Use of a cosmid recombination system in mutational analysis of herpes simplex virus type 1 genes UL14 to UL17

by

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SUMMARY

The HSV-1 genome is 152 kbp in length and contains at least 74 distinct genes. About half of the genes are individually not essential for viral growth in cell culture but most appear to be required for efficient viral replication and pathogenesis in experimental animal systems. The availability of the complete DNA sequence of HSV-1 since 1988 has made it possible to target any gene for mutation, including those that had been previously inaccessible to conventional genetic analysis.

A cosmid-based recombination system for producing HSV-1 mutants has been developed recently. The inserts from a set of five cosmids, each about 40 kbp in size, which together contain the entire HSV-1 genome, are co-transfected into BHK C13 cells to yield *wt* virus *via* recombination processes. One cosmid containing a gene of interest can be mutated, and replacement of the *wt* cosmid in the set by the mutated cosmid results in mutant progeny after co-transfection. This approach has a number of advantages over traditional methods of mutagenesis, particularly in allowing minimal alterations to be introduced and in permitting the generation of mutants in the absence of a *wt* background.

The aim of this project was to expand the use the cosmid-based system of mutagenesis to analyse HSV-1 genes UL14, UL15, UL16 and UL17 and to investigate the phenotypes of mutants obtained.

The technique used takes advantage of the observation that digestion of covalently closed DNA molecules in the presence of ethidium bromide by restriction enzymes with multiple recognition sites preferentially yields full length linear molecules after a single site is cleaved. An appropriate cosmid (cos24) was linearised with the restriction enzyme *Asp*718, which cleaves at the sequence G'GTACC, leaving a 5' overhang. Treatment with T4 DNA polymerase in the presence of the four deoxynucleotide triphosphates, followed by religation, resulted in a 4 bp insertion, thus altering the reading frame of the gene from that point onwards. *Asp*718 has seven sites in cos24, and mutant cosmids were made with a 4 bp insertion in UL14, UL15 and UL17, in addition to UL6 and UL7.

Since these genes may be essential in cell culture, a number of candidate complementing cell lines were produced by three different strategies. These were generated using either cos24 (containing genes UL1 to UL21), a plasmid containing genes UL14 to UL18, or

plasmids containing UL14, UL15 or UL17 individually. Overall, 122 candidate cell lines were produced, and 40 were tested to see if they could complement the mutation by transfection with the appropriate mutant cosmid sets. BHK C13 cells were also transfected. Following transfection, all of the progeny generated from these cell lines and BHK C13 cells were *wt* revertants, presumably owing to loss of the 4 bp insertion by intramolecular recombination. This indicates that the cell lines tested are not able to complement the defects and that UL14, UL15, and UL17 are essential for viral replication in cell culture.

Proteins whose sequences are present in databases may be identified by newly developed techniques involving mass spectrometry. Proteins are separated by SDS-PAGE and electroblotted onto a membrane, then proteins of interest are excised and treated with trypsin. The peptides produced are subjected to laser desorption mass spectrometry, and the resulting masses from the spectrum are compared to the predicted tryptic products from proteins in a database. Using this method, evidence was obtained that the protein products of UL17 and UL14 may be present in small amounts in HSV-1 virions.

Two independent UL16$^-$ viruses were available in the laboratory at the commencement of this work, and a further four independent UL16$^-$ mutants were also produced. These six mutants were characterised in cell culture and the effects of the lesions on neurovirulence and latency were investigated using a mouse model.

The mutants produce smaller plaques on BHK C13 cells and yield less virus than *wt* and a revertant. The small plaque size and low yield were dependent on the particular site of the mutation within UL16. The mutants were able to adsorb as efficiently as *wt*, but were slightly reduced in their ability to bind and penetrate cell, they were not significantly temperature sensitive or cell type-specific. Particle counts by electron microscopy revealed that they have a high particle to plaque forming unit (pfu) ratio (i.e. they produce the same amount of particles but fewer are infectious).

Analysis of the mutants was carried out using a mouse model. After inoculation of virus into the cranium, *wt* virus produced from the parental cosmid set was fully neurovirulent but the UL16$^-$ viruses were less virulent than *wt* (i.e. they killed fewer animals at each dose). Footpad inoculations were carried out to investigate the ability of the mutants to replicate at the periphery and to establish and reactivate from latency. The mutants were able to establish and reactivate from latent infection, but less efficiently than *wt.*

In conclusion, although UL16 mutants are viable in cell culture they have a high particle to pfu ratio, yield less virus than *wt* and produce smaller plaques. The results from experiments involving infection of mice indicate that the UL16 protein has a role in neurovirulence and latency.

