section 3.2.5 indicate that even when lipofecting cells with a mixture of just two plasmids, a small proportion of the cells express the gene product of one plasmid but not the other.

Cotransfection of cells with pE5, pE52 and pNΔ023, pNΔ043, pNΔ097 or pNΔ165 resulted in cytoplasmic patterns of fluorescence for UL52 expression (**panels n-q**). A cytoplasmic pattern of UL52 distribution was also observed in cells coexpressing UL5, UL52 and the UL8 internal deletion mutant 8ΔN1 (**panel r**).

Similar results were obtained when transfected cells were stained with the anti-UL5 antibody and anti-rabbit IgG fluorescein conjugate. Cells cotransfected with pE5, pE52 and either pCΔ3', pCΔ004 or pNΔ005 exhibited nuclear patterns of fluorescence for UL5 expression (**Figure 30, panels a, b** and **c**). In contrast predominantly cytoplasmic fluorescence was observed in cells cotransfected with pE5, pE52 and pCMV10 (**panel d**) and in cells expressing UL5, UL52 and any of the other N- and C-terminally truncated UL8 proteins (CΔ033, CΔ497, NΔ023 and NΔ165 are shown in

Figure 30. Effect of mutant UL8 proteins on the intracellular localisation of UL5.

Cells were co-lipofected with a mixture of pE5, pE52 and a third plasmid as indicated. 24 hr post-transfection the cells were fixed, incubated with a 1:50 dilution of C-terminal anti-peptide rabbit serum against UL5, and stained with a 1:80 dilution of anti-rabbit IgG fluorescein-conjugated antibody. Cells were viewed under x50 magnification.


a) pCΔ3′
b) pCΔ004
c) pNΔ005
d) pCMV10
e) pCΔ033
f) pCΔ497
g) pNΔ023
h) pNΔ165
i) p8ΔN1

panels e-h; data not shown for the other plasmids). A cytoplasmic pattern of UL5 distribution was also observed in cells cotransfected with pE5, pE52 and p8ΔN1 (**panel i**).

In summary, deletion of four amino acids from the C-terminus of UL8 generates a protein which, when coexpressed with UL5 and UL52, remains capable of facilitating efficient nuclear localisation of UL5 and UL52, whereas deletion of 33 or more C-terminal amino acids results in a protein which is unable to facilitate the efficient nuclear localisation of these proteins. Likewise, deletion of five amino acids from the N-terminus of UL8 does not affect its ability to facilitate efficient nuclear localisation of UL5 and UL52, but deletion of 23 or more N-terminal amino acids renders UL8 incapable of this function. Although amino acids close to both termini of UL8 are necessary for efficient nuclear localisation of the complex, they are not sufficient since the protein specified by p8ΔN1, which lacks amino acids 78 to 339 of UL8, fails to facilitate efficient nuclear localisation of UL5 and UL52.

3.4.4 Intracellular localisation of UL8 deletion mutants

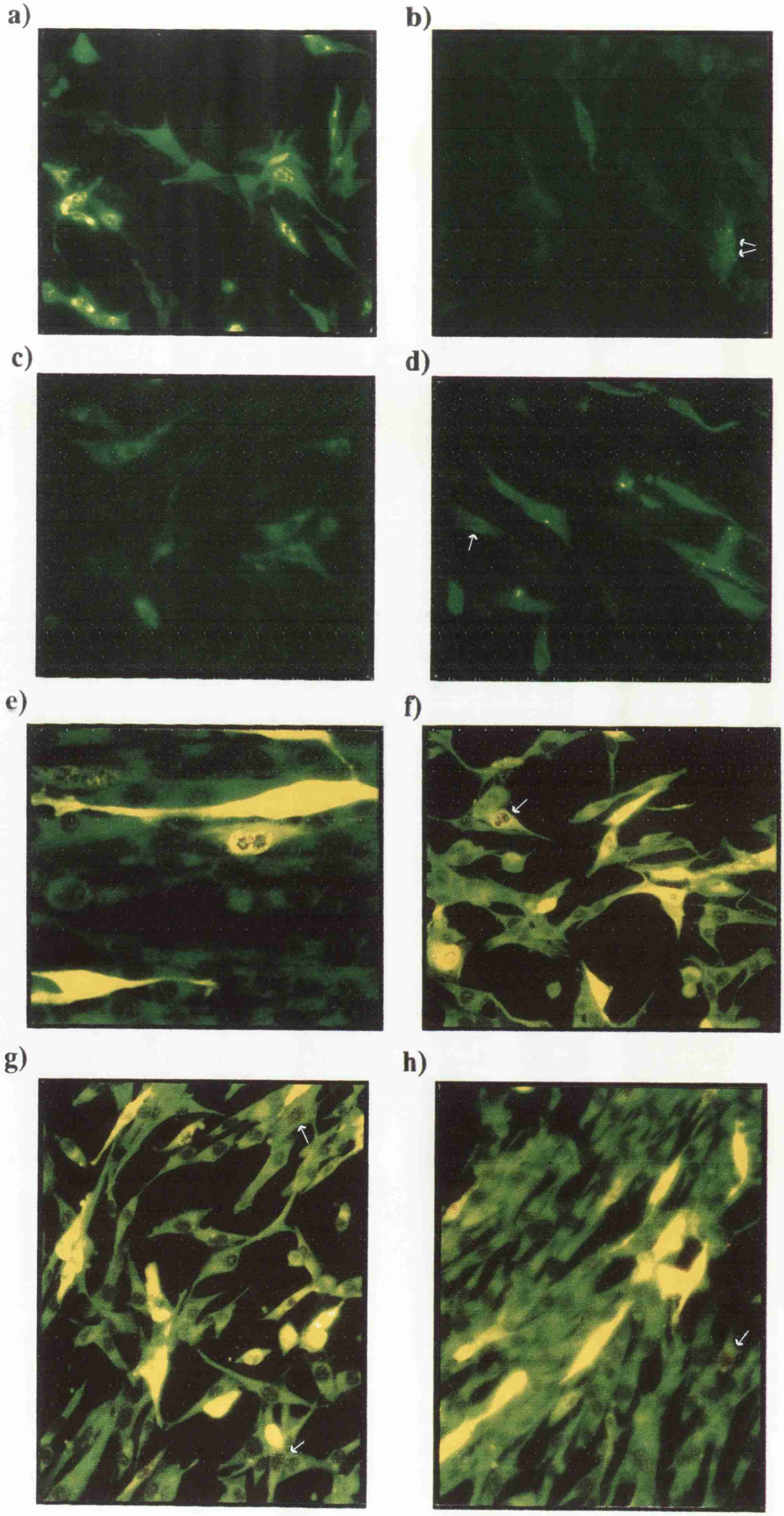
For selected UL8 mutants, the intracellular location when expressed alone was assessed, to determine whether their ability to facilitate nuclear localisation of UL5 and UL52 corresponded to an intrinsic ability to localise to the nucleus. Cells lipofected with pE8, pCΔ004, pCΔ033 or p8ΔN1 were fixed 24 hr later and incubated with MAb 0814 (for detecting CΔ004 or CΔ033) or MAb 0817 (for detecting UL8 or 8ΔN1) followed by fluorescein-conjugated anti-mouse-IgG antibody. Representative results are shown in **Figure 31 a-d**.

In agreement with the results presented in Section 3.2.3, the distribution of UL8 was cytoplasmic in approximately 70% of fluorescing cells, with the remaining 30% exhibiting predominantly nuclear fluorescence for UL8 (**panel a**). CΔ004 exhibited patterns of localisation similar to *wt* UL8, with approximately 50% of the cells showing cytoplasmic fluorescence and approximately 50% showing nuclear fluorescence (**panel b**, with arrows marking the cells in which CΔ004 is nuclear). In contrast, no nuclear fluorescence was observed in cells expressing CΔ033 (**panel c**).

Figure 31. Intracellular localisation of mutant UL8 proteins

Cells were lipofected with pE8 (**panel a**), pCΔ004 (**panel b**), pCΔ033 (**panel c**) or p8ΔN1 (**panel d**), or were co-lipofected with pE52 and either pE8 (**panel e**), pCΔ004 (**panel f**), pCΔ033 (**panel g**) or pNΔ005 (**panel h**). 24 hr later the cells were fixed and incubated with the following primary antibodies: a 1:200 dilution of MAb 0817 (**panels a** and **d**); a 1:200 dilution of MAb 0814 (**panels b** and **c**); a 1:200 dilution of MAb 0811 and a 1:100 dilution of anti-UL52 serum (**panels e-h**). Following staining with a 1:80 dilution of fluorescein-conjugated anti-mouse-IgG antibody (**panels a-d**) or with 1:80 dilutions of fluorescein-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG antibodies (**panels e-h**), cells were mounted under coverslips and viewed under x25 magnification (x50 for **panel e**).

This figure has been colour photocopied, resulting in loss of some of the original colour contrast, so white arrows have been used in **panels b, c** and **f-h** to draw attention to cells exhibiting nuclear fluorescence of UL8-related polypeptides.


a)
b)
c)
d)
e)
f)
g)
h)

Surprisingly, although the majority of cells expressing 8ΔN1 exhibited cytoplasmic fluorescence, nuclear 8ΔN1 was seen in approximately 20% of fluorescing cells (**panel d**, with a cell exhibiting nuclear fluorescence marked with an arrow).

In addition, an interesting observation arose from a double staining experiment designed to follow the colocalisation of mutant UL8 proteins with UL52. Cells were lipofected with pE52 and either pE8 or one of the mutated plasmid constructs, fixed 24 hr later and incubated with MAb 0811 and anti-UL52 serum, followed by fluorescein-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG antibodies. *Wt* UL8 and all of the mutant UL8 proteins were seen to colocalise with UL52 in the cytoplasm of cells expressing both proteins (data not shown). However, some of the cells on the plate transfected with pE8 and pE52 expressed UL8 alone, or UL52 alone, and in those expressing UL8, the protein was located in the nuclei (**Figure 31 e**). This had also been observed in previous double staining experiments, as described in Sections 3.2.4 and 3.2.5.

Examination of the cells lipofected with pE52 and each of the UL8 mutants revealed that, in cells not expressing UL52, proteins CΔ004, CΔ033 and NΔ005 could, like *wt* UL8, be detected in the nucleus, whereas co-expression of these proteins with UL52 resulted in cytoplasmic fluorescence (**Figure 31 f, g** and **h**, with arrows indicating cells in which the mutated UL8 proteins are nuclear). For the other mutants, no cells expressing the truncated UL8 protein in the absence of UL52 were observed, so the location of these proteins when expressed alone could not be followed.

From these preliminary results it can be concluded that CΔ004 and NΔ005 resemble *wt* UL8 in both their intracellular location when expressed alone and their ability to affect the localisation of co-expressed UL5 and UL52. Whilst many of the UL8 mutants failed to affect the localisation of co-expressed UL5 and UL52, it appears that at least some of them may retain *wt* patterns of intracellular localisation when expressed alone (*e.g.* CΔ033 and 8ΔN1). Although these experiments require to be repeated, they suggest that the failure of some UL8 deletion mutants to facilitate nuclear localisation of UL5 and UL52 may not be due to an intrinsic incapacity to enter or be retained in the nucleus.

3.4.5 Solubility of UL8 deletion mutants

A possible explanation for the failure of some of the UL8 mutants to function in the transient DNA replication assay or to facilitate the nuclear localisation of UL5 and UL52 may be that the mutant polypeptides are insoluble in transfected cells. To investigate this, total protein preparations and soluble extracts (the supernatants following centrifugation of lysates at 100,000g for 30 min) were prepared as described in Sections 2.2.10a-b, from BHK cells lipofected with the relevant plasmids. Total proteins and soluble extracts from equivalent numbers of transfected cells were analysed in parallel by SDS-PAGE and Western blotting, and the UL8-related proteins were detected using appropriate antibodies. [In some experiments an aliquot of Nonidet-P40 lysate was removed from each sample before centrifugation and was used as an indicator of the proteins present in the extract prior to the removal of insoluble matter, in place of the total protein preparation.]

Figure 32a shows Western blots of total proteins and soluble extracts from BHK cells lipofected with the plasmids indicated. It can be seen that all of the N-terminal mutants were detected in the soluble fractions. However, the UL8 polypeptides with deletions larger than 5 amino acids, *e.g.* NΔ023 (**lanes 7** and **8**), appear to be recovered relatively less efficiently in the soluble fraction, indicating that they may be slightly reduced in solubility.

Figures 32b and **c** show Western blots of Nonidet-P40 lysates from BHK cells lipofected with the plasmids indicated, before and after centrifugation. The mutants CΔ004, CΔ033, CΔ041, CΔ079 (**Figure 32b, lanes 3-10**) and 8ΔN1 (**Figure 32c, lanes 2-3**) were detected in the soluble fractions although again they appeared less soluble than *wt* UL8. Only CΔ497, the mutant with the largest deletion, was undetectable in the soluble extracts (**Figure 32c, lanes 9-10**) whilst CΔ280 and CΔ359 were barely detectable (**lanes 4-7**).

Since the mutant proteins are all recovered, albeit to varying degrees, in the soluble fraction of cell extracts, their inability to behave as *wt* UL8 protein in DNA replication or cellular localisation studies is unlikely to be a simple consequence of insolubility.

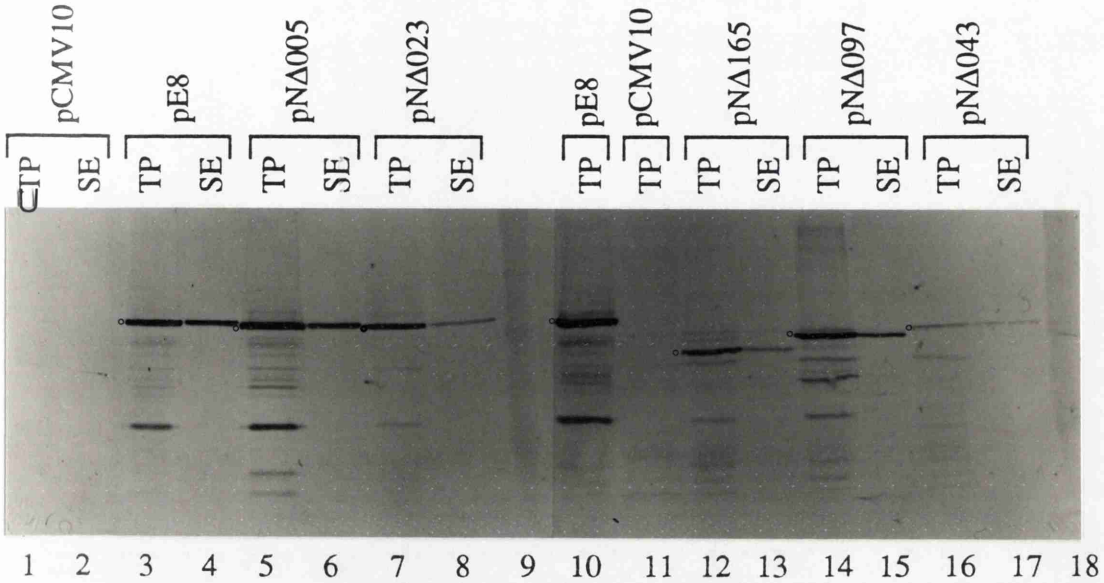
Figure 32. Solubility of mutated UL8 proteins.

a) Total protein preparations (TP) and soluble extracts (SE) from BHK cells lipofected with the plasmids indicated were subjected to SDS-PAGE and Western blotting. UL8-related protein was detected using a 1:2000 dilution of monoclonal antibody 0817.

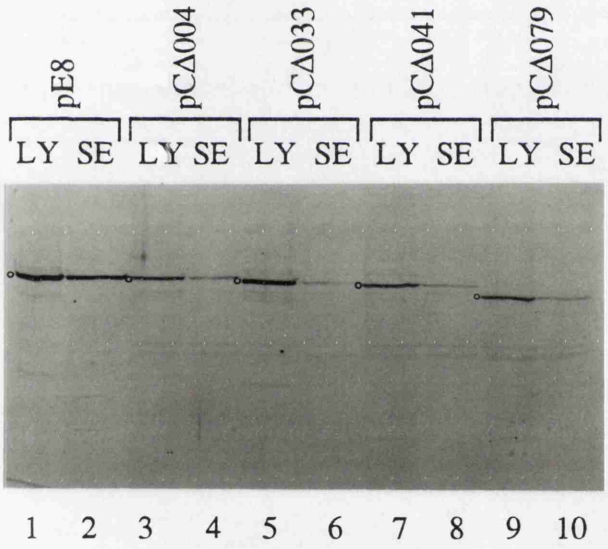
b) and **c)** Nonidet-P40 lysates were prepared from BHK cells lipofected with the plasmids indicated. Samples taken pre- and post-centrifugation of the lysates (LY and SE respectively) were subjected to SDS-PAGE and Western blotting. UL8-related protein was detected using a 1:2000 dilution of monoclonal antibody 0814 (**b**) or anti-UL8 polyclonal serum 094 (**c**).