CONTENTS

Chapter 3 - Methods

Abbreviations

A	adenine
ACV	acyclovir
AIDS	acquired immune deficiency syndrome
APS	ammonium persulphate
ATP	adenosine triphosphate
Ara A	arabinoside A
BPB	bromophenol blue
bp	base pairs
BSA	bovine serum albumin
BUdR	5-bromodeoxyuridine
C	cytosine
cAMP	cyclic adenosine monophosphate
CAV	cell-associated virus
cDNA	complementary DNA
Ci	Curie
CIP	calf intestinal phosphatase
cpe	cytopathic effect
cpm	counts per minute
cos	cosmid
CRV	cell-released virus
Da	daltons
°	degrees Celsius
DATD	N,N'-diallyltartardiamide
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
ddATP	2'3'-dideoxyadenosine-triphosphate
ddCTP	2'3'-dideoxycytidine-triphosphate
ddGTP	2'3'-dideoxyguanosine-triphosphate
ddTTP	2'3'-dideoxythymidine-triphosphate
DE	delayed early
del	deletion
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxynucleotide triphosphates
DR	direct repeat
DRG	dorsal root ganglion
DTT	dithiothreitol
dUTPase	deoxyuridine triphosphate nucleotidohydrolase
E	early
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
EM	electron microscopy
EtBr	ethidium bromide
EtOH	ethanol
FCS	foetal calf serum
FP	foot pad
G	guanine

g	gram
gp	glycoprotein
h	hour
HCF	host cell factor
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid
HRP	horseradish peroxidase
hsp	heat shock protein
IC	intracranial
ICP	infected cell polypeptide
IE	immediate early
IFN	interferon
IP	intraperitoneal
Ig	immunoglobulin
IPTG	isopropyl-β-D-thiogalactoside
IR_L	internal long repeat
IR_S	internal short repeal
IU	intraurethral
IV	intravaginal
k	kilo (i.e. 10^3)
kbp	kilobase pair
L	late
LL	leaky late
L-particle	light particle
l	litre
LAT	latency-associated transcript
M	molar
mg	milligram
min	minute
ml	millilitre
mM	millimolar
moi	multiplicity of infection
Mr	relative mobility
mRNA	messenger RNA
µCi	microcurie
µg	microgram
µM	micromolar
NBCS	new born calf serum
neo	neomycin (G418)
NGF	nerve growth factor
NPT	non-permissive temperature
NP40	Nonidet P40
OBP	origin-binding protein
Oct	octamer-binding protein
OD	optical density
ORF	open reading frame
ori_L	origin of replication in U_L
ori_S	origin of replication in R_S
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming unit
pi	post-infection

PI	pre-immume
PMSF	phenylmethylsulphonylfluoride
poly (A)	polyadenylic acid
PT	permisive temperature
PVP	polyvinylpyrrolidone
R	purine base
Rl	large subunit of ribonucleotide reductase
R2	small subunit of ribonucleotide reductase
RE	restriction enzyme
RNA	ribonucleic acid
RNase A	ribonuclease A
rpm	revolutions per minule
RR	ribonucleotide-reductase
RT	room temperature
SDS	sodium dodecyl sulphate
snRNP	small nuclear ribonucleoprotein particle
ssb	single-stranded DNA binding protein
syn	syncitial
T	thymidine
TBF	TATA box-binding factor
TCA	trichloroacetic acid
TEMED	N, N, N', N' -tetramethlyethylene diamine
TFA	trifluoroacetic acid
TFIIB	transcription factor IIB
TG	trigeminal ganglion
TIF	transinducing factor
Tris	Tris (hydroxymethyl) aminomethane
TK	thymidine kinase
TR_L	long terminal repeat
TR_S	short terminal repeat
ts	temperature sensitive
Tween 20	polyoxyetheylene sorbitan monolaurate
U	unique
U_L	long unique
U_S	short unique
UV	ultraviolet
V	volts
vhs	virion host shutoff
Vmw	molecular weight in kDa of HSV-induced polypeptides
vol	volume
VP	virion protein
v/v	volume/volume
wt	wild type
w/v	weight/volume
w/w	weight/weight
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Cell lines

BHK C13	baby hamster kidney cells clone 13
BSC-1	monkey epithelial cell line
CV1	monkey fibroblastoid cell line
HeLa	human epithelial cell line
HFL cells	human foetal lung cell line
MDCK	canine epithelial cell line
MeWo	human fibroblastoid cell line
Vero cells	African green monkey kidney cell line

Herpesviruses

Virus name	Common abbreviation	ICTV designation
Herpes simplex virus type 1	HSV-1	HHV-1
Herpes simplex virus type 2	HSV-2	HHV-2
Varicella-zoster virus	VZV	HHV-3
Epstein-Barr virus	EBV	HHV-4
Human cytomegalovirus	HCMV	HHV-5
Human herpesvirus 6	HHV-6	HHV-6
Human herpesvirus 7	HHV-7	HHV-7
Kaposi's sarcoma-associated herpesvirus	KSHV	(HHV-8)
Equine herpesvirus 1	EHV-1	EHV-1
Equine herpesvirus 2	EHV-2	EHV-2
Herpesvirus saimiri	HVS	SHV-2
Channel catfish virus	CCV	IHV-1
Simian herpesvirus	B virus	CHV-1
Bovine herpesvirus 2	BHV-2	BHV-2
Pseudorabies virus	PRV	SHV-1
Cottontail rabbit herpesvirus	LHV-1	LHV-1
Marek's disease virus	MDV	GHV-2
Murine cytomegalovirus	MCMV	MHV-1[a]
Herpesvirus ateles	HVA	AHV-2
Herpesvirus of turkey	HVT	MHV-1[b]

[a] Murid herpesvirus 1. [b] Meleagrid herpesvirus 1.

One and three letter abbreviations for amino acid residues

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

List of Figures and Tables

[a] ap=after the page number shown; fp=facing the page number shown

Chapter 1

Introduction

1.1. THE FAMILY HERPESVIRIDAE

1.1.1. General properties

The Herpesviridae is a large family of eukaryotic viruses. To date over 100 have been identified, infecting vertebrate hosts ranging from fish to man (Roizman and Sears, 1993) and probably at least one invertebrate host, the oyster (Comps and Cochennec, 1993). The viruses exhibit a high degree of host specificity and a wide range of pathological properties. A single host species can be host to several herpesviruses - for example, there are eight herpesviruses associated with humans. Most herpesviruses have been discovered in husbanded animals where large groups live in close contact.

Herpesviruses are defined by the structural features of their virions. A large linear double-stranded DNA molecule is located within an icosahedral capsid approximately 100-110 nm in diameter. The capsid is embedded in an amorphous proteinaceous matrix (the tegument), which is in turn surrounded by a lipid envelope containing viral glycoproteins (Fig. 1). During productive infection, viral DNA is transcribed, replicated and packaged into capsids in the nucleus. The capsids then acquire tegument and envelope prior to leaving the infected cell. In addition, all herpesviruses examined to date have the ability to establish latent infections in their natural host, and subsequent reactivation of virus can occur to cause a second round or even recurrent rounds of recrudescent disease.

1.1.2. Biological properties

The herpesviruses are versatile and well adapted pathogens, causing diseases whose pathology may range from inapparent to life-threatening.

It is common for primary or recrudescent infections to be inapparent or slight in the natural setting. Under certain conditions, however, particularly those resulting in a degree of immune suppression, herpesviruses can cause serious or even fatal disease. For example, 25% of mortality in bone marrow recipients results from interstitial pneumonitis caused by HCMV infection (White and Fenner, 1986).

Several herpesviruses have been implicated in various types of cancer. For example, MDV causes a T-cell lymphoma in chickens (Fenner *et al.*, 1987), and EBV is implicated in Burkitt's lymphoma and nasopharyngeal carcinoma in humans (Epstein *et al.*, 1964; Evens and Niederman, 1991).