Bands representing UL8-related polypeptides are marked •

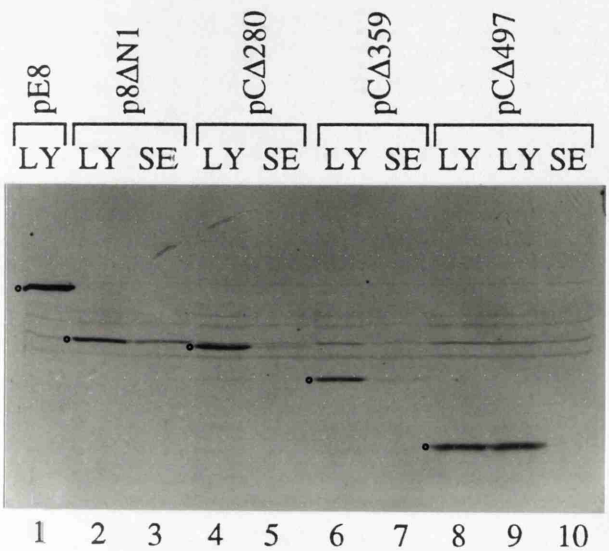
a)


pCMV10
pE8
pNΔ005
pNΔ023
pE8
pCMV10
pNΔ165
pNΔ097
pNΔ043
TP SE TP SE TP SE TP SE TP TP TP SE TP SE TP SE
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

b)


pE8
pCΔ004
pCΔ033
pCΔ041
pCΔ079
LY SE LY SE LY SE LY SE LY SE
1 2 3 4 5 6 7 8 9 10

c)


pE8
p8ΔN1
pCΔ280
pCΔ359
pCΔ497
LY LY SE LY SE LY SE LY LY SE
1 2 3 4 5 6 7 8 9 10

Further evidence that some of the truncated UL8 proteins can remain soluble and fold correctly comes from the co-immunoprecipitation experiments described in Section 3.5. Mutant proteins CΔ033, CΔ280, NΔ165 and 8ΔN1, expressed in insect cells using recombinant baculoviruses, are immunoprecipitable from soluble extracts of the insect cells, and exhibit the ability to interact with both UL5 and UL52 as demonstrated by their co-immunoprecipitation with these proteins from insect cell extracts.

3.4.6 Discussion

Taking the results of these various assays together, some preliminary conclusions can be drawn about how the structure of the UL8 protein relates to its functions.

Deletion of five N-terminal amino acids does not significantly affect the protein's ability to function in HSV-1 origin-dependent DNA synthesis or in facilitating nuclear localisation of the complex, whereas removal of 23 or more residues from the amino terminus of UL8 abolishes both of these functions. This indicates that residues lying close to, but not at, the N-terminus are vital for the functional integrity of UL8, but does not reveal whether they are essential for interactions with UL5 or UL52, or for some other UL8 function (*e.g.* facilitating uptake of the complex into the nucleus).

Interestingly, deletion of four amino acids from the C-terminus of UL8 reduces but does not abolish replicative ability in the transient transfection assay, apparently without affecting the ability to facilitate nuclear localisation of the helicase-primase complex. Deletion of 33 residues from the UL8 C-terminus abolishes both DNA synthesis in the transient transfection assay and efficient nuclear localisation of the complex. This data can be explained in light of the recent finding that UL8 interacts with the UL30 component of the HSV-1 polymerase, and that the region of UL8 required for this interaction is defined by the CΔ033 mutant (H.S. Marsden, *et al.*, manuscript in preparation). The failure of CΔ033 to support HSV-1 origin-dependent DNA synthesis is therefore likely to be explained

by failure to interact with UL30 (although CΔ033 also fails to localise the helicase-primase complex to the nucleus efficiently). The limited DNA replication supported by CΔ004 possibly indicates reduced (but not abrogated) ability to interact with UL30. This could be tested by determining whether CΔ004 and UL30 coprecipitate from insect cells infected with appropriate recombinant baculoviruses, and by assessing the activity of CΔ004 in coupled primase-polymerase assays (Tenney *et al.*, 1994).

With the exception of CΔ004, the replicative ability of the UL8 deletion mutants correlates directly with their ability to facilitate efficient nuclear localisation of UL5 and UL52. This raises the question as to whether the failure of the UL8 mutants to function in the transient replication assay is caused solely by the inability of the helicase-primase to accumulate efficiently in the nucleus. Ways of addressing this will be considered in Chapter 4.

As well as being screened for ability to support DNA replication, the UL8 mutants were also tested for ability to interfere with origin-dependent DNA synthesis. This was done because some mutant proteins, whilst having lost an essential property of the *wt* protein, retain the ability to interact with other cellular components (*e.g.* with the *wt* version of itself, or with other proteins, or with DNA) such that they exert dominant inhibition over the *wt* protein. In the case of DNA replication proteins, dominant negative mutations may result in a reduction in or complete absence of DNA synthesis. For example, it has been shown that the C-terminal DNA binding domain of UL9 protein (UL9CT), although functionally incapable of replacing *wt* UL9 in the transient replication assay, exerts a dominant inhibitory effect on DNA synthesis when co-expressed with all seven *wt* replication proteins (Stow, 1992; Stow *et al.*, 1993; Perry *et al.*, 1993). This inhibition most likely occurs as a result of UL9CT competing with *wt* UL9 protein for binding to viral origins of replication, with the UL9CT-origin interaction preventing the replication of that template molecule (Stow *et al.*, 1993).

Since UL8 forms protein-protein interactions with all but one of the other HSV-1 DNA replication proteins (refer to Section 1.4.3), UL8 mutants have the potential to exert a dominant inhibitory effect on HSV-1 DNA synthesis. One possible mechanism by which a UL8 molecule might exert a dominant inhibitory phenotype

might be if it could interact with UL9 at the origins but not with UL30, thus failing to recruit the DNA polymerase into an initiation complex.

The failure of the mutant UL8 proteins to localise the helicase-primase complex to the nucleus may be sufficient to explain why none exhibited a strongly dominant inhibitory phenotype. Presumably sufficient *wt* UL8 can complex with UL5 and UL52 to provide functional helicase-primase complexes which are taken into the nucleus, irrespective of whether the mutant UL8 proteins can also interact with UL5 and UL52.

Section 3.5 CONSTRUCTION AND ANALYSIS OF RECOMBINANT BACULOVIRUSES

3.5.1 Introduction

Although the immunofluorescence experiments indicated that the majority of the UL8 mutants did not have the ability to localise UL5 and UL52 efficiently to the cell nucleus (Section 3.4.3), it was not known whether this resulted directly from an inability to bind to UL5 and/or UL52. To determine whether the mutant UL8 proteins could interact with UL5 and UL52, it was decided to employ a co-immunoprecipitation assay.

In initial experiments BHK cells were cotransfected with plasmids pE5, pE8 and pE52, labelled extracts were prepared and precipitated with monoclonal antibody against UL8. Under these conditions UL8 could be weakly observed but no coprecipitation of UL5 or UL52 was detected (data not shown). This failure was probably due to the relatively low level of heterologous gene expression compared with the total protein content of the cells and/or a low proportion of cells expressing the transfected genes. To circumvent these problems it was therefore decided to employ the baculovirus expression system, which had previously been successfully used to analyse interactions between UL5, UL8 and UL52 (McLean *et al.*, 1994).

Several of the UL8 mutations were selected to be expressed by the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) so that their abilities to interact with UL5 and UL52 could be tested in co-immunoprecipitation assays. The mutations chosen were CΔ280 and NΔ165 (to divide UL8 into three approximately equal regions) and 8ΔN1 (which contains both terminal regions but lacks an internal segment of UL8). The mutant CΔ033 was also chosen to be expressed using the baculovirus system for use in a collaborative project with Dr H. Marsden's group, investigating potential interactions between the carboxy-terminus of UL8 and the HSV-1 UL30 protein.

3.5.2 Construction of recombinant baculoviruses

Each gene to be expressed was cloned into the bacterial plasmid transfer vector pAcYM1, downstream of the strong late polyhedrin gene promoter and flanked by baculovirus sequences. When these modified transfer vectors were introduced into insect cells along with *Bsu*36I-cleaved AcPAK6 DNA, homologous recombination between the vector flanking sequences and the baculovirus genome resulted in insertion of the foreign genes into infectious recombinant viruses.

a) Cloning of mutant UL8 genes into pAcYM1

Plasmid pAcYM1 (Matsuura *et al.*, 1987) derives from a pUC8-based plasmid into which an *Eco*RI fragment from the AcNPV genome has been inserted. pAcYM1 is 9.2 kbp in size and contains the complete promoter, untranslated leader sequence and first nucleotide of the initiation codon for the polyhedrin gene, but lacks the rest of the polyhedrin ORF and the first thirteen untranslated nucleotides downstream of the gene (**Figure 33**). There is a unique *Bam*HI site located downstream of the polyhedrin promoter in pAcYM1 into which foreign genes can be cloned.

The UL8 ORF in plasmid p8ΔN1 was already flanked by *Bam*HI sites but plasmids pCΔ033 and pCΔ280 were lacking a *Bam*HI site downstream of the UL8 ORF and plasmid pNΔ165 was lacking a *Bam*HI site upsteam of the UL8 ORF. It was therefore necessary to attach a linker containing a *Bam*HI site (5'-CGGATCCG-3') at the appropriate end of the UL8 ORF of the latter three plasmids, so that the mutated UL8 genes could be cloned as *Bam*HI fragments into the *Bam*HI site of pAcYM1.

Plasmids pCΔ033 and pCΔ280 were linearised at the *Xba*I site downstream of the UL8 ORF and plasmid pNΔ165 was linearised at the *Eco*RI site upstream of the UL8 ORF. The 5' overhanging ends of the linearised plasmids were filled in using T4 DNA polymerase to give blunt-ended DNA. *Bam*HI linkers were ligated to these blunt ends, after which the DNA was cleaved with *Bam*HI and the products were resolved by agarose gel electrophoresis. *Bam*HI-cleaved p8ΔN1 DNA was also resolved by agarose gel electrophoresis. For each mutant, the smaller of the two DNA fragments

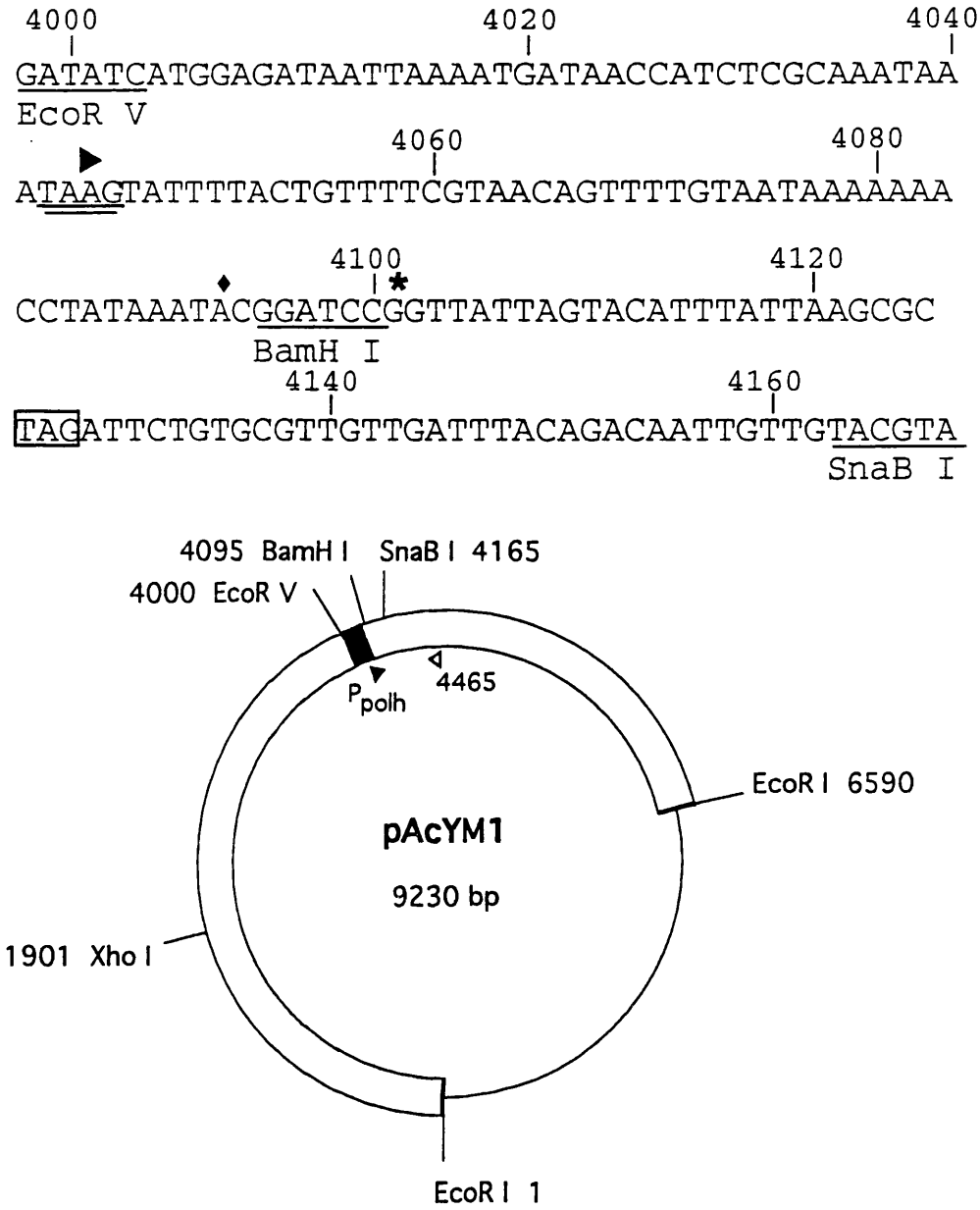


Figure 33. Map of plasmid pAcYM1.

The plasmid backbone (pUC derivative) is represented by a single line and the AcNPV sequences by a double line. The polyhedrin promoter (P_{polh}) is indicated by a solid box. The site of transcription initiation (▶,position 4044) and the 3' end of the mRNA (◁, position 4465) are marked. Selected restriction enzyme sites are also indicated. The sequence spanning the *Bam*HI insertion site is given above the circular map. The 5' end of the mRNA (▶), the first nucleotide of the normal polh initiation codon (♦), and the nucleotide normally 14 residues downstream of the polh ORF (*) are marked. The boxed TAG is utilised as a termination codon for the CΔ280 ORF (refer to page 118). Adapted from King & Possee, 1992.

(containing the UL8 ORF) was excised, purified and ligated to pAcYM1 which had been cleaved with *Bam*HI and treated with CIP.

It should be noted that in the case of mutant CΔ280, the termination codon which was in frame with the ORF in plasmid pCΔ280 was downstream of the *Xba*I site, and hence was not maintained upon cloning of the CΔ280 ORF into plasmid pAcYM1. There is, however, a termination codon in frame with the cloned CΔ280 ORF, within the 3' untranslated nucleotides of the polyhedrin gene in pAcYM1 (**Figure 33**, marked with an open box). This means that instead of the three additional amino acids that constitute the C-terminus of CΔ280 protein encoded by plasmid pCΔ280 (P^{470}SLD; see **Table 2**), the CΔ280 protein expressed using the recombinant baculovirus is predicted to contain 13 amino acids C-terminal to the final proline (P^{470}SLADPVISTFIKR). For the other three mutants, the termination codons were maintained through the cloning procedure.

Small scale plasmid preparations, made from *E. coli* DH5 cells transformed with the above ligation products, were screened for the presence of two correctly sized fragments following digestion with *Bam*HI (*e.g.* **Figure 34, lanes 6, 7, 8** and **9**). The orientation of the insert in these plasmids was determined by further restriction enzyme digests. The presence of a unique *Eco*RV site 95 bp upstream of the *Bam*HI insertion site in plasmid pAcYM1 (**Figure 33**), and a single *Eco*RV site near the 5' end of the UL8 ORF, enabled the orientation of the CΔ033, CΔ280 and 8ΔN1 inserts in the plasmids to be determined from the sizes of the fragments produced following digestion with *Eco*RV. As can be seen from **Figure 35**, with the insert in the correct orientation for expression an *Eco*RV fragment of 130 bp is expected, whereas a much larger fragment is produced if the insert is in the opposite orientation. Since the 5' end of the UL8 ORF was deleted in mutant NΔ165, the orientation of the insert in this plasmid was determined using *Sma*I and *Xho*I sites within the UL8 ORF in conjunction with a unique *Xho*I site 2194 bp upstream of the *Bam*HI insertion site in plasmid pAcYM1 (**Figure 35**). This produces diagnostic fragments of 2580 bp (correct orientation) or 2964 bp (inverted). **Figure 36** shows the fragments obtained following digestion of representative plasmids containing the UL8 ORF in the correct

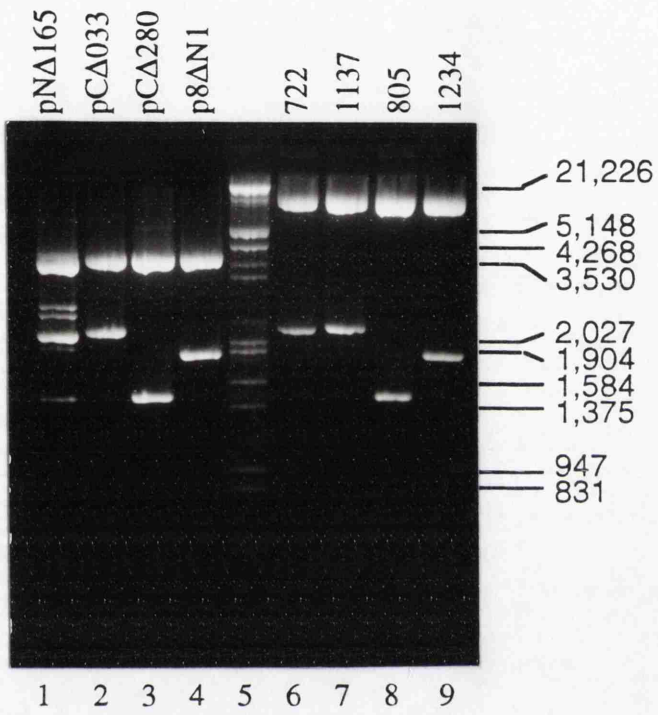
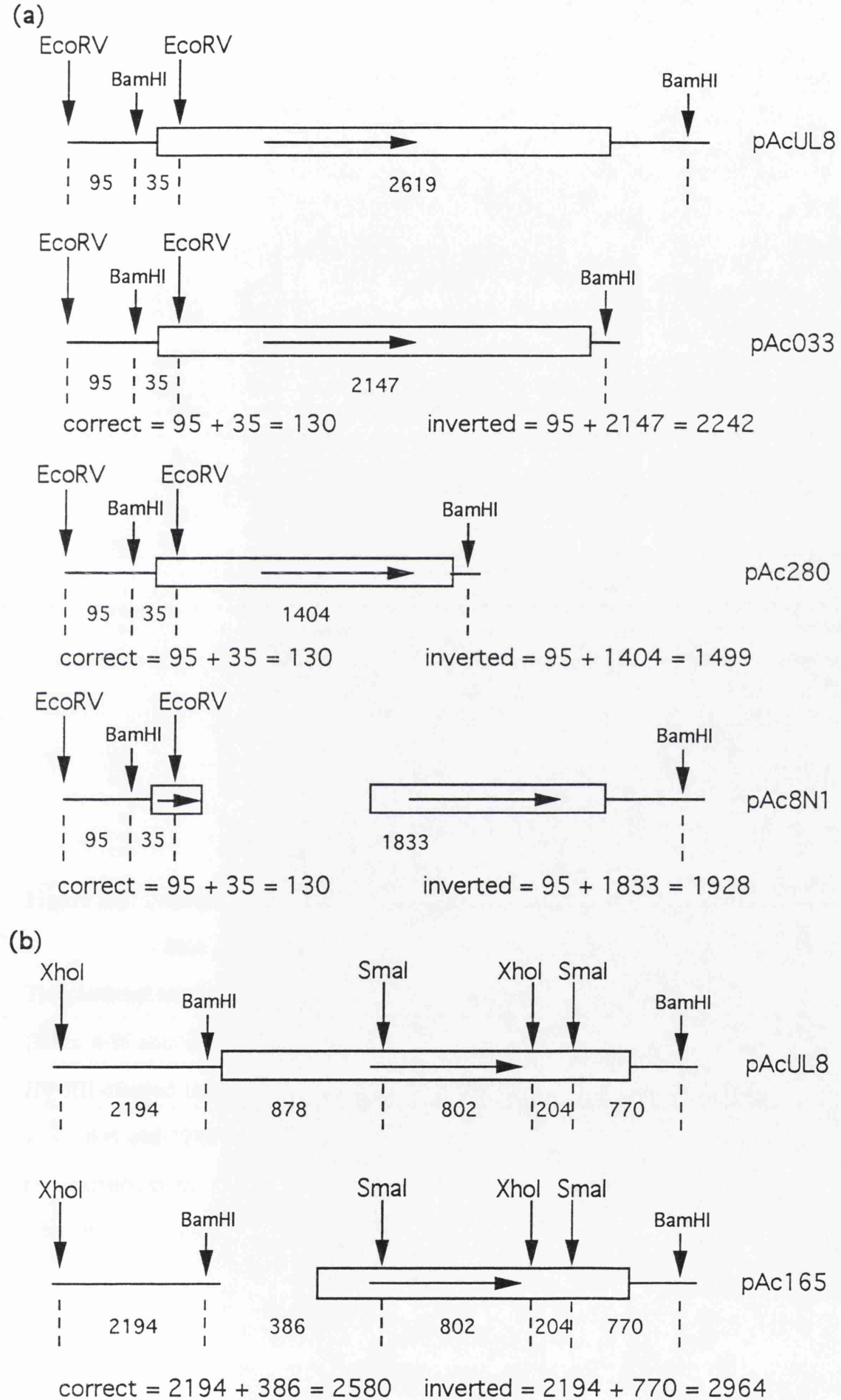


Figure 34. Cloning of UL8 ORF-containing fragments from pCMV10-based plasmids into plasmid pAcYM1.

The plasmids indicated were cleaved with the following restriction enzymes: *Bam*HI plus *Eco*RI (lane 1); *Bam*HI plus *Xba*I (lanes 2 & 3) and *Bam*HI alone (lanes 4 and 6-9) and the products were resolved by agarose gel electrophoresis with *Eco*RI plus *Hin*dIII-cleaved lambda DNA (lane 5; sizes of fragments indicated in bp). In lane 1, *Eco*RI star activity generated some fainter additional fragments to those expected.

Figure 35. Determining the orientation of inserts cloned into pAcYM1.

A region of pAcYM1 is shown, with the UL8, CΔ033, CΔ280, 8ΔN1 and NΔ165 ORFs (boxed arrows) cloned in as *Bam*HI inserts, generating plasmids pAcUL8, pAc033, pAc280, pAc8N1 and pAc165, respectively. The locations of the *Eco*RV (**a**), *Xho*I and *Sma*I (**b**) sites used to determine the orientation of the inserts are given. The predicted sizes (in bp) of fragments generated following digestion with these enzymes are also given, both for the insert in the correct orientation for expression and in the inverted orientation.


(a)
EcoRV
EcoRV
BamHI
BamHI
pAcUL8
95
35
2619
EcoRV
EcoRV
BamHI
BamHI
pAc033
95
35
2147
correct = 95 + 35 = 130
inverted = 95 + 2147 = 2242
EcoRV
EcoRV
BamHI
BamHI
pAc280
95
35
1404
correct = 95 + 35 = 130
inverted = 95 + 1404 = 1499
EcoRV
EcoRV
BamHI
BamHI
pAc8N1
95
35
1833
correct = 95 + 35 = 130
inverted = 95 + 1833 = 1928
(b)
XhoI
SmaI
XhoI
SmaI
BamHI
BamHI
pAcUL8
2194
878
802
204
770
XhoI
SmaI
XhoI
SmaI
BamHI
BamHI
pAc165
2194
386
802
204
770
correct = 2194 + 386 = 2580
inverted = 2194 + 770 = 2964

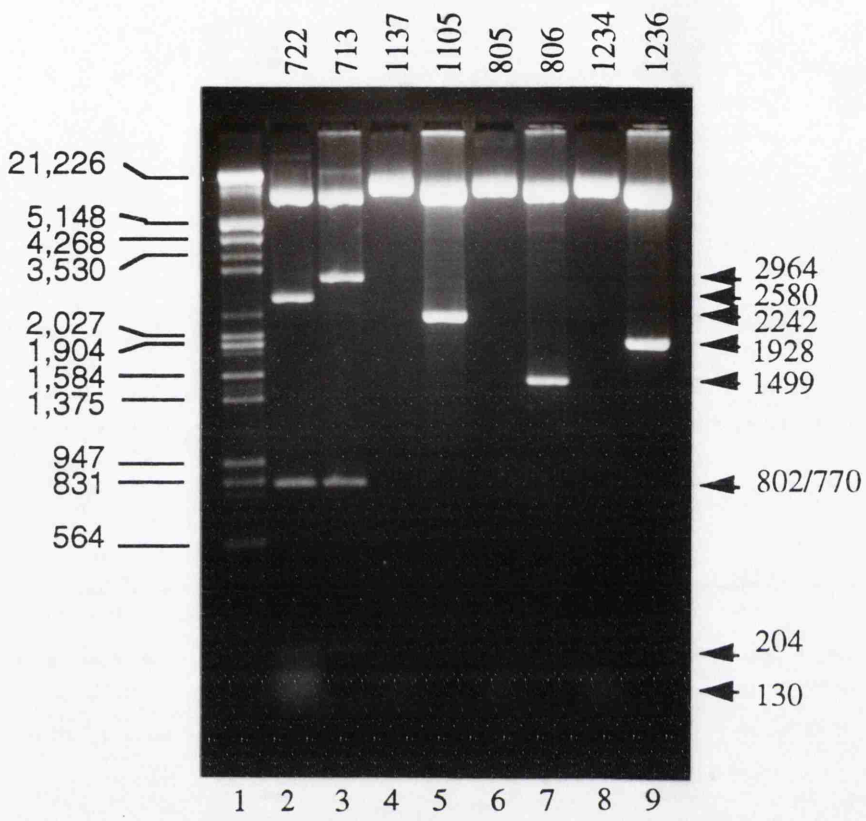


Figure 36. Determination of the orientation of the UL8 ORFs cloned into pAcYM1.

The plasmids indicated were cleaved with *Sma*I plus *Xho*I (lanes 2 & 3) or with *Eco*RV (lanes 4-9) and were electrophoresed through an agarose gel along with *Eco*RI plus *Hin*dIII-cleaved lambda DNA (lane 1; fragment sizes indicated in bp). Plasmids 722, 1137, 805 and 1234 contain the mutant UL8 ORFs NΔ165, CΔ033, CΔ280 and 8ΔN1 respectively, in the correct orientation for expression. These inserts are in the opposite orientation in plasmids 713, 1105, 806 and 1236, respectively.

(**lanes 2, 4, 6** and **8**) or inverted (**lanes 3, 5, 7** and **9**) orientations. Large scale preparations were made of those plasmids with the insert in the correct orientation for expression (plasmids 722, 805, 1137 and 1234; renamed pAc165, pAc280, pAc033 and pAc8N1, respectively).

b) Isolation of recombinant viruses

Cotransfection of insect cells with pAcYM1 derivatives and *Bsu*36I-cleaved AcPAK6 DNA results in almost 100% of the progeny being recombinant (Kitts *et al.*, 1990; Bishop, 1992; Kitts & Possee, 1993). AcPAK6 is a derivative of *wt* AcNPV in which the *E. coli lacZ* gene, encoding the ß-galactosidase protein, has been inserted into the genome at the polyhedrin locus (Kitts & Possee, 1993). In addition to a naturally occurring *Bsu*36I site within the *lacZ* gene, AcPAK6 has two additional *Bsu*36I sites, one in adjacent upstream sequences and one in an essential gene downstream of the polyhedrin locus. This means that the large *Bsu*36I fragment of AcPAK6 DNA (representing approximately 95% of the viral genome) cannot recircularise to give viable virus, so there is selection for recombination with pAcYM1 to produce infectious circular genomes and hence progeny virus containing the foreign gene (**Figure 37**). As virtually all the progeny from cotransfection are recombinant, the recombinants are readily purified by performing limiting dilutions (O'Reilly *et al.*, 1992) and can be conveniently screened for expression of the foreign gene by SDS-PAGE analysis.

70% confluent monolayers of *S.f.* cells in 35mm plates were lipofected with a mixture of *Bsu*36I-cleaved AcPAK6 DNA and one of the four plasmids pAc165, pAc280, pAc033 or pAc8N1. 3 days post-lipofection the supernatant virus was removed from the plates and, following amplification by two rounds of infection of *S.f.* cells in linbro wells, was purified by performing limiting dilutions on microtitre wells of *S.f.* cells as described in section 2.2.3. The virus in supernatants from infected microtitre wells was tested for ability to express the mutated UL8 protein by infecting *S.f.* cells in linbro wells, harvesting the cells 72 hr p.i. and preparing total protein samples. The protein samples were analysed by SDS-PAGE and Coomassie brilliant

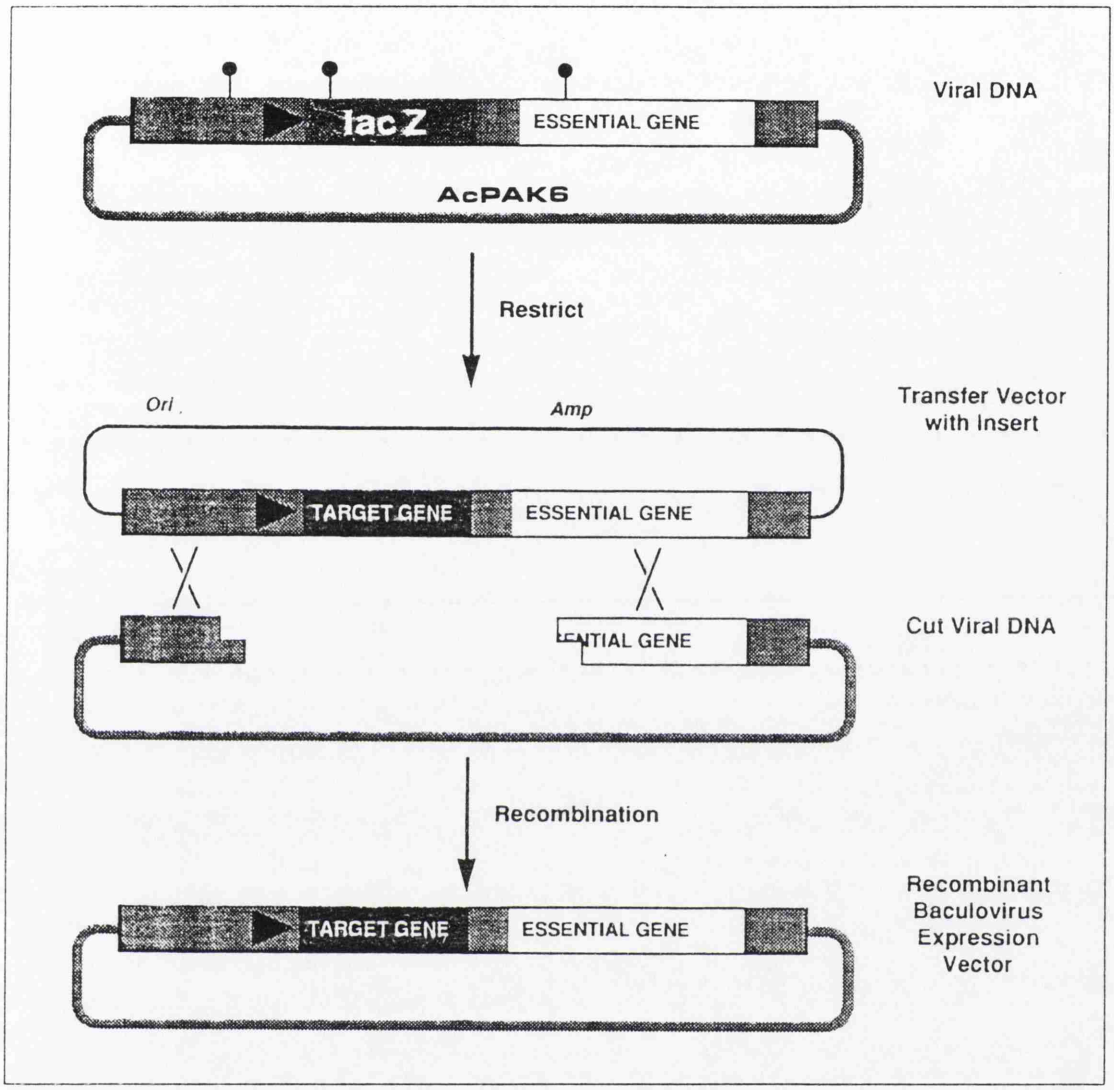


Figure 37. Generation of recombinant baculoviruses.

Following cotransfection of insect cells with *Bsu*36I-cleaved AcPAK6 DNA and a pAcYM1 derivative (containing cloned target gene), recombination between homologous baculovirus sequences in the viral genome and the plasmid generates infectious circular genomes and inserts the target gene into the AcPAK6 genome.

† *Bsu*36I restriction site, ▶ polyhedrin promoter.

Adapted from Kitts & Possee (1993).

blue staining for expression of the desired UL8 protein. **Figure 38a** shows that bands corresponding to proteins of the expected size were clearly visible for mutants Ac280 (**lanes 2-5**), Ac165 (**lanes 10-13**) and Ac033 (**lanes 15-18**), but not for Ac8N1 (**lanes 6-9**). Testing of supernatants from additional microtitre wells infected with the latter virus revealed a faint band of the expected size for 8ΔN1 protein (**Figure 38b, lanes 1-3**). To confirm that this band corresponded to the mutant UL8 protein, a duplicate SDS-PAGE gel was Western blotted and incubated with the polyclonal anti-UL8 serum 094 (**Figure 38c**). This antibody recognised a band of the expected size for 8ΔN1 protein in **lanes 1** and **3**.

Comparison of the relative positions of the bands representing polypeptides 8ΔN1 and CΔ280 in insect cell extracts (**Figure 38c, lanes 3** and **4**) with those from BHK cell extracts (**Figure 32c, lanes 2** and **4**) suggests that the CΔ280 protein expressed using the recombinant baculovirus is not significantly larger than that expressed from plasmid pCΔ280. Therefore the predicted termination codon in the baculovirus sequence downstream of the UL8 ORF in Ac280 is likely to be utilised (predicted sizes of polypeptides - 8ΔN1: 488 amino acids; baculovirus-expressed CΔ280: 483 amino acids; plasmid-expressed CΔ280: 473 amino acids).