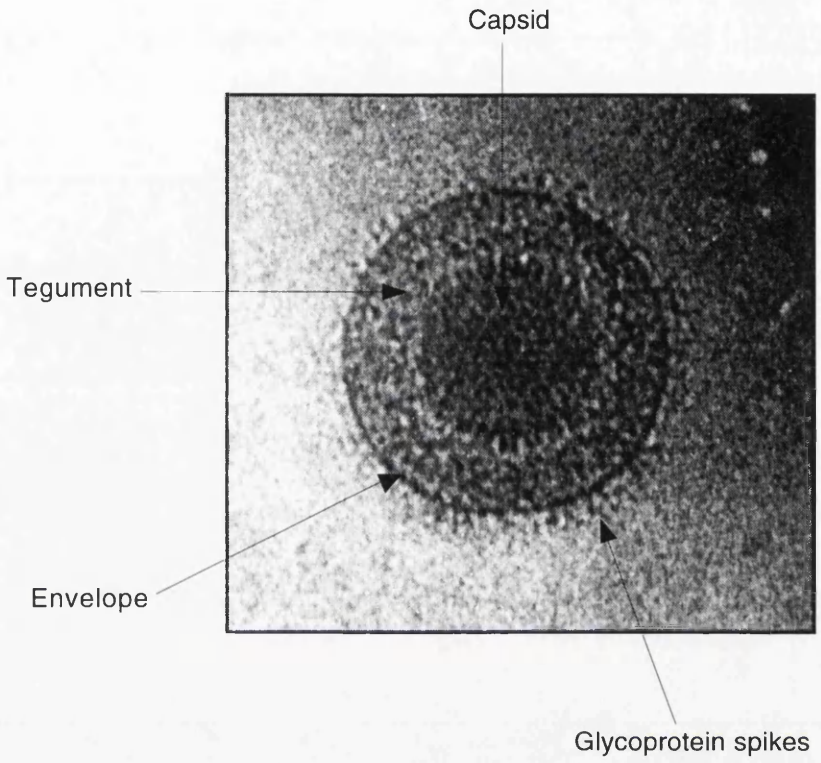


Fig. 1. HSV-1 virion structure

Electron cryomicrograph of an HSV-1 virion. Three distinct substructures can be seen; the capsid (including the core), the surrouning tegument and the outer lipid envelope which contains glycoprotein spikes (magnification is approximately 230,000). Original photograph provided by F.J. Rixon.

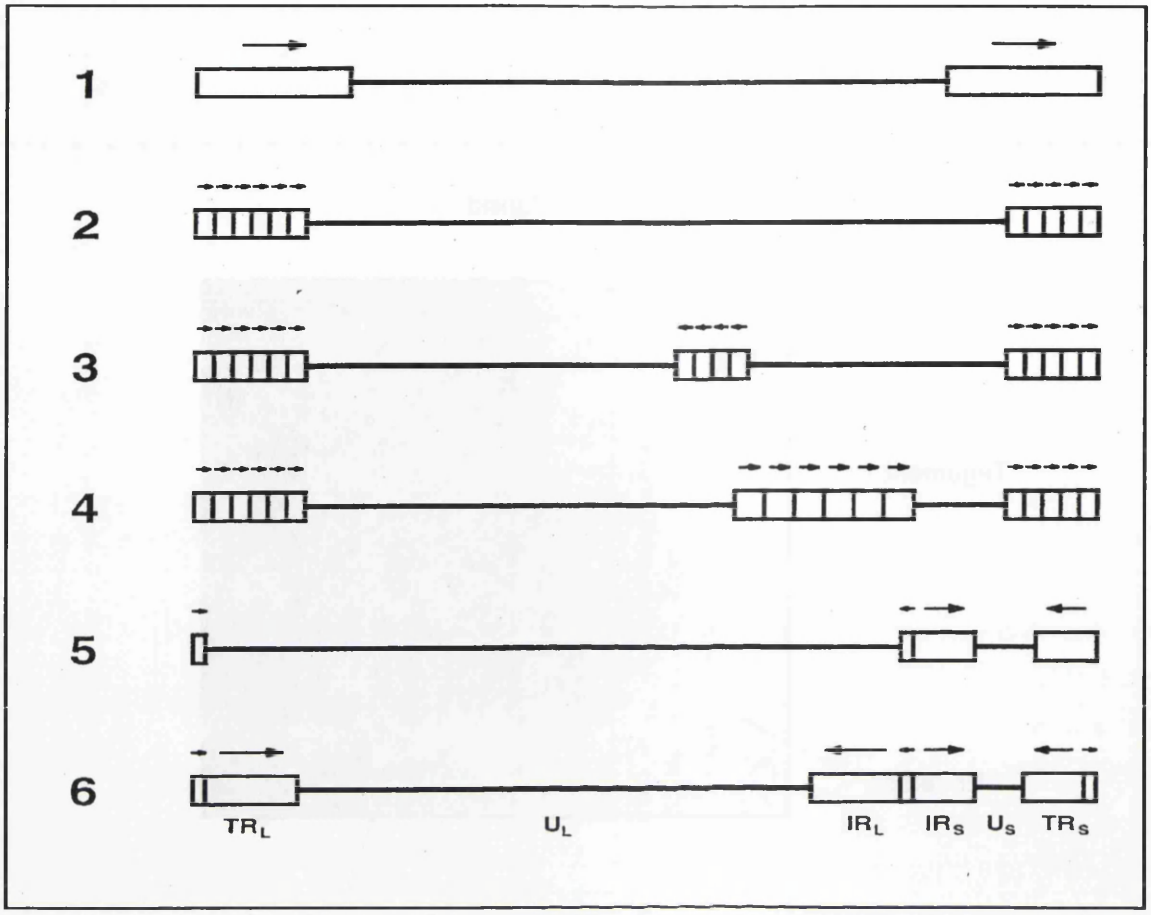


Fig. 2. Types of herpesvirus genome structure
The six types of herpesvirus genome structure are shown (See Table 1 for description and examples). The genomes are not drawn to scale. Unique and repeat regions are shown as horizontal lines and rectangles, respectively. The orientations of repeats are shown by arrows. The nomenclature used to designate regions of the HSV-1 genome is shown at the foot of the figure. Reproduced from Davison and Clements, 1996.