Virus supernatants corresponding to the infections shown in **lanes 16, 11**, and **4** of **Figure 38a** and **lane 3** of **Figure 38b** were selected and passaged further to generate working stocks of recombinant viruses Ac033, Ac165, Ac280 (**Figure 38b, lanes 5, 6** and **4**, respectively) and Ac8N1 (not shown).

3.5.3 The truncated UL8 proteins CΔ033, CΔ280 and NΔ165 are immuno-precipitated by MAb0811 and coprecipitate with UL5 and UL52

Co-immunoprecipitations were performed to test the truncated UL8 proteins for their ability to form a tripartite complex with UL5 and UL52. Labelled extracts were prepared as described (Section 2.2.10.f) from insect cells triply infected with 10 pfu/cell of AcUL5 and AcUL52, and 5 pfu/cell of AcUL8, Ac033, Ac165 or Ac280. Labelled extracts were also prepared from mock-infected cells, cells singly infected with AcUL8, Ac033, Ac165 or Ac280 and cells doubly infected with AcUL5 and

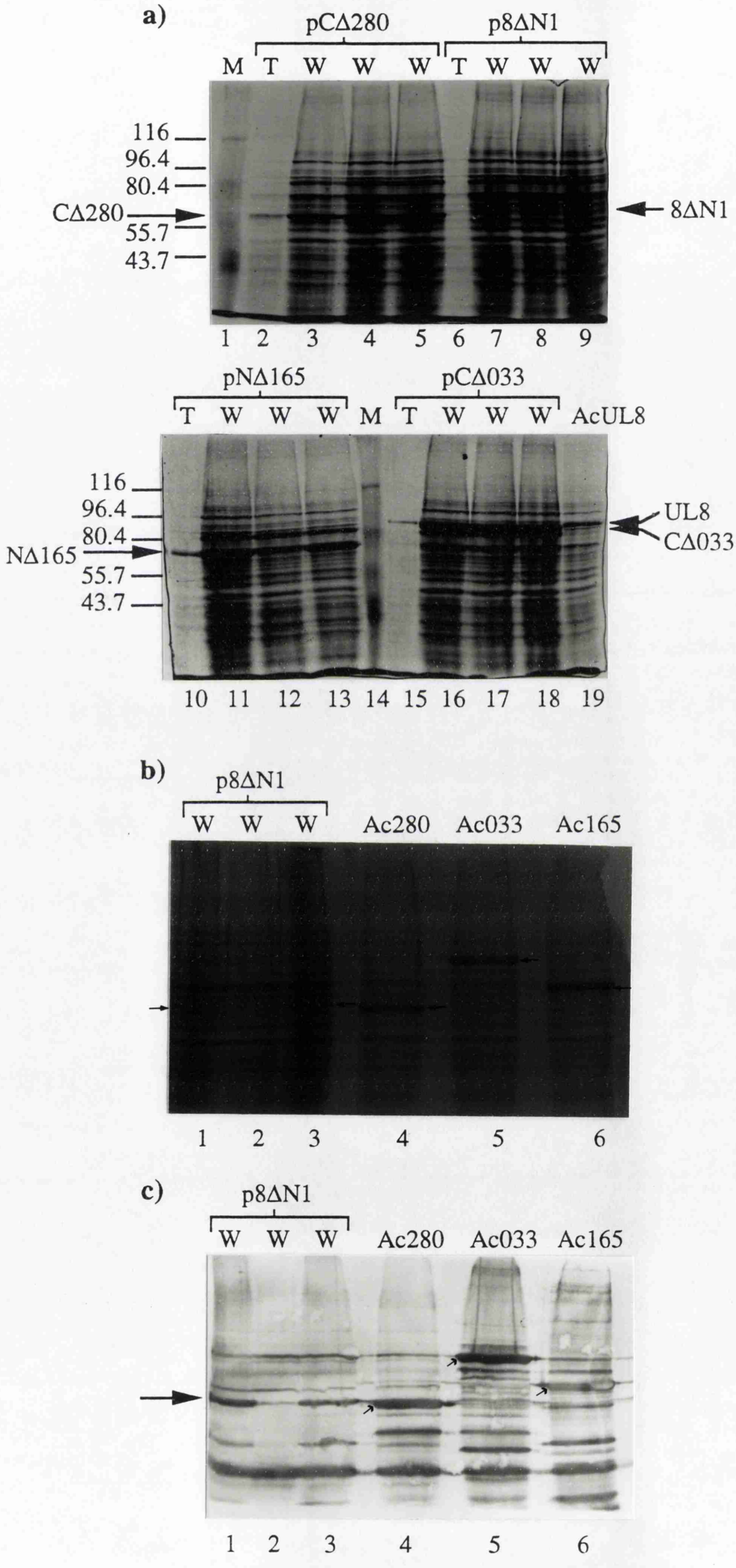
Figure 38. Analysis of total protein samples from infected insect cells for the expression of UL8 proteins.

a) SDS-PAGE analysis of total protein samples from insect cells infected with progeny before (**T**) and after (**W**) purification by limiting dilutions on microtitre plates. Progeny viruses arose from cotransfection of insect cells with AcPAK6 DNA and the plasmids indicated. Progeny from three separate microtitre wells were tested for each putative recombinant. Lane 19 shows a total protein sample from cells infected with AcUL8, with the position of *wt* UL8 protein marked. The predicted positions of the mutant UL8 proteins are indicated. The apparent sizes of molecular weight markers (Sigma; lanes 1 & 14) are also marked.

b) SDS-PAGE analysis of total protein samples from insect cells infected with recombinant viruses Ac280, Ac033 or Ac165 (**lanes 4-6**) or with purified progeny virus resulting from cotransfection of cells with AcPAK6 DNA and plasmid pAc8N1 (**lanes 1-3** correspond to three different microtitre wells from those in **a**).

c) Western blot of a duplicate of the SDS-polyacrylamide gel in **b**. The blot was incubated with a 1:2000 dilution of polyclonal anti-UL8 serum 094 followed by incubation with a 1:7500 dilution of anti-rabbit IgG alkaline phosphatase-conjugated antibody and colour development. The arrow indicates the position of a band in lanes 1 and 3 which corresponds to the expected size of 8ΔN1.

In **b** and **c**, bands corresponding to mutant UL8 polypeptides are indicated by arrows


a)
pCΔ280
p8ΔN1
M T W W W T W W W
116
96.4
80.4
CΔ280
55.7
43.7
8ΔN1
1 2 3 4 5 6 7 8 9
pNΔ165
pCΔ033
T W W W M T W W W AcUL8
116
96.4
80.4
NΔ165
55.7
43.7
UL8
CΔ033
10 11 12 13 14 15 16 17 18 19
b)
p8ΔN1
W W W Ac280 Ac033 Ac165
1 2 3 4 5 6
c)
p8ΔN1
W W W Ac280 Ac033 Ac165
1 2 3 4 5 6

AcUL52. The labelled extracts were then incubated with MAb 0811. This monoclonal antibody recognises an epitope between amino acids 166 and 253 of UL8 (Section 3.3.3), which is present in all three mutants.

Protein-antibody complexes were precipitated using protein-A sepharose beads as described (Section 2.2.10.g). The immunoprecipitated proteins were resolved on an 8.5% polyacrylamide gel, which was fixed, treated with En^{3}Hance, dried and exposed to autoradiographic film.

The autoradiograph in **Figure 39** shows the total protein content of the labelled extracts (**lanes 1-10**) and the proteins which were precipitated from these extracts by MAb0811 (**lanes 11-20**). **Lanes 13-16** demonstrate that MAb0811 precipitates *wt* UL8 and the three truncated UL8 proteins from singly infected insect cells.

Both UL5 and UL52 were precipitated by MAb0811 from triply infected cells in the presence of *wt* UL8 protein or any one of the three truncated UL8 proteins (**lanes 17-20**). Precipitation of UL5 and UL52 is dependent on the presence of *wt* or truncated UL8 protein since no UL5 or UL52 protein could be detected when the antibody was reacted with the doubly infected cell extract (**lane 12**).

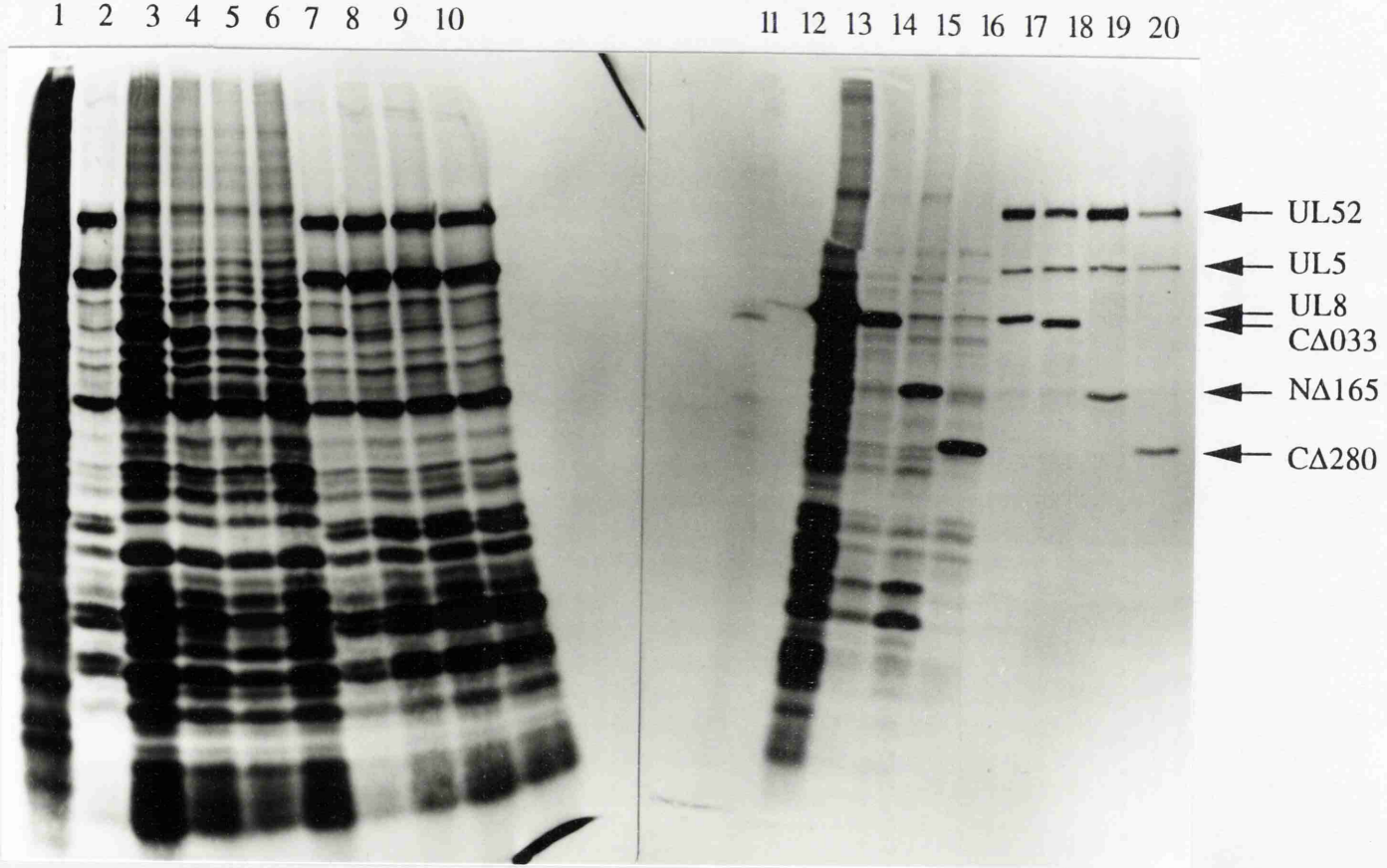
These results show that each of the three truncated UL8 proteins CΔ033, CΔ280 and NΔ165, can interact with at least one other component of the helicase-primase complex.

The immunoprecipitations in **lanes 12** and **17-20** appear 'cleaner' than in **lanes 13-16** because of the higher m.o.i. in the former infections (25 pfu per cell for the triple infections, 5 pfu per cell in the single infections). The higher m.o.i. causes more efficient shut-off of host cell macromolecular synthesis, resulting in fewer total counts being incorporated in the protein samples (compare **lanes 2** and **7-10** with **lanes 3-6**). However, relatively few proteins are precipitated from mock-infected cells (**lane 11**) despite the efficient labelling of proteins in the cell extract (**lane 1**), indicating that the bands in **lanes 13-16** are dependent upon the presence of AcUL8 or derivatives thereof. The bands may therefore represent complexes between UL8 and other baculovirus or cell proteins or, in the case of faster migrating proteins, are more probably breakdown products of UL8. Interestingly, these bands are almost

Figure 39. The truncated UL8 proteins CΔ033, CΔ280 and NΔ165 are immunoprecipitated by MAb0811 and coprecipitate with UL5 and UL52.

Lanes 1-10 show labelled extracts prepared 40 hr p.i., from insect cells mock-infected or infected with the baculoviruses indicated below. Lanes 11-20, respectively, show proteins immunoprecipitated from these extracts by MAb 0811. Proteins were resolved by SDS-PAGE. The positions of the UL52, UL5, UL8, CΔ033, NΔ165 and CΔ280 polypeptides are indicated.

Lane		Baculoviruses
1	11	mock-infected
2	12	AcUL5 + AcUL52
3	13	AcUL8
4	14	Ac033
5	15	Ac165
6	16	Ac280
7	17	AcUL5 + AcUL52 + AcUL8
8	18	AcUL5 + AcUL52 + Ac033
9	19	AcUL5 + AcUL52 + Ac165
10	20	AcUL5 + AcUL52 + Ac280


1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
UL52
UL5
UL8
CΔ033
NΔ165
CΔ280

completely absent in **lanes 17-20**, possibly reflecting the relatively smaller number of labelled cellular proteins available to complex with UL8 as a result of the higher m.o.i, or indicating that UL8 is more stable and/or less 'sticky' when complexed with UL5 and UL52.

3.5.4 The truncated UL8 proteins CΔ033, CΔ280 and NΔ165 interact separately with both UL5 and UL52

Since the UL5 and UL52 proteins can interact separately with *wt* UL8 protein (McLean *et al.*, 1994), the three truncated UL8 proteins were tested for their ability to coprecipitate with UL5 alone or UL52 alone. Insect cells were infected with 10 pfu/cell of either AcUL5 or AcUL52, plus 5 pfu/cell of AcUL8, Ac033, Ac165, or Ac280. Labelled extracts were prepared from these cells and singly infected controls (**Figure 40, lanes 1-10**) and incubated with MAb0811 as before.

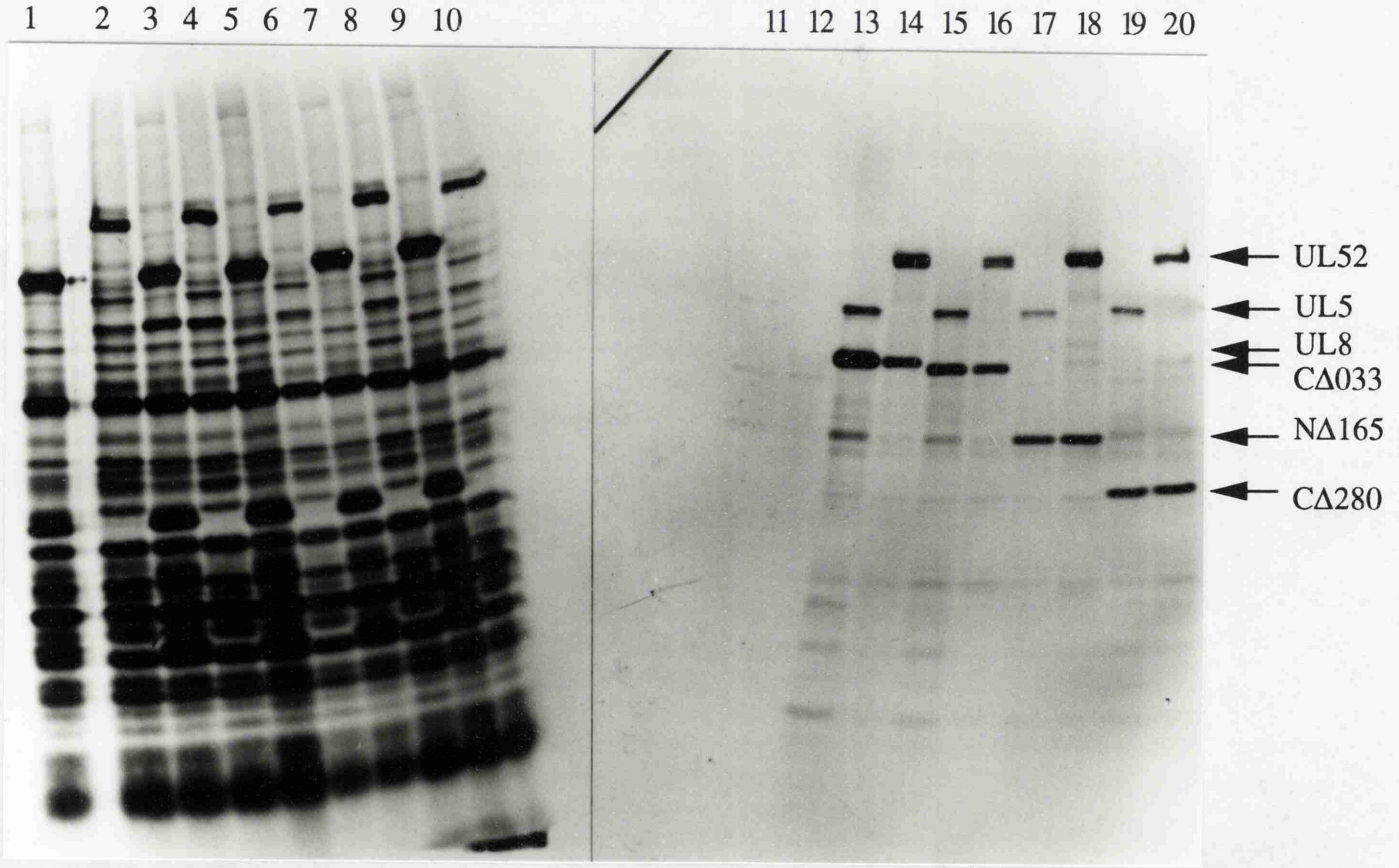
The autoradiograph in **Figure 40** demonstrates that MAb0811 coprecipitates UL5 with UL8 (**lane 13**) and UL52 with UL8 (**lane 14**) from insect cells doubly infected with the appropriate viruses, in agreement with the previous results of McLean *et al.* (1994). Deletion of 33 or 280 amino acids from the C-terminus of UL8 does not affect its ability to coprecipitate with UL5 (**lanes 15** and **17**) or UL52 (**lanes 16** and **18**). The truncated UL8 protein in which the amino-terminal 165 amino acids had been deleted was also capable of coprecipitating with UL5 (**lane 19**) and with UL52 (**lane 20**) from extracts of doubly infected cells. UL5 and UL52 were not precipitated when control extracts from cells infected with AcUL5 alone or AcUL52 alone were incubated with the antibody MAb0811 (**lanes 11** and **12**).

This experiment shows that each of the three truncated UL8 proteins interacts directly with both the UL5 protein and with the UL52 protein, indicating that the N-terminal 165 amino acids and C-terminal 280 amino acids (although not necessarily both together) are dispensable for intersubunit interactions.

Figure 40. Sequences at either end of UL8 are dispensable for interaction with UL5 or with UL52.

Lanes 1-10 show the labelled extracts prepared 40 hr p.i., from insect cells infected with the baculoviruses indicated below. The corresponding immunoprecipitations using MAb 0811 are shown in lanes 11-20, respectively. Proteins were resolved by SDS-PAGE. The positions of the UL52, UL5, UL8 and mutant polypeptides are indicated.

Lane		Baculoviruses
1	11	AcUL5
2	12	AcUL52
3	13	AcUL5 + AcUL8
4	14	AcUL52 + AcUL8
5	15	AcUL5 + Ac033
6	16	AcUL52 + Ac033
7	17	AcUL5 + Ac165
8	18	AcUL52 + Ac165
9	19	AcUL5 + Ac280
10	20	AcUL52 + Ac280


1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
UL52
UL5
UL8
CΔ033
NΔ165
CΔ280

3.5.5 The UL8 mutant 8ΔN1 interacts with the other two components of the helicase-primase complex

In order to investigate the interactions of the mutant protein 8ΔN1 it was necessary to use a different monoclonal antibody, since the epitope recognised by MAb0811 is located in a region of UL8 which is deleted in the mutant 8ΔN1 (Section 3.3.3). The antibody MAb0817, which binds to an epitope within the carboxy-terminal 33 amino acids of the UL8 protein (Section 3.3.3) was therefore selected for use in the following experiments.

Labelled extracts were prepared as before from insect cells infected with 5 pfu/cell of AcUL8 or Ac8ΔN1, either alone or in double infections with 10 pfu/cell of AcUL5 or AcUL52, or in triple infections with both AcUL5 and AcUL52. Labelled extracts were also prepared from mock-infected cells and cells infected with AcUL5 alone, AcUL52 alone, and both AcUL5 and AcUL52.

Figure 41 shows the labelled extracts before (**lanes 1-12**) and after reaction with the antibody. The results demonstrate that MAb0817, like MAb0811, precipitates *wt* UL8 from singly infected cells (**lane 16**) and coprecipitates UL8 with UL5 or UL52 and both UL5 and UL52 from doubly and triply infected cells respectively (**lanes 14, 15** and **13**). UL5 and UL52 were detected only at very low levels in immunoprecipitations in the absence of UL8 (**lanes 17, 18** and **19**).

The UL8 deletion mutant 8ΔN1 is precipitated by MAb 0817 from cells infected with Ac8ΔN1 alone (**lane 21**). 8ΔN1 also coprecipitates with UL5 and UL52 from doubly infected insect cells (**lanes 22** and **23**) and with both UL5 and UL52 from triply infected cell extracts (**lane 24**). Thus amino acids 78-339 of UL8 appear to be dispensable for intersubunit interactions within the helicase-primase complex.

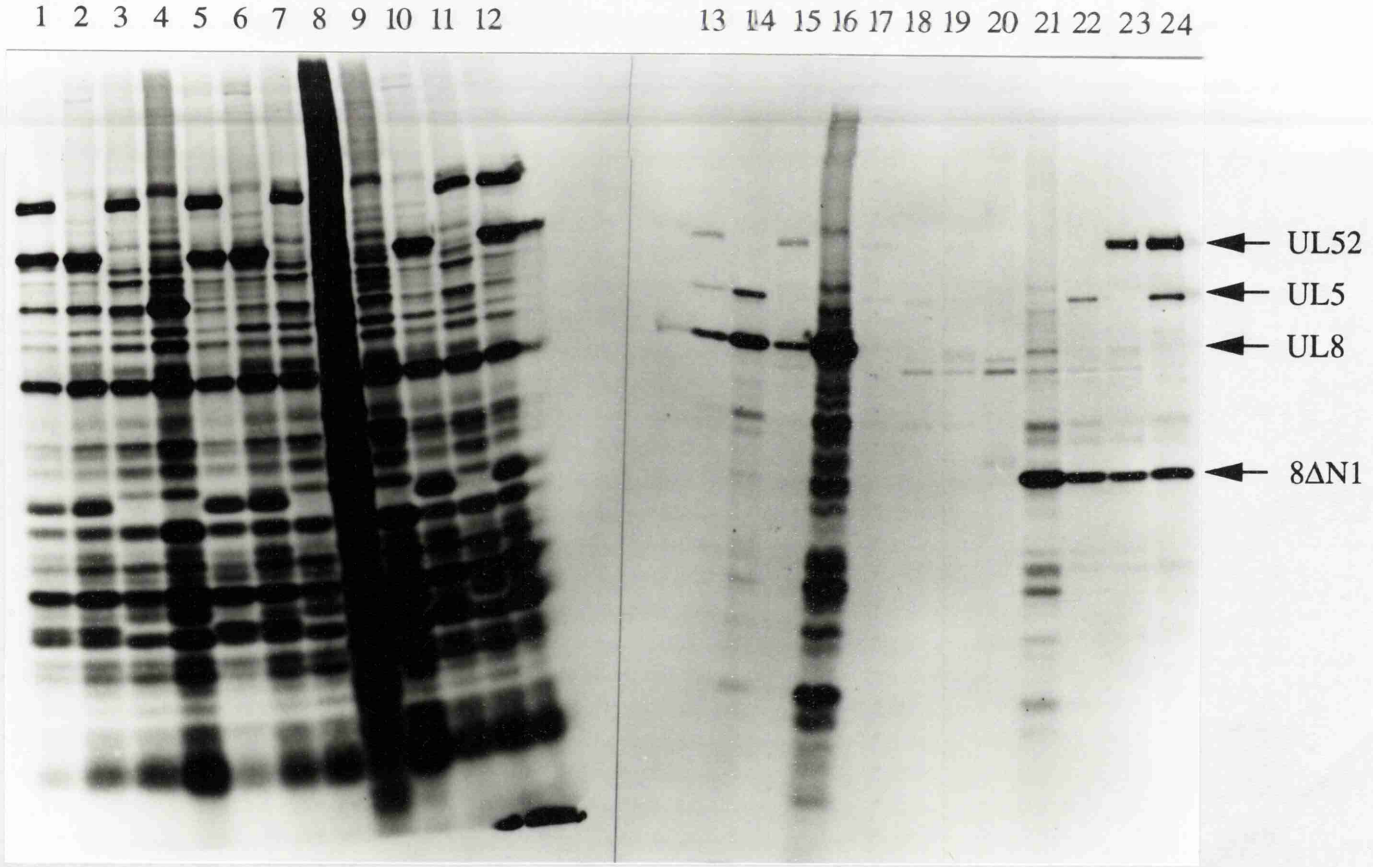
3.5.6 Discussion

The ability of the four UL8 mutants to coprecipitate with UL5 and UL52 from extracts of baculovirus infected cells indicates that at least a proportion of the protein is soluble and retains some degree of correct folding. Furthermore, the 8ΔN1 protein was not immunoprecipitated from infected cell extracts using MAb 0811 (data not

Figure 41 The mutant 8ΔN1 interacts with the other two components of the helicase-primase complex.

Lanes 1-12 show the labelled extracts prepared 40 hr p.i., from insect cells mock-infected or infected with the baculoviruses indicated below. The corresponding immunoprecipitations using MAb 0817 are shown in lanes 13-24, respectively. Proteins were resolved by SDS-PAGE. The positions of the UL52, UL5, UL8 and 8ΔN1 polypeptides are indicated.

Lane		Baculoviruses
1	13	AcUL8 + AcUL5 + AcUL52
2	14	AcUL8 + AcUL5
3	15	AcUL8 + AcUL52
4	16	AcUL8
5	17	AcUL5 + AcUL52
6	18	AcUL5
7	19	AcUL52
8	20	mock-infected
9	21	Ac8N1
10	22	Ac8N1 + AcUL5
11	23	Ac8N1 + AcUL52
12	24	Ac8N1 + AcUL5 + AcUL52


1 2 3 4 5 6 7 8 9 10 11 12
13 14 15 16 17 18 19 20 21 22 23 24
UL52
UL5
UL8
8ΔN1

shown), demonstrating that precipitation of this protein is dependent upon specific recognition by the MAb (the epitope(s) recognised by MAb 0811 lie within amino acids 166-253, which are deleted in 8ΔN1).

The co-immunoprecipitation experiments show that the four UL8 mutants can each interact separately with UL5 and UL52. Taken together, these mutants carry deletions covering most of the UL8 protein, apart from a region spanning amino acids 340-470. Either the sequences of UL8 which are responsible for interacting with the other two components of the helicase-primase complex are confined within the region from amino acids 340-470, or intersubunit interactions involve more than one site on the UL8 protein such that coprecipitation is still detected even though some of the sequences involved (for example near the N- or C-terminal ends) are deleted.

It would be of interest, therefore, to determine the ability of mutants CΔ359 and CΔ497 (which consist of amino acids 1-391 and 1-253, respectively) to coprecipitate with UL5 and UL52. In particular, since the deletion in CΔ497 encompasses amino acids 340-470, coprecipitation of UL5 and UL52 with the CΔ497 protein would demonstrate that intersubunit interactions were not confined to this region of UL8. To this end, these and other truncated UL8 ORFs were transferred into pAcYM1, in preparation for generating recombinant baculovirus stocks for immunoprecipitation studies, although lack of time prevented this experiment from being completed. It should, however, be noted that earlier studies showed that the CΔ359 and CΔ497 proteins were very poorly soluble when expressed in BHK cells (Section 3.4.5). If this were also the case in insect cells it would prevent the role of the deleted sequences being assessed. An alternative route might therefore be to make a construct specifically expressing amino acids 340-470, to determine whether this region is sufficient for interaction with UL5 and UL52.

In addition, it should be noted that three of the monoclonal antibodies (MAbs 0801, 0813 and 0814) capable of recognising UL8 by immunofluorescence showed reduced staining upon co-expression of UL8 with UL5 and UL52 (section 3.3.4). This suggests that formation of the heterotrimeric complex masks the epitopes recognised by these MAbs. Pre-incubation of these three MAbs with extracts of insect cells

infected with AcUL8, prior to mixing with an extract from insect cells infected with AcUL5 and/or AcUL52, might therefore inhibit the co-immunoprecipitation of UL5 and/or UL52 with *wt* UL8 by preventing intersubunit interactions. This would provide evidence that the epitopes recognised by the MAbs were at or near surfaces involved in intersubunit interactions.

The region of UL8 containing epitopes for one of the antibodies, MAb 0814, was mapped to amino acids 470-554. It is possible that amino acids constituting the epitope are directly involved in interactions with UL5 and UL52, or alternatively this epitope lies sufficiently close to a region involved in intersubunit interactions (such as amino acids 340-470), that recognition by the antibody is sterically inhibited by the UL5 and/or UL52 proteins. The locations of epitopes recognised by the other two MAbs, 0801 and 0813, have not been mapped but, since both MAbs precipitate *wt* UL8 from infected insect cells (refer to **Table 4**), it might be possible to locate the epitopes by testing the MAbs for ability to precipitate the UL8 deletion mutants. These MAbs should therefore prove useful in further defining the regions of UL8 which are essential for interactions with UL5 and UL52.

CHAPTER 4 DISCUSSION

Section 4.1 INTRACELLULAR LOCALISATION OF THE HELICASE-PRIMASE COMPLEX

It had previously been demonstrated, by immunofluorescent staining of BHK cells infected with HSV-1 recombinants, that all three components of the helicase-primase complex (UL5, UL8 and UL52) must be co-expressed in order for their efficient localisation to the nucleus, whereas the UL9 protein efficiently enters the nucleus when expressed alone (Calder *et al.*, 1992). A potential role for the UL8 protein in facilitating nuclear uptake or retention of the helicase-primase complex was therefore suggested (Calder *et al.*, 1992). To investigate the intracellular location of mutated UL8 proteins, without the requirement for constructing recombinant herpesviruses, a plasmid-based assay for intracellular localisation of the helicase-primase complex was developed. This assay, coupled with the availability of monoclonal antibodies to UL8, permitted the findings of Calder *et al.* (1992) to be extended, demonstrating that co-expression of the three components of the helicase-primase complex in the absence of all other viral proteins is sufficient for their nuclear localisation, and that UL8 can apparently enter the nucleus in the absence of UL5 and UL52. Prior to discussing the findings obtained with mutated UL8 proteins in this assay, it is first necessary to consider the process of nuclear localisation and the mechanisms by which UL8 may facilitate nuclear accumulation of the helicase-primase complex.

Nuclear protein import is a selective process (for reviews see Dingwall & Laskey, 1991; Silver, 1991; Nigg *et al.*, 1991). Following synthesis in the cytoplasm, proteins containing a nuclear localisation signal (NLS) enter the nucleus through pore complexes. Nuclear pore complexes (NPCs) are large proteinaceous structures in the nuclear envelope. They provide aqueous channels with a functional diameter of 9-10 nm, allowing diffusion of ions, small molecules, and proteins with molecular masses up to 40-60 kDa (Peters, 1986). For larger karyophilic proteins, delivery to, and

binding with, the NPCs may be mediated by cytoplasmic NLS-binding proteins (NBPs), and is followed by translocation through the pore in an ATP-dependent process. Although small proteins do not require a NLS for entering the nucleus, the finding that histone H1 (21 kDa) exhibits saturable import kinetics suggests that even small nuclear proteins may enter the nucleus by active transport (Breeuwer & Goldfarb, 1990).

A NLS is defined by two criteria: deletion or mutation causes cytoplasmic accumulation of a normally nuclear protein; and when fused to a non-nuclear protein, the NLS directs the protein to the nucleus. There is no good primary sequence consensus for NLSs, but they are generally less than twelve amino acids long and rich in the basic amino acids arginine and lysine. The SV40 large T antigen minimal NLS ($PKK^{128}KRKV$) is considered the prototypic NLS, although additional amino acids within the T antigen are required for fully efficient nuclear localisation (Kalderon *et al.*, 1984; Rihs & Peters, 1989). Other types of NLS have also been described, such as the influenza virus nucleoprotein NLS (AAFEDLRVLS), which has only a single basic residue (Davey *et al.*, 1985).

Dingwall & Laskey (1991) proposed a consensus NLS that was bipartite, comprising two basic amino acids (lysine or arginine), a spacer region most commonly ten amino acids long, and a basic cluster in which three out of five amino acids must be lysine or arginine. A database search of eukaryotic cellular proteins identified this motif in 56% of nuclear proteins and less than 5% of nonnuclear ones, whereas only 17% of nuclear proteins contained five consecutive basic residues as in SV40 T antigen. Thus the bipartite motif appears to be the most frequently occurring type of NLS, although it should be noted that clusters of basic amino acids are also found in DNA-binding domains of nuclear proteins, which could be an alternative explanation for their relative abundance in nuclear proteins (Dingwall & Laskey, 1991). Whether the bipartite motif is as common amongst viral nuclear proteins has not been reported, but the NLS of influenza virus polymerase basic protein 1 and of the adenovirus DNA-binding protein both consist of two basic domains separated by a 16 and 37 amino acid spacer, respectively (Nath & Nayak, 1990; Morin *et al.*, 1989).

The suggested structure of the bipartite motif involves looping out of the spacer region, bringing the two basic domains closer together, possibly so as to mimic shorter basic NLSs such as that of the SV40 T antigen (Dingwall & Laskey, 1991). The bipartite motif may therefore function even when inverted relative to the orientation of the polypeptide sequence, since the spacer would still loop out to bring the two basic domains together. In contrast, reversing the order of amino acids in the NLS of SV40 T antigen renders it nonfunctional (Adam *et al.*, 1989).