Table 1. Herpesvirus genome structures

Class	Description	Isomers	Example
1	Single direct repeat at termini.	1	CCV; HHV-6; MCMV
2	Multiple direct repeats of a short sequence at the termini in variable copy number.	1	HVS
3	As 2, with a variable number of the terminal repeats in inverse orientation internally.	4	LHV-1
4	As 2, with multiple direct repeats of a different sequence in variable copy number internally.	1	EBV
5	Unique regions U_S and U_L flanked by inverted repeats TR_L/IR_L and TR_S/IR_S.	2 major, 2 minor or 2 only	PRV;VZV; EHV-1
6	Unique regions U_S and U_L flanked by inverted repeats TR_L/IR_L and TR_S/IR_S. Short sequence (*a* sequence) repeated directly at the termini and indirectly at the junction between IR_L and IR_S.	4 equimolar	HSV-1; HSV-2; HCMV

Although herpesviruses normally infect a single species, some can cause more severe disease in non-natural hosts. For example, B virus, which is related to HSV-1, infects monkeys and has similar pathological effects to HSV-1 infection in humans, but causes fatal ascending paralysis and encephalitis in humans (Fenner *et al.*, 1987). Also, PRV, a herpesvirus of swine, can infect cattle, sheep, goats, dogs and cats. Infection of cattle results in "mad itch", and death can occur a few hours after symptoms appear. PRV-infected dogs show many classic symptoms of rabies, and infection is invariably fatal (Fenner *et al.*, 1987).

1.1.3. Genome features

Herpesviruses genomes range in size from 125-250 kbp and contain 70 to 200 genes (Roizman and Sears, 1990, 1993). All genomes investigated in sufficient detail contain direct or inverted repeat sequences located internally or at the genome termini. The six main types of genome structures, differing in the size and relative arrangement of repeated and unique sequences, are shown in Fig. 2 and Table 1.

Herpesvirus genomes range in base composition from 31-75% G+C (Roizman and Batterson, 1985; McGeoch, 1989; Roizman and Sears, 1993) and G+C residues may be distributed non-uniformly throughout the genome: for example, the terminal repeats in HVS have a much higher G+C content than the unique region. Table 2 summarises the features of the ten herpesvirus genomes whose complete sequences have been published.

1.1.4. Classification and evolution

Classification of herpesviruses into three subfamilies, the *Alpha-*, *Beta* and *Gamma-herpesvirinae,* by the International Committee on Taxonomy of Viruses (ICTV) is based on biological criteria (Roizman *et al.*, 1981). The subfamilies have been divided further on the basis of additional biological and genetic data (Roizman *et al.*, 1992). Members of the *Alphaherpesvirinae* are characterised by a short replicative cycle, which results in the rapid spread of infection in cell culture and the mass destruction of susceptible cells. They tend to have a wide host range in cell culture, and most form latent infections in sensory ganglia. They are subdivided into the Simplexvirus and Varicellovirus genera, the former including HSV-1, HSV-2 and BHV-2, and the latter VZV, EHV-1 and PRV. The *Betaherpesvirinae* have a long replicative cycle, a narrow host range in cell culture, and can establish latent infections in secretory glands, lymphoreticular cells and kidney cells. Infection results in the production of characteristically enlarged infected cells (cytomegalia). They are divided into three genera: Cytomegalovirus, Muromegalovirus and Roseolovirus, exemplified by HCMV,

Table 2. Genetic content of the fully sequenced herpesviruses

Virus	ICTV designation	Subfamily	Genus	Genetic group	Genome structure	Size (kbp)	G+C %	References
HSV-1	HHV-1	*Alphaherpesvirinae*	Simplexvirus	α_1	6	152	67	McGeoch *et al.*, 1988.
VZV	HHV-3	*Alphaherpesvirinae*	Varicellovirus	α_2	5	125	46	Davison and Scott, 1986.
EHV-1	EHV-1	*Alphaherpesvirinae*	Varicellovirus	α_2	5	150	57	Telford *et al.*, 1992
HCMV	HHV-5	*Betaherpesvirinae*	Cytomegalovirus	β_1	6	230[a]	57	Chee *et al.*, 1990.
HHV-6	HHV-6	*Betaherpesvirinae*	Roseolovirus	β_2	1	160	43	Gompels *et al.*, 1995.
EBV	HHV-4	*Gammaherpesvirinae*	Lymphocryptovirus	γ_1	4	172[b]	60	Baer *et al.*, 1984.
HVS	SHV-2	*Gammaherpesvirinae*	Rhadinovirus	γ_2	2	155[c]	46	Albrecht *et al.*, 1992.
EHV-2	EHV-2	*Gammaherpesvirinae*	Rhadinovirus	γ_2	1	184	57	Telford *et al.*, 1995.
CCV	IHV-1	None	None	None	1	134	56	Davison, 1992

[a] Laboratory isolate, clinical isolates are 13 kbp larger (Quinnan *et al.*, 1983; Kemble *et al.*, 1996).