Analysis of the aligned amino acid sequences of UL8 homologues in HSV-1, VZV and EHV-1 (**Figure 42**) does not reveal a single NLS, defined as either five consecutive basic residues or as the consensus bipartite motif, that is positionally conserved between the three proteins. However all three proteins do contain sequences matching the bipartite motif, in either the correct or inverted orientation, with spacers ranging from 9 to 36 residues long (**Figure 42**). The presence of these basic clusters only indicates the potential for an NLS in UL8. Demonstrating that any of them actually function to promote nuclear localisation may be complicated by the tendency of UL8 protein to accumulate independently in the nucleus in only a proportion of transfected cells. UL8 polypeptides with specific mutations designed to disrupt the potential NLS motifs could be assessed by indirect immunofluorescence of transfected cells, although for any regions of UL8 where mutation results in a complete inability to localise to the nucleus, proof of function as an NLS would necessitate fusing this region to a non-nuclear protein and demonstrating nuclear accumulation. The ability of such UL8 mutants to form a trimeric complex with UL5 and UL52 would indicate that the defect in nuclear localisation was not merely caused by extensive refolding of the mutant UL8 protein.

In comparison, the finding that UL9 localises efficiently to the nucleus not only in recombinant herpesvirus-infected cells but also in transfected cells, without the aid of other viral proteins, indicates that it must contain an NLS. Although no part of UL9 has yet been reported to function as an NLS, analysis of the aligned amino acid sequences of UL9 homologues from HSV-1, VZV and EHV-1 reveals the presence of a sequence, matching the bipartite NLS motif, at equivalent positions in the HSV-1

Figure 42. Alignment of the amino acid sequences of three alphaherpesvirus UL8 homologues.

The amino acid sequences of the HSV-1 gene UL8, EHV-1 gene 54 and VZV gene 52 products were aligned using the Pileup program (Genetics Computer Group). The consensus line shows residues identical in all three sequences in red, and residues identical in two out of the three sequences in black. Potential bipartite NLS motifs are underlined, with relevant arginine (R) and lysine (K) residues highlighted in green.

```
          1                                                          60
  HSV-1   ..MDTADIVW VEESVSAITL YAVWLPPR.. AREYFHALVY FVCRNAAGEG RARFAEVSVT
  EHV-1   ...MEGSVEW FNGHVCATSI YSLWTDPHHP GH..LQALVY MLCRRGSD.Y TAEFCHVPVS
  VZV     MDATQITLVR ESGHICAASI YTSWTQSGQL TQNGLSVLYY LLCKNSCGKY VPKFAEITVQ
Consensus --------VW --GHVCA-SI Y--WT-P--- ----L-ALVY -LCRN--G-Y -A-FAEV-V-

         61                                                         120
  HSV-1   ATELRDFYGS ADVSVQAVVA AARAATTPA. ..ASPLEPLE NPTLWRALYA CVLAALERQT
  EHV-1   GELLKRGARD ASLVTPARVA SAAQTA..AV PGCWPLAPLG NAMLWKSVYG GITAALKRAV
  VZV     QEDLCRYSRH GGSVSAATFA SICRAASSAA LDAWPLEPLG NADTWRCLHG TALATLRRVL
Consensus -E-L-R--R- A--V--A-VA SA--AA--A- --AWPLEPLG NA-LWR-LYG --LAAL-R--

         121                                                        180
  HSV-1   GPVALFAPLR IGSDPRTGLV VKVERASWGP PAA.....PR AALLVAEANI DIDPMALAAR
  EHV-1   GSFAFYQPLV LGINTQTGLL VTLRPAASAG EGGGDHVSPR AAIVNVSVEV DLDPAGIEAS
  VZV     GFKSFYSPVT FETDINTGLL LKTIPDEHAL NNDN...TPS TGVLRANFPV AIDVSAVSAC
Consensus G--AFY-PL- -G-DT-TGLL VK--PA--A- --------PR AA-L-A---V DIDP-A--A-

         181                                                        240
  HSV-1   VAEHPDARLA WARLAAIRDT .PQCASAASL TVNITTGTAL FAREYQTLAF PPIKKEGAFG
  EHV-1   AASSTGSSLA RARLCTLRDG YFLSKRDIAL EVEIATKEVS FYRKYDSVQQ PANKRRGDMA
  VZV     NAHTQGTSLA YARLTALKSN .GDTQQQTPL DVEVITPKAY IRRKYKSTFS PPIEREGQTS
Consensus -A---G-SLA -ARL-ALRD- ---------L -VEI-T--A- F-RKY-S--- PPIKREG---

         241                                                        300
  HSV-1   DLVEVCEVGL RPRGHPQRVT ARVLLPRDYD YFVSAGEKFS APALVALFRQ WHTTVHAA..
  EHV-1   DLFVVHERTL LLGGC.KRMG VKVLLPRTFD CLVASSQSVS GLAAMALYKQ WHATLFSVEL
  VZV     DLFNLEERRL VLSGN.RAIV VRVLLPCYFD CLTTDSTVTS SLSILATYRL WYAAAFGK..
Consensus DLF-V-ER-L -L-G---R-- VRVLLPR-FD CLV--S---S -LA--ALYRQ WHAT-F----

         301                                                        360
  HSV-1   PGALAPVFAF LGPEFEVRGG PVPYFAVLGF PGWPTFTVPA TAESARDLVR GAAAAYAALL
  EHV-1   PDTVVQIFAY LGPELNPCGE EVDYCCFVGF PGLPTLK... ASSSTTEAVR DAMAAYRLSD
  VZV     PGVVRPIFAY LGPELNPKGE DRDYFCTVGF PGWTTLR... TQTPAVESIR TATEMYMETD
Consensus PG-V-PIFAY LGPELNP-GE -VDYFC-VGF PGWPTL---- T--SA-E-VR -A-AAY---D

         361                                                        420
  HSV-1   GAWPAVG..A RVVLPPRAWP GVASAAAGCL LPAVREAVAR WHP.....AT KIIQLLDPPA
  EHV-1   GLWPALGMSA FHFLAPWDPE DRWPGESEAK RVEGAVHRLQ LGTEDDWGAG RVSCILESDA
  VZV     GLWPVTGIQA FHYLAPWGQH PPLPPRVQDL IGQIPQDTGH ADATVNWDAG RISTVFKQPV
Consensus GLWPA-G--A FH-LAPW--- ---P-----L ---------- ------W-AG RIS--L--PA

         421                                                        480
  HSV-1   AVGPVWTARF CFPGLRAQLL AAL..ADLGG SGLADPHGRT GLARLDALVV AAPSEPWAGA
  EHV-1   VMQGPWFAKF DFSAFFPTLY LLLFPANERL AEVVRLRARG QHPTLKLALV .....SFFGG
  VZV     QLQDRWMAKF DFSAFFPTIY CAMFPMHFRL GKIVLARMRR GMGCLKPALV .....SFFGG
Consensus --Q--W-AKF DFSAFFPTLY -ALFPA--RL ---V--R-R- G---LK-ALV -----SFFGG

         481                                                        540
  HSV-1   VLERLVPDTC NACPALRQLL GGVMAAVCLQ IEETASSVKF AVCGGDGGAF WGV...FNVD
  EHV-1   LQHINPVAY RSIIALSN.. .....GISKR LEHEVNQRGF AICTYVKDGF WGAAGNLPSD
  VZV     .LRHILPSIY KAIIFIAN.. .....EISLC VEQTALEQGF AICTYIKDGF WGIFTDLHTR
Consensus -L-HI-P--Y -AIIAL-N-- ------ISL- -E-TA---GF AICTY-KDGF WG----L--D

         541                                                        600
  HSV-1   PQDADAASGV IEDARRAIET AVGAVLRAN. AVRLRHPL.. CLALEGVYTH AVAWSQAGVW
  EHV-1   SVSYADALVY AEELRSAAQK AALGHVS.EM GFSLPEGVHL NLRLEGLFTD AISWSTHCYW
  VZV     NVCSDQARCS ALNLAATCER AVTGLLRIQL GLNFTPAMEP VLRVEGVYTH AFTWCTTGSW
Consensus -V--D-A--- AE-LR-A-E- AV-G-LR--- G--L------ -LRLEGVYTH A--WST-G-W

         601                                                        660
  HSV-1   FWNSRDNT.D HLGGFPLRGP AYTTAAGVVR DTLRRVLGLT TACVPEEDAL TARGLM....
  EHV-1   LYNRFTKM.E DFVGFPAKSG ....AGRAAK ASLSALLPLV AAVCDSSDMS TLHQSV....
  VZV     LWNLQTNTPP DLVGVPWRSQ ....AARDLK ERLSGLLCTA TKIRERIQEN CIWDHVLYDI
Consensus LWN--TNT-- DLVGFP-RS- ----AAR--K --LS-LL-L- TA-----D-- T----V----

         661                                                        720
  HSV-1   .........E DACDRLILDA FNKRLDAEYW SVRVSPFEAS DPLPPTAFRG GALLDAEHYW
  EHV-1   .........R GACEQLVAGA FAERNNPQFW STR.TGIESS TLLPPAVYRN GSLLDRDCGQ
  VZV     WAGQVVEAAR KTYVDFFEHV FDRRYTPVYW SLQ.EQNSET KAIPASYLTY GHMQDKDYKP
Consensus ---------R -AC--L---A F--R--P-YW S-R----E-S --LPP---R- G-LLD-D---

         721                                                        780
  HSV-1   RRVVRVCPGG GESVGVPV.. DLYPRPLVLP PVDCAHHLRE ILREIELVFT GVLAGVWGEG
  EHV-1   REIVLTRKHD CESPSPVP.W TLFPPPLVLG RIDCMVYLTS IFKTYLSMLN RAISASCD.A
  VZV     RQIIMVRNPN PHGPPTVVYW ELLPSCACIP PIDCAAHLKP LIHIFVTIIN HLLDAHNDFS
Consensus R-IV-VR--- -ESP--VV-W -L-P-PLVLP PIDCA-HL-- I--T-----N --L-A--D--

         781              799
  HSV-1   GKFVYPFDDK MS...FLFA
  EHV-1   DESMN.VDFP ISDYAFLFT
  VZV     SPSLKFTDDP LASYNFLFL
Consensus --S----DDP -S-Y-FLF-
```

UL9 (K^{793}REFAGARFKLR) and EHV-1 gene 53 (R^{829}KLFAGPRYKLR) products. The second and fifth lysine/arginine residues of this motif are not conserved in the VZV gene 51 product (R^{775}HSFSQQRYKIT), although a nearby cluster of lysines (K^{740}NLKKK) may compensate for this. The location of these potential NLSs in the C-terminal DNA-binding domain is in agreement with the ability of this domain to exert dominant inhibition of *wt* UL9 in transient assays of DNA replication, since this property would be expected to require the protein to be nuclear (Stow *et al.*, 1993). Interestingly, insertion of four amino acids at position 799 of UL9 to give the sequence KREFAGAPEFRRFKLR results in reduced ability of the protein to interact with ori$_S$ site II in gel retardation experiments but a complete failure to promote ori$_S$-dependent replication in insect cells (Arbuckle, 1993). It is not yet known whether this mutation causes failure of the protein to localise to the nucleus or whether it affects another property of the protein.

Nuclear targeting sequences may be of different potencies and may be additive in their effects, since the presence of additional copies of an NLS in a molecule increases the initial rate and final steady-state level of nuclear accumulation (Dworetzky *et al.*, 1988; Dingwall & Laskey, 1991). It is possible that any NLSs present in UL8 are relatively weak, hence the inefficient nuclear localisation of UL8 when expressed alone. This may also explain why nuclear entry of UL8 was detected in the transfection assay in which expression was for 24 hr from the particularly strong HCMV major IE promoter, but not with *ts*K/UL8 where expression was for a shorter time and from a weaker promoter.

It might be the case that binding of UL8 with UL5 and UL52 results in a sufficient number of weak NLSs within the complex to allow efficient transport to the nucleus. A search for potential NLSs revealed that a single bipartite motif, with a relatively long spacer of 63 amino acids, is conserved in the UL5 homologues of HSV, VZV and EHV-1. HSV-1 UL52 also contains a bipartite motif with a spacer of 31 residues, but the upstream pair of basic residues are not conserved in its homologues in VZV and EHV-1, although it is possible that an extra basic residue located within two residues of the downstream basic region in these two proteins may compensate for the

absence of the upstream residues. The functional importance of these residues in nuclear targeting is not known. Indeed, since UL5 and UL52 appear cytoplasmic when expressed alone, it is possible that they do not contain any functional NLSs at all, relying instead on binding with UL8 for nuclear transport, although if this were the case one might expect pairwise combinations of UL5 and UL8, or UL52 and UL8, to localise to the nucleus. Other proteins which do not possess their own NLS but enter the nucleus via cotransport with another protein have been described (*e.g.* Zhao & Padmanabhan, 1988) and this is consistent with complex formation occurring in the cytoplasm without disassembly or unfolding taking place on nuclear entry.

NLSs must be exposed on the surface of proteins to allow interaction with components of the import machinery. For example, placement of the SV40 T antigen NLS in the buried hydrophobic domain of pyruvate kinase does not alter the normally cytoplasmic location of this protein (Roberts *et al.*, 1987; Nelson & Silver, 1989). This provides another potential explanation for the requirement for all three components of the helicase-primase to be co-expressed for their efficient nuclear localisation. Interactions between all three proteins may be required for a conformational change to unmask a buried NLS, permitting nuclear import of the complex and explaining why pairwise combinations of the helicase-primase subunits are not capable of nuclear localisation. It could also be envisaged that a single nuclear localisation signal, comprising a basic region on the surface of the complex, includes amino acids from all three subunits.

NLSs may be modulated by other factors such as phosphorylation of flanking sequences or binding to cytoplasmic anchoring proteins. Two residues outside the minimal NLS of SV40 T antigen are required for an enhanced rate of nuclear uptake and are potential substrates for casein kinase II (Rihs *et al.*, 1991). In addition, potential cdc2 kinase sites occur near the bipartite motif in many of the sequences identified by the database search. cdc2 is a cell-cycle dependent kinase, prompting speculation that phosphorylation of these sites may play a role in cell-cycle dependent nuclear import (Dingwall & Laskey, 1991). Phosphorylation of components of the helicase-primase is not, however, thought to occur.

The cytoplasm contains many proteins, smaller than the approximately 40 kDa diffusional limit of the nucleus, that are nevertheless excluded from the nucleus. These proteins may be retained in the cytoplasm through interaction with cytoplasmic anchoring proteins. Such anchoring proteins may also be involved in the regulation of nuclear protein import by binding in such a way as to mask the NLS and prevent interaction with NBPs (reviewed by Silver, 1991; Nigg *et al.*, 1991). Hence UL5 and UL52 may be bound to anchoring proteins in the cytoplasm, perhaps masking their NLSs, but complex formation with the other two components of the helicase-primase might disrupt this interaction, releasing the proteins from the cytoplasmic anchor and exposing an NLS for translocation to the nucleus.

It has been noted that ß-galactosidase hybrids carrying the SV40 T antigen minimal NLS enter the nucleus but remain close to the periphery of the nucleoplasm, suggesting that although the NLS may be sufficient for nuclear import, distribution and retention in the nucleus may depend on specific protein-protein or protein-DNA interactions. Indeed, Schmidt-Zachmann *et al.* (1993) measured the rates of nuclear export of a variety of protein constructs following injection into Xenopus oocyte nuclei and found that whilst export of *wt* nucleolin was barely detectable even after 24 hr, inactivating its bipartite NLS resulted in 90% of the mutant protein being exported to the cytoplasm within 24 hr, implying that the *wt* protein undergoes efflux from the nucleus and NLS-dependent re-import. Moreover, pyruvate kinase, which is normally 90% cytoplasmic, was about 45% cytoplasmic at equilibrium when fused to the SV40 T antigen NLS, and further addition of the domains of nucleolin required for its retention in the nucleus strongly inhibited the nuclear export of the pyruvate kinase fusion protein. Since a cytoplasmic protein is unlikely to possess a specific nuclear export signal, export is unlikely to be signal mediated. Finally, they found that removal of nuclear retention signals from lamin B_2 converted it from a non-shuttling nuclear protein to a shuttling protein. These experiments support the view that nuclear accumulation of a protein can be reinforced by selective binding following nuclear import, and that without selective binding, some nuclear proteins escape and shuttle repeatedly between the nucleus and the cytoplasm.

Different components of the helicase-primase might contribute signals required for different steps in the transport process, perhaps one for docking with, and translocation across, the NPC, and one for intranuclear targeting and retention. If UL8 were to contain the nuclear import signal, and the retention signal was formed by the association of UL5 with UL52 (perhaps involving an interaction with a specific nuclear component), then this could explain the observed locations of the proteins, since UL8 would be capable of entering the nucleus, but would leach out again in the absence of UL5 and UL52, and UL5 and UL52 would remain cytoplasmic without the import signal provided by UL8. However, this model fails to explain why UL5/UL8 and UL52/UL8 are more cytoplasmic than UL8 expressed alone.

Why should all components of the helicase-primase complex be necessary for efficient nuclear localisation, rather than being individually imported into the nucleus? One possibility is that whatever mechanism is operating ensures that the three proteins are present in the nucleus in a 1:1:1 ratio, irrespective of the levels at which the corresponding genes are expressed. It may be undesirable, for example, to have the enzymatically active UL5/UL52 subassembly present in the nucleus, where it could interfere with the properly regulated process of viral DNA replication.