[b] Repeat elements are present in variable number. Deletion of 12 kbp relative to other EBV strains (Parker *et al.*, 1990).

[c] Repeat elements are present in variable number.

Table 3. Mis-classified viruses

Virus	Biological classification	Genetic classification	Reference
HHV-6	*Gammaherpesvirinae*	*Betaherpesvirinae*	Lawrence *et al.*, 1990
MDV	*Gammaherpesvirinae*	*Alphaherpesvirinae*	Buckmaster *et al.*, 1988
HVT	*Gammaherpesvirinae*	*Alphaherpesvirinae*	Buckmaster *et al.*, 1988
EHV-2	*Betaherpesvirinae*	*Gammaherpesvirinae*	Telford *et al.*, 1993
CCV	*Alphaherpesvirinae*	None	Davison, 1992

MCMV and HHV-6, respectively. The *Gammaherpesvirinae* exhibit a restricted host range and the duration of the reproductive cycle is variable. Latent infection often occurs in lymphoid tissue. The Lymphocryptovirus genus includes EBV which shows specificity for B-lymphocytes, and the Rhadinovirus genus includes HVA and HVS, which are restricted to New World primates and grow in T-lymphocytes.

For practical purposes, the use of biological criteria in herpesvirus classification has been superseded by genetic data derived from sequence analysis. This involves consideration of similarities between encoded proteins and the gene order, and has been facilitated by the availability of complete DNA sequences for several herpesviruses (see Table 2) and substantial incomplete sequence information for many other herpesviruses. Genetic data largely support the biological classification scheme, and relatively few viruses have been wrongly classified. There are five instances where a virus which has similar biological properties to members of one subfamily is most closely related genetically to another subfamily (Table 3). Three of these five viruses were initially classified as members of the *Gammaherpesvirinae* because they are lymphotropic.

Genetic data provide an insight into viral evolution. Herpesviruses are highly divergent, and it is clear that evolution of the herpesviruses has proceeded through mutational and recombinational processes. The latter category involves large scale genomic rearrangements, gene duplications, generation of repeated sequence elements and changes in the extent and copy number of these elements. Gene deletion and importation of non-herpesvirus genes also appears to have occurred (McGeoch, 1989; McGeoch and Cook, 1994).

A common evolutionary origin for the herpesviruses is strongly supported by the observation that the three subfamilies contain a subset of about 40 "core" genes that are conserved by criteria of similarities in encoded amino acid sequences or genomic position (McGeoch, 1989; Davison, 1993). McGeoch and Cook (1994) and McGeoch *et al.* (1995) presented data consistent with the idea that herpesviruses have evolved with their hosts, and have proposed an evolutionary timescale (Fig. 3). CCV, however, lacks specific sequence similarities to mammalian and avian herpesviruses, but recent data on capsid structure (Booy *et al.*, 1996) and virion protein composition (Davison and Davison, 1995) support the view that mammalian, avian and fish herpesviruses share a common evolutionary source.