Section 4.2 MUTATIONAL ANALYSIS OF THE UL8 PROTEIN

At the start of this study UL8 was known to be essential for HSV-1 DNA synthesis, forming a complex with UL5 and UL52 and facilitating nuclear localisation of this complex, but the UL8 protein did not appear to contribute to the helicase and primase activities of the complex and no clues as to its function could be gleaned from its amino acid sequence. For this reason a mutational study, relating the structure of UL8 to its known functions, was undertaken. Despite more recent advances demonstrating that UL8 can affect the activity of the helicase-primase complex in biochemical assays and that it interacts with other components of the viral replicative machinery, the exact function(s) of the UL8 protein in HSV DNA synthesis remain elusive, and nothing is known of the regions of the protein required for these functions.

A comparison of the aligned amino acid sequences of three alphaherpesvirus UL8 gene homologues (HSV-1 gene UL8, VZV gene 52 and EHV-1 gene 54) reveals approximately 16% identity between all three proteins, although 27-34% of amino acids are identical by pairwise comparisons within this alignment (*e.g.* 33.7% identity between VZV and EHV-1) and there are regions of the sequence where the identity is higher (**Figure 42**).

The UL8 protein interacts with four of the six other HSV-1 DNA replication proteins (**Table 7**), suggesting that the role of this protein in DNA synthesis may be more dependent upon the formation of protein-protein interactions than on the presence of catalytic or DNA-binding domains. However, one of the regions of UL8 showing best conservation amongst the alpha-herpesviruses exhibits good similarity to regions of the homologous HCMV and EBV proteins (products of UL102 and BBLF2/3, respectively; **Figure 43**; Fixman *et al.*, 1992). This conserved region might therefore be a candidate for involvement in an enzymatic function, for example modulating primer synthesis and utilisation (Sherman *et al.*, 1992; Tenney *et al.*, 1994).

The specificity of protein-protein interactions within the replication complex is illustrated by the finding that, although the complete set of HSV replication proteins (minus UL9) is functionally equivalent to the complex formed by the corresponding

Protein	Evidence
UL5 UL52	form heterotrimeric complex with UL8 (*e.g.* Crute & Lehman, 1991; McLean *et al.*, 1994)
UL9	coprecipitates with UL8 (McLean *et al.*, 1994)
UL29	stimulates UL5/UL8/UL52 > UL5/UL52 (Tenney *et al.*, 1995)
UL30	coprecipitates with UL8 (Marsden *et al.*, in preparation)
UL42	no evidence for an interaction

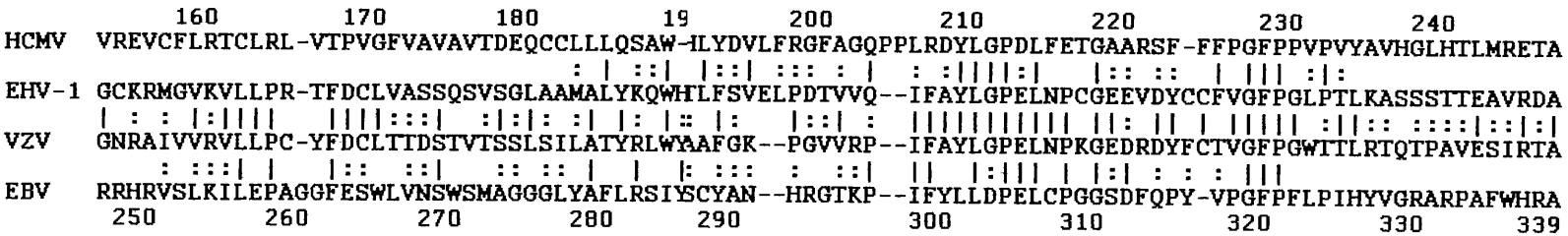
Table 7. Interactions between UL8 and the other six HSV-1 DNA replication proteins.

The table gives a brief summary of the available evidence for interactions between UL8 and the other six virally encoded replication proteins. Refer to Sections 1.4.2d and 1.4.3 for a fuller description of these interactions.

Figure 43: **Alignment of alpha-, beta-, and gamma-herpesvirus UL8 homologues reveals a conserved sequence block.**

Amino acids 153-247 of the HCMV UL102 protein, and amino acids 247-339 of the EBV BBLF2 protein, are aligned with the equivalent region of the alphaherpesvirus UL8 homologue with which they share the highest degree of identity (amino acids 246-340 of EHV-1 gene 54 product and amino acids 250-342 of VZV gene 52 product, respectively).

(|) indicates identical residues, (:) indicates conservative changes.



In pairwise comparisons, HCMV and EHV-1 share 30.8% identity in a 52 amino acid overlap,

EBV and VZV share 29.2% identity in a 72 amino acid overlap,

and EHV-1 and VZV share 48.4% identity in a 95 amino acid overlap.

six EBV proteins in a transient assay of EBV ori-Lyt-dependent replication (providing the EBV transactivators Zta and Rta are present), individual proteins within the complex may not be readily interchangeable (Fixman *et al.*, 1995). Moreover, the interaction of UL8 with UL9 (McLean *et al.*, 1994), represents an interaction confined to the alpha-herpesviruses. It is therefore perhaps not surprising that the amino acid sequence varies significantly between UL8 homologues.

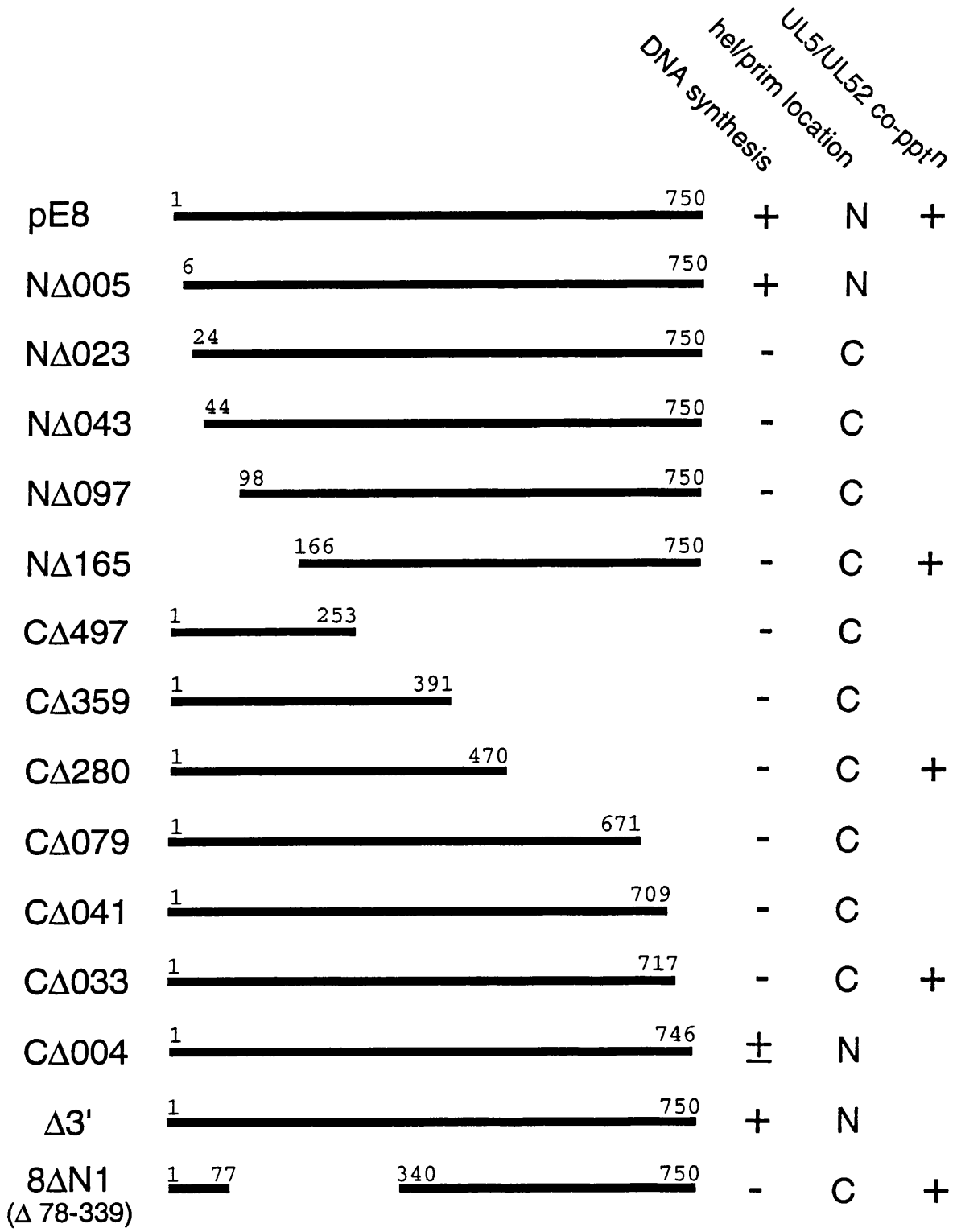
Given the overall low sequence conservation and the lack of information regarding important structural elements within UL8, it was decided that an initial analysis of the HSV-1 UL8 protein should employ a random set of N- and C-terminally truncated versions of the protein. Conveniently located *Not*I restriction sites enabled construction of an additional mutant containing an internal deletion, but maintaining both the N- and C-termini of UL8. The mutants obtained are represented diagrammatically in **Figure 44**, which also summarises the ability of these mutants to support HSV-1 origin-dependent DNA synthesis and facilitate nuclear localisation of the helicase-primase complex in transfected cells, and to coprecipitate with UL5 and UL52 from insect cells infected with the appropriate recombinant baculoviruses.

4.2.1 Complex formation and intracellular localisation of mutated UL8 proteins

The results from the transfection assays demonstrate that a deletion of five amino acids from the N-terminus of UL8 can be tolerated without affecting the protein's ability to support HSV-1 origin-dependent DNA synthesis and to facilitate nuclear localisation of the helicase-primase complex, whereas larger deletions of 23 to 165 amino acids abolish both of these activities. Interestingly, the VZV gene 52 and EHV-1 gene 54 products are identical to UL8 at positions 15, 19 and 22 (**Figure 42**), in support of the region between amino acids 5 and 23 being important to the structure and/or function of the protein.

A similar situation was observed for the C-terminally truncated proteins. Removal of the four most C-terminal amino acids of UL8 does not appear to affect the protein's ability to facilitate efficient nuclear localisation of the helicase-primase complex, although this protein supports less than *wt* levels of HSV-1 origin-dependent

Figure 44: Summary of UL8 deletion mutants and their properties. The name of each mutant is given on the left of the diagram, with the proteins represented by black bars and the amino acids defining the limits of each polypeptide numbered according to their position in *wt* UL8. Δ3' carries a full-length UL8 ORF but has a region of the 3' untranslated sequence deleted. The amino acids deleted from 8ΔN1 are given in brackets. Some properties of these mutants are given on the right of the diagram. Ability to support HSV-1 origin-dependent DNA synthesis in transient transfection assays is either wild-type (+), absent (-) or reduced (±). Location of the helicase-primase complex in transfected cells is either nuclear (N) or cytoplasmic (C). For those mutants expressed by recombinant baculoviruses, ability to coprecipitate with UL5 and UL52 from extracts of insect cells is indicated (+).


DNA synthesis
hel/prim location
UL5/UL52 co-pptn
pE8
1
750
+
N
+
NΔ005
6
750
+
N
NΔ023
24
750
-
C
NΔ043
44
750
-
C
NΔ097
98
750
-
C
NΔ165
166
750
-
C
+
CΔ497
1
253
-
C
CΔ359
1
391
-
C
CΔ280
1
470
-
C
+
CΔ079
1
671
-
C
CΔ041
1
709
-
C
CΔ033
1
717
-
C
+
CΔ004
1
746
±
N
Δ3'
1
750
+
N
8ΔN1
(Δ 78-339)
1
77
340
750
-
C
+

DNA synthesis in the transfection assay. Larger C-terminal deletions of 33 to 497 amino acids abolished both transient replicative ability and efficient nuclear localisation of the helicase-primase complex in transfected cells. Curiously, amino acids 718-746 of UL8, which are present in CΔ004 but deleted in CΔ033, are not well conserved amongst the alphaherpesvirus homologues. The functional contribution of these amino acids will be considered later.

The failure of the UL8 mutants to facilitate efficient nuclear localisation of UL5 and UL52 is presumably not due solely to an inability to form a heterotrimeric complex, since the truncated UL8 proteins containing the largest N-terminal deletion (of 165 amino acids) and the third largest C-terminal deletion (of 280 amino acids), when expressed by recombinant baculoviruses, coprecipitate with both UL5 and UL52 from appropriately infected insect cells. One would therefore predict that the four other N-terminal mutants and all of the C-terminal mutants with deletions of less than 280 amino acids would also coprecipitate with UL5 and UL52. Why, then, do complexes consisting of UL5, UL52 and mutated UL8 proteins fail to accumulate in the nucleus?

As discussed in section 3.5.6, it is possible that amino acids in two separate regions of UL8 (*e.g.* from both the N-terminal portion and the C-terminal portion) participate in the interactions with both UL5 and UL52, such that deletion of one region affects intersubunit interactions without abolishing formation of a complex. If this is the case, formation of a complex which does not present the appropriate NLS might result *e.g.* a buried NLS may not be unmasked or an NLS formed by regions of the three subunits may not be generated (see section 4.1).

If, however, as previously suggested (section 3.5.6) the region from amino acids 340-470 of UL8 is solely responsible for intersubunit interactions, then the observation that the mutants form a complex with UL5 and UL52 that fails to localise to the nucleus suggests that either UL8 does not facilitate nuclear import of UL5 and UL52 simply by contributing an NLS to the complex, or that amino acids between residues 6 and 23, and 718 and 746, each contribute to the NLS such that the NLS in the mutants is non-functional. Amino acids 6-23 and 718-746 might also be necessary for the correct folding of UL8, with their deletion resulting in a conformation in which

the NLS is masked. These possibilities could be addressed by assessing the intracellular localisation of UL8 deletion mutants when expressed alone. Nuclear fluorescence in any of the cells would suggest that a particular mutant contains an intact and functional NLS, and might enable the mapping of this signal in UL8.

The fact that the mutant 8ΔN1, whilst capable of coprecipitating with UL5 and UL52, also fails to facilitate efficient nuclear localisation of the helicase-primase complex argues against amino acids 6-23 and 718-746 together forming an NLS, and this is supported by the lack of basic residues in these regions of UL8. However the alternative possibilities, that 8ΔN1 has a masked NLS or is unable to establish the full subunit interactions with UL5 and UL52 required to form a functional NLS, cannot be excluded.

Clearly further experiments are required to explain the intracellular localisation of UL8 and the helicase-primase complex. Firstly it is important to assess the ability of the mutants CΔ359 and CΔ497 to coprecipitate with UL5 and UL52, to determine whether there are one or two regions of UL8 involved in intersubunit interactions. The preliminary experiments described in section 3.4.4, which suggest that mutant UL8 proteins such as CΔ033 are capable of localising to the nucleus but fail to facilitate nuclear accumulation of UL5 and UL52, also need to be extended to include the other UL8 mutants, in an attempt to define NLS-containing regions of the protein.

4.2.2 Mutated UL8 proteins in DNA synthesis

Replicative ability of the mutated UL8 proteins was found to correlate well with ability to facilitate efficient nuclear localisation of UL5 and UL52, and most of the mutants can (or are predicted to be able to) form a complex with UL5 and UL52. The possibility therefore arises that these mutant complexes are biochemically active but fail to function in the transient assay of DNA replication solely due to their failure to enter the nucleus. It would therefore be interesting to "force" the UL8 mutants into the nucleus of transfected cells by attaching a strong NLS to one or all of the subunits of the helicase-primase complex and assess their ability to function in the transient replication assay. In addition, biochemical characterisation of tripartite complexes

containing mutant UL8 proteins could be performed to determine whether the mutant proteins retain the functionality of the wild-type UL8 product. Appropriate assays would be to examine the stimulation of synthesis, and/or utilisation, of RNA primers (Tenney *et al.*, 1994; Sherman *et al.*, 1992), stimulation of coupled primase-polymerase activity (Sherman *et al.*, 1992) and stimulation of DNA synthesis on an artificial forked substrate (Klinedinst & Challberg, 1994). In each case the complex containing the mutant protein would be compared to a *wt* UL5/UL8/UL52 assembly and the UL5/UL52 subassembly. Biochemical assays could also be used to screen the tripartite complexes containing mutant UL8 proteins for ability to inhibit the *wt* complex. This would address the question of whether the mutants examined in Table 5 (section 3.4.2) failed to act as dominant inhibitors of DNA replication because of inefficient nuclear localisation of the helicase-primase complex.

Biochemical assays with CΔ004 in particular, might explain its impairment compared with *wt* UL8 in the transient DNA replication assay. Since the immunofluorescence studies are qualitative rather than quantitative, it remains possible that the CΔ004/UL5/UL52 complex, although capable of localising to the nucleus, does so less efficiently than *wt* UL8/UL5/UL52, and this alone is responsible for the reduced levels of plasmid amplification. However, since the C-terminus of UL8 is involved in interactions with the UL30 component of the polymerase (Marsden *et al.*, manuscript in preparation), it is also possible that the reduced ability of CΔ004 to support DNA synthesis might result from a reduced ability of this protein to interact with UL30. Since MAb 0811 coprecipitates UL30 with UL8 from extracts of insect cells infected with appropriate recombinant baculoviruses (G. McLean, personal communication), co-immuno-precipitation studies utilising a baculovirus recombinant expressing the CΔ004 protein could be performed to assess the contribution of the C-terminal four amino acids to the interaction between UL8 and UL30.

Recombinant baculoviruses expressing the range of N- and C-terminally truncated UL8 proteins could also be used in co-immunoprecipitation studies to identify regions of UL8 responsible for the previously reported interaction with UL9 (McLean *et al.*, 1994). Although a preliminary experiment was performed using

extracts from *S.f.* cells infected with AcUL9 and AcUL8, Ac033, Ac165, Ac280 or Ac8N1, the results were inconclusive and the experiment requires to be repeated.

4.2.3 Conclusions

The picture which emerges from my studies is that known functions of UL8 can not as yet be associated with particular stretches of amino acids. However, it is clear that residues close to each terminus are indispensable, despite being relatively less well conserved than some internal regions of the protein: amino acids 6-23 and 718-746 of UL8 are, respectively, 17% and 3% identical to the equivalent regions of the homologous VZV and EHV-1 proteins, compared with 28% for the region spanning amino acids 266-325 of UL8. This region also shows the highest degree of conservation with the UL8 homologues in HCMV and EBV.

The production of the deletion mutants has provided the basis for a number of future experiments, the most immediate of which have already been described in the discussion sections of this thesis. In the longer term, the next step to obtain further information relating UL8 structure to function might be to create and assess a set of mutations consisting of small (*e.g.* 2-4 amino acid) insertions distributed throughout the UL8 protein. These mutations might hopefully exert more localised effects on the conformation of the protein, and may therefore help in identifying functional domains within UL8. In particular it would be useful to isolate mutants capable of supporting efficient nuclear localisation of the helicase-primase complex, but impaired in ability to support DNA synthesis, to further elucidate the roles of this protein in viral genome replication. Additional information concerning nuclear localisation of UL8 and the helicase-primase complex might also be obtained by targetting mutations specifically to potential NLS motifs within UL8. Site-directed mutagenesis within the region 266-325, which is conserved amongst the alpha-, beta- and gamma-herpesvirus homologues of UL8, should provide information on the function of this part of the protein. Ultimately the determination of the crystallographic structure of free UL8 and its complex with UL5 and UL52 may be necessary to provide further insights into UL8

function and to resolve many of the questions concerning its involvement in nuclear localisation.

The nuclear localisation studies of the *wt* helicase-primase complex can also be taken further. Confocal microscopy, in combination with staining for ND10-associated proteins PML and Sp100, might be employed to ascertain whether the helicase-primase complex colocalises in transfected cells with ND10, the nuclear domains at which viral DNA accumulates and begins to replicate in cells infected with HSV-1 (Maul *et al.*, 1996), or whether other viral proteins or origin of replication signals are required.

Section 4.3 THE ROLE OF UL8 IN HSV-1 DNA SYNTHESIS

In spite of the growing amount of data available on properties of the HSV-1 UL8 protein, it is not yet clear precisely what functions this essential protein contributes to viral DNA synthesis. It is clear that UL8 is necessary for the efficient nuclear localisation of UL5 and UL52, and its involvement in prereplicative site formation (Liptak *et al.*, 1996) and ability to coprecipitate with other viral replication proteins, most notably UL9 (McLean *et al.*, 1994), suggests that UL8 may play a key role in the assembly of the replication complex prior to the initiation of viral DNA synthesis. UL9 and the SV40 T antigen both function as an origin-binding protein and DNA helicase and, like the primosome-loading function of SV40 T antigen, UL9 may interact with UL8 to load the helicase-primase complex at the origin, allowing priming of DNA synthesis.

Moreover, the ability of UL8 to stimulate primer synthesis by UL5/UL52, to work in concert with mDBP to stimulate the helicase-primase complex, and possibly to stimulate lagging strand DNA synthesis itself (Sherman *et al.*, 1992; Tenney *et al.*, 1994, 1995), suggests that the binding of UL8 to other viral replication proteins is not only important structurally for formation of the replication complex, but that UL8 also regulates functions of this complex by affecting the biochemical activities of its components.

Our current understanding of events occurrring at the eukaryotic DNA replication fork is derived primarily from studies involving the SV40 cell-free DNA replication system (Li & Kelly, 1984, 1985; Stillman & Gluzman, 1985; Wobbe *et al.*, 1985; Waga *et al.*, 1994). Based upon the very close correspondence between the mammalian cell enzyme activities required for SV40 origin-dependent DNA synthesis *in vitro* and the activities specified by the HSV replication proteins, it is thought likely that the overall mechanisms in the two systems are similar.

Figure 45a shows the current model of a eukaryotic cell DNA replication fork (Waga & Stillman, 1994) and in **Figure 45b** this model has been adapted to show how UL8 might interact with other components of the replicative machinery to coordinate leading and lagging strand synthesis in HSV-1. In both models, the lagging strand is looped around so that the lagging- and leading-strand polymerase complexes can move along the DNA in the same direction, in the direction of the replication fork.

Origin-dependent replication of the SV40 genome depends upon the host cell replicative machinery and one virally encoded gene product: the T antigen. In the presence of ATP, a double hexamer of T antigen binds to the SV40 origin and causes structural distortion of the DNA (Dean *et al.*, 1992). The cellular replication protein A (RPA), a three-subunit single-stranded DNA-binding protein, then binds, allowing more extensive unwinding of the DNA by the T antigen. The T antigen-RPA complex recruits the cellular polymerase α (pol α)/primase complex (reviewed by Stillman, 1989).

The precise nature of origins of DNA replication in higher eukaryotes is not yet clear. Whether the replication origins of animal viruses, lower eukaryotes and prokaryotes, all of which consist of well-characterised sequences that interact with specific proteins, are appropriate models for the replication origins of higher eukaryotes remains to be determined (reviewed by DePamphilis, 1993). It is clear, however, that DNA replication initiates at preferred, non-random sites within eukaryotic chromosomes and proceeds by the replication fork mechanism described for viral and prokaryotic systems. Likewise, although cellular DNA helicases have been identified in higher eukaryotes, their functions have not been defined. Therefore, for

Figure 45. Models for multi-protein complexes at DNA replication forks.

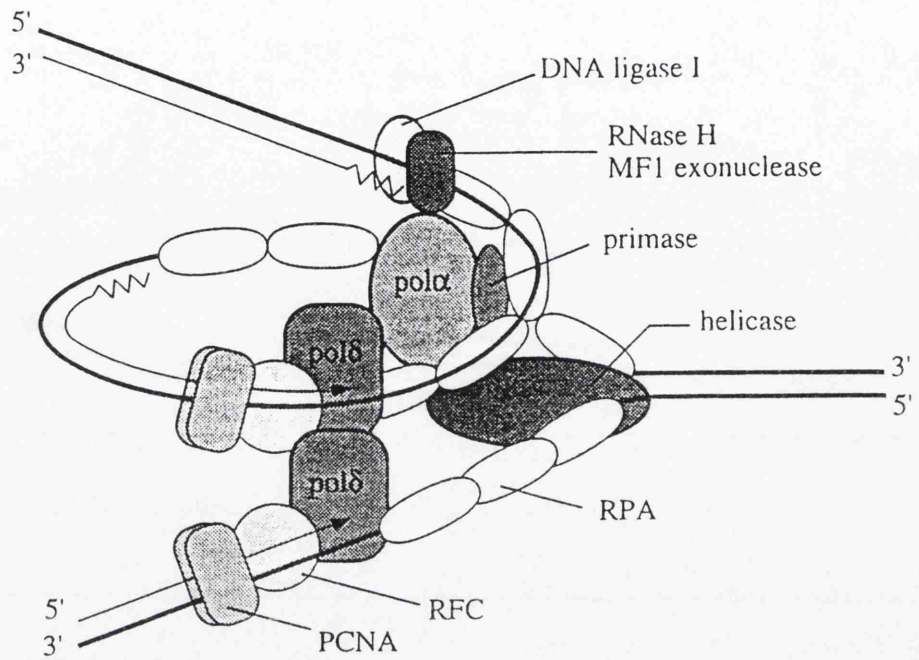
(a) Model for a multi-protein complex at a eukaryotic cell DNA replication fork. Template DNA is represented by thick lines, and newly synthesised DNA by thin lines, with arrows indicating the direction of synthesis. One molecule of polymerase-α/primase complex is about to synthesise an initiator RNA/DNA on the lagging-strand template proximal to the replication fork. Two molecules of polymerase-∂ are shown, one on the lagging-strand template is elongating the DNA strand from the initiator DNA (jagged line) previously synthesised by polymerase-α/primase, and the other on the leading strand template is continuously replicating the leading strand. During SV40 DNA replication, the DNA helicase at the fork is SV40 T antigen.

This diagram was adapted from Waga & Stillman, 1994.

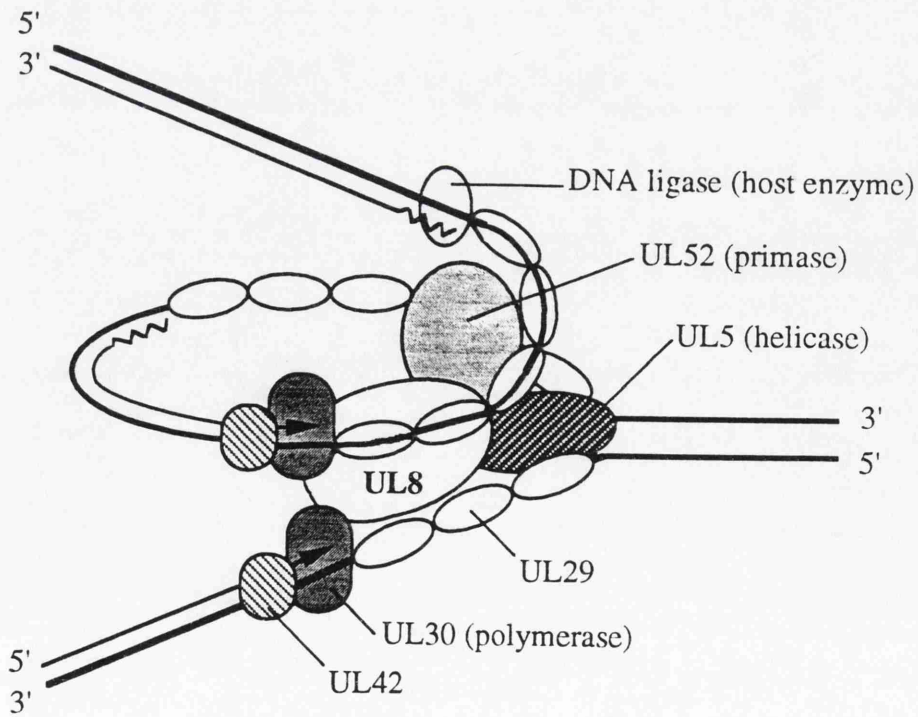
(b) Model for a multi-protein complex at a HSV-1 DNA replication fork.

The model described above was redrawn, with the cellular replication proteins (except DNA ligase) being replaced by the virally encoded replication proteins, to represent protein complexes present during HSV-1 DNA replication. In this model, the primase activity of the UL5/UL8/UL52 heterotrimer is about to synthesise an RNA primer on the lagging-strand template proximal to the replication fork. Two molecules of the heterodimeric polymerase (UL30/UL42) are shown, one on the lagging-strand template is elongating the DNA strand from the RNA primer (jagged line) previously synthesised by the UL5/UL8/UL52 complex, and the other on the leading strand template is continuously replicating the leading strand. The helicase activity at the fork is provided by the UL5/UL8UL52 complex (UL5 subunit). The RNase H/exonuclease activity required to remove RNA primers on the lagging strand is provided by the UL30 component of the HSV-1 polymerase.

(a)


5'
3'
DNA ligase I
RNase H
MF1 exonuclease
primase
polα
helicase
polδ
3'
5'
polδ
RPA
5'
3'
RFC
PCNA

(b)


5'
3'
DNA ligase (host enzyme)
UL52 (primase)
UL5 (helicase)
3'
5'
UL8
UL29
UL30 (polymerase)
5'
3'
UL42

the purposes of the model of cellular DNA replication in **Figure 45a**, it is assumed that, following initial unwinding of the DNA by an as yet undefined mechanism, a cellular helicase functionally analogous to the SV40 T antigen acts, in concert with RPA, to extend the unwound region and bind the pol α/primase complex.

The pol α/primase complex consists of a catalytic core subunit (170 kDa), two associated primase subunits (50 and 60 kDa) and an additional polypeptide (70-75 kDa) of uncertain function. Pol α achieves moderate processivity and relatively high fidelity, despite the apparent absence of an active 3'-5' exonuclease (Kornberg & Baker, 1992a). Pol α/primase synthesises an RNA primer and then extends the RNA by synthesizing a short initiator DNA (iDNA). The replication factor C (RFC) protein binds to the 3' end of iDNA and loads proliferating cell nuclear antigen (PCNA) and DNA polymerase ∂ (pol ∂) on to the template, displacing pol α/primase from the template DNA and thus preventing it from extending the iDNA further (Waga & Stillman, 1994).

This polymerase switching mechanism occurs at the initiation of leading-strand DNA replication and following priming of each Okazaki fragment by the pol α/primase, resulting in coordinated replication of both strands by two molecules of pol ∂ (Waga & Stillman, 1994). Each pol ∂ 'molecule' consists of two subunits (125 and 48 kDa), possesses an intrinsic 3'-5' exonuclease proofreading activity, and is more highly processive than pol α (Kornberg & Baker, 1992a). PCNA is a 36 kDa auxiliary protein which increases pol ∂ processivity by 40-fold, and is required for initiation of DNA replication in the cell cycle (Tan *et al.*, 1986; Bravo *et al.*, 1987).

Completion of Okazaki fragment synthesis involves removal of the RNA by RNase H and the 5'-3' exonuclease activity of MF1, synthesis of DNA by the RFC/PCNA/pol ∂ holoenzyme, and ligation of the DNA by DNA ligase I (Waga & Stillman, 1994). Topoisomerase I activity is absolutely required *in vitro* for the replication of SV40 DNA, to relieve superhelical tension ahead of the proceeding replication fork. Since the resultant SV40 daughter strands were not separated *in vitro*, it is likely that topoisomerase II activity is also required *in vivo* (Sundin & Varshavsky, 1981).

Although similar enzymatic activities are postulated to be active at the HSV and eukaryotic DNA replication forks (**Figures 45a** and **b**), there are also significant differences in the organisation of these components in the two systems, and in some of these aspects HSV-1 replication more closely resembles prokaryote organisms. Most notably, the HSV-1 primase activity is not constitutively associated with the polymerase, as in the eukaryotic pol α/primase complex, but with the helicase activity (UL5/UL8/UL52 assembly). The primase may thus resemble prokaryotic systems by cycling on and off the lagging-strand template, a process in which UL8 could potentially be involved, since UL8 regulates primase activity on the lagging strand (Sherman *et al.*, 1992; Tenney *et al.*, 1994). There is also no evidence for a polymerase switching mechanism in HSV-1 and there is no equivalent of pol α synthesising an initiator DNA. In addition to synthesising long DNA strands, the HSV-1 polymerase holoenzyme (UL30/UL42) is able to directly extend the RNA primer synthesised by the UL5/UL8/UL52 complex. UL30/UL42 nevertheless also resembles pol ∂ in being highly processive and possessing a 3'-5' exonuclease for proof-reading.

Although it is not clear whether UL8 interacts with UL30 on both the leading and lagging strands, such an interaction might provide a mechanism for the coordination of HSV-1 leading- and lagging- strand DNA synthesis. It might also be the case that interaction between UL8 and UL30 on the lagging strand prevents dissociation of the lagging strand polymerase from the replication complex at the end of synthesising each Okazaki fragment. This would provide a mechanism to explain why the observed stimulation by UL8 of lagging strand synthesis is greater than its stimulatory effect on primer synthesis (Tenney *et al.*, 1994). Evidence for the importance of specific protein-protein interactions in co-ordinating synthesis of the two strands at a replication fork is provided by the observation that although bacteriophage T4 DNA polymerase can substitute for the pol ∂ complex during *in vitro* leading strand synthesis, it is unable to perform coordinated leading- and lagging-strand synthesis (Waga & Stillman, 1994).

Figure 45b thus illustrates how UL8 might interact with UL5 to regulate helicase activity, with UL52 to regulate primase activity on the lagging strand, and

with UL30 on one or both strands to regulate lagging strand synthesis and/or co-ordinate leading and lagging strand DNA synthesis.

There is no requirement in the HSV system for the specific RNase H and 5'-3' exonuclease MF1 proteins, since this activity is provided by the UL30 component of the HSV-1 polymerase complex. In this respect the HSV-1 polymerase resembles the *E. coli* polymerase I. Upon reaching the RNA primer of the previous Okazaki fragment, the RNAse H activity of UL30 would presumably remove the primer, permitting the polymerase to fill in the gap. DNA ligase activity, necessary to link the Okazaki fragments together, is probably provided by a cellular component.

It is, however, important to stress that more experimental data is needed before it is known how accurately this model reflects the actual events at the HSV-1 replication fork. In particular several aspects will only become testable once a cell-free system for HSV-1 origin-dependent DNA synthesis is developed.

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