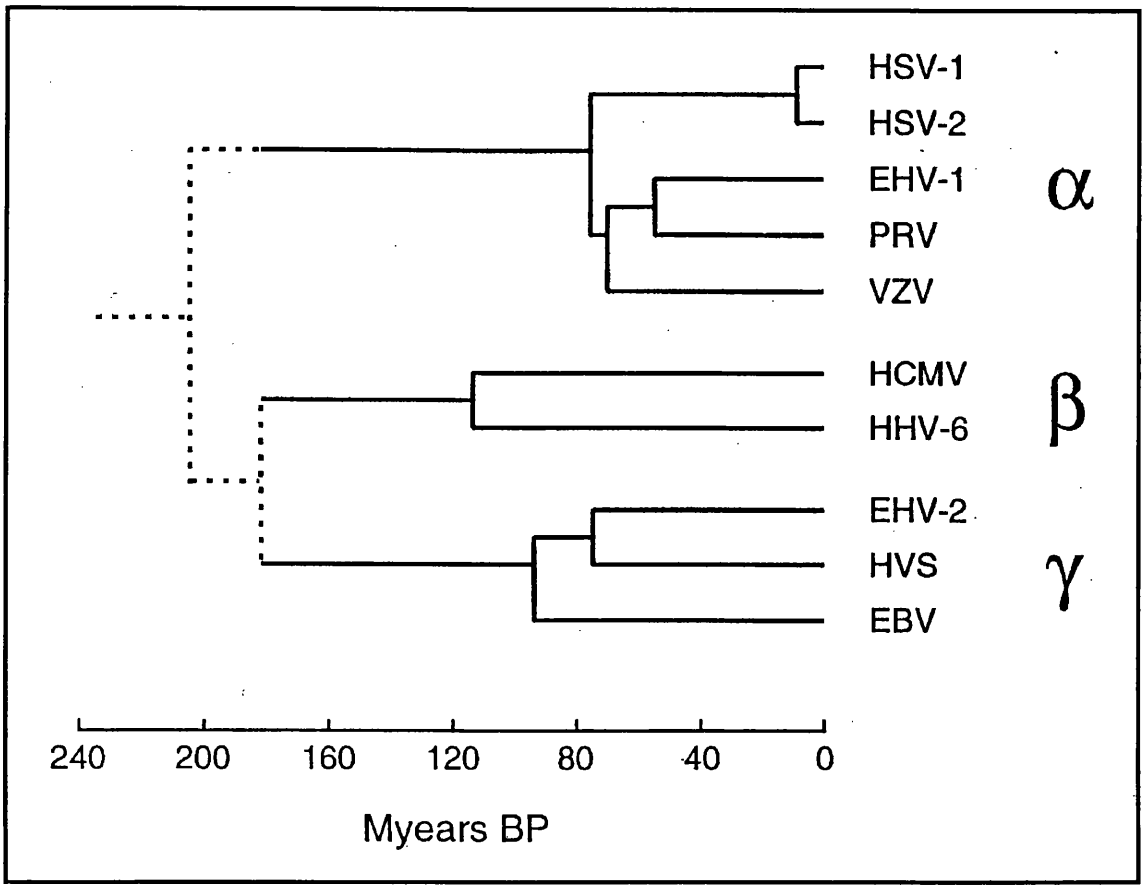


Fig. 3. Phylogenetic tree

A phylogenetic tree for the herpesviruses derived from sequence comparisons, with a time scale (millions of years before present) based on the hypothesis that the viruses have cospeciated with their hosts. Broken lines indicate regions of lower confidence. Reproduced from McGeoch *et al.* (1995).

Table 4. Human herpesviruses

Virus	ICTV Designation	Subfamily	Genus	Genetic group	Genome type	Size (kbp)	G+C %
HSV-1	HHV-1	*Alphaherpesvirinae*	Simplexvirus	α_1	6	152259	67
HSV-2	HHV-2	*Alphaherpesvirinae*	Simplexvirus	α_1	6	~ 154000	69
VZV	HHV-3	*Alphaherpesvirinae*	Varicellovirus	α_2	5	124884	46
EBV	HHV-4	*Gammaherpesvirinae*	Lymphocryptovirus	γ_1	4	172282[a]	60
HCMV	HHV-5	*Betaherpesvirinae*	Cytomegalovirus	β_1	6	229354[b]	57
HHV-6	HHV-6	*Betaherpesvirinae*	Roseolovirus	β_2	1	159321	43
HHV-7	HHV-7	*Betaherpesvirinae*	Roseolovirus	β_2	1	144861	40
KSHV	HHV-8	*Gammaherpesvirinae*	Not yet designated	γ_2	Not known	Not known	Not known

[a] Repeat elements are present in variable numbers. The sequenced strain has a deletion of 12 kbp relative to other strains (Parker *et al.*, 1990).

[b] Laboratory isolate: Clinical isolates are 13 kbp larger (Quinnan *et al.*, 1983; Kemble *et al.*, 1996).

1.2. HUMAN HERPESVIRUSES

1.2.1. Biological properties

Features of the eight different herpesviruses that have been recognised in humans are summarised in Table 4.

Primary infection with HSV-1 is usually asymptomatic, and infection is virtually universal in human populations. In rare circumstances, however, severe primary symptoms can occur, including conjunctivitis, keratitis and acute necrotizing encephalitis. A lifelong latent infection is established in neurons of the sensory ganglia and reactivation can be periodically induced following a number of stimuli including stress, exposure to UV light and immune suppression (Hill *et al.*, 1978), giving rise to oro-facial lesions. HSV-2 is transmitted sexually, and causes genital lesions on reactivation, but the clinical pathologies associated with HSV-1 and HSV-2 are not strictly separated (Nahmias *et al.*, 1991). HSV-2 reactivation usually occurs more frequently than that of HSV-1 (Timbury, 1991; Whitley, 1990).

VZV produces two different diseases: varicella (chickenpox) upon primary infection and zoster (shingles) after reactivation (Gelb, 1990). Chickenpox is a generally benign disease characterised by fever and a vesicular rash, and is seasonally distributed, the highest incidence occurring in late winter and early spring. The virus is transmitted by inhalation of infectious respiratory secretions. Complications are rare, but the disease may be more severe in adults, where pneumonia is common. Latency is normally established in the dorsal root or cranial nerve ganglia, and reactivation, which usually occurs many years after primary infection, results in painful vesicles in the area of skin innervated by the affected ganglion. The incidence of reactivation increases with age (Timbury, 1991).

HCMV infection is normally symptomless, but infection of the foetus *via* the mother can cause deafness and mental retardation. HCMV is one of the commonest opportunistic infections of immunocompromised individuals, and is the major viral complication in bone marrow and organ transplantation, where the virus can be acquired from the donor organ and result in retinitis and pneumonia (Timbury, 1991). The virus is thought to be spread by salivary contact but may be transmitted sexually (Emery and Griffith, 1990). Unlike latent infections established by HSV-1 and VZV, those of HCMV are more like chronic infections because virus can be readily recovered from saliva, urine, semen or cervical secretions for long periods of time. The intermittent nature of virus shedding and fluctuations in antibody levels suggest that

asymptomatic reactivation occurs throughout life. HCMV establishes latency in lymphocytes or polymorphonuclear leukocytes (Ho, 1991).

EBV was initially detected in lymphoblasts from Burkitt's lymphoma (Epstein, 1964), a highly malignant B-cell lymphoma common in African children which shows a striking geographical distribution and is virtually confined to areas where there is endemic malaria. EBV was later found to be the causative agent of infectious mononucleosis (glandular fever), particularly in young adults, and in the immunocompromised individual EBV can result in severe lymphoproliferative disease where organs are infiltrated by immature B lymphocytes. EBV has also been associated with nasopharyngeal carcinoma, since viral DNA has been isolated from malignant epithelial cells of the tumour. This tumour also shows a marked geographical and racial distribution and it is particularly common among southern Chinese people. The selective distribution of these tumours suggests the influence of genetic and environmental factors as well as EBV infection in the progression towards disease (Miller, 1990; Timbury, 1991).

HHV-6 was initially isolated from the peripheral blood leukocytes of AIDS patients and patients with lymphoproliferative disorders (Salahuddin *et al.*, 1986). It grows in B and T cells *in vitro*. Infection is widespread and there is a high incidence of antibody in the normal population. Infection is thought to occur early in life *via* saliva (Harnett *et al.*, 1990). The virus is the causative agent of exanthema subitum (roseola infantum), which is characterised by fever and a mild facial rash in young children (Yamanishi *et al.*, 1988). The virus is genetically related to HCMV (Lawrence *et al.*, 1990) and consists of two closely related genotypes, HHV-6 A and B, of which only the latter has been associated with roseola (Dewhurst *et al.*, 1993).

HHV-7 was isolated from $CD4^+$ T cells of healthy individuals by Frenkel *et al.* (1990). Infection is widespread and is acquired early in childhood. HHV-7 is related to HHV-6 (Berneman *et al.*, 1992), and is also associated with roseola (Portolani *et al.*, 1995).

KSHV (HHV-8) was identified by Chang *et al.* (1994) as a new herpesvirus associated with Kaposi's sarcoma. The DNA from this virus has also been recovered from rare body cavity-based lymphomas (Cesarman *et al.*, 1995).

1.2.2. Genome structures

The genome structures of the human herpesviruses are shown in Fig. 4. They represent four of the six types of genome structure previously described in Section 1.1.3. The structure of the KSHV genome is unknown.

1.3. THE GENOME OF HSV-1

1.3.1. General properties

The genome of HSV-1 strain 17 is composed of a linear molecule of double-stranded DNA containing 152,260 bp (McGeoch *et al.*, 1985; 1986; 1988). The DNA is not methylated (Low *et al.*, 1969) and has an average base pair composition of 68.3% G+C, although the G+C content is not uniform throughout the genome, being higher in the repeat regions. There is a single unpaired base at each 3' terminus (Mocarski and Roizman, 1982), and there is no protein covalently linked to the termini.

1.3.2. Genome structure

The HSV-1 genome consists of two covalently linked components, L and S (Sheldrick and Berthelot, 1974; Delius and Clements, 1976; Roizman, 1979). The L and S components consist largely of unique sequences, U_L (107.9 kbp) and U_S (13 kbp), each flanked by inverted repeats, R_L (9 kbp) and R_S (6.5 kbp) (McGeoch *et al.*, 1988). The sequences of R_L and R_S are unrelated with the exception of a direct repeat (the *a* sequence) of approximately 400 bp located at the termini (see Fig. 4). One copy of the *a* sequence is present at the S terminus and although one copy is usually present at the L terminus, some molecules contain more. The *a* sequence is also present at the L-S joint, usually as a single copy but sometimes as more (Wagner and Summers, 1978). Recombination between the repeat sequences results in inversion of U_L and U_S (Sheldrick and Berthelot, 1974), and DNA extracted from virions consists of four equimolar populations differing with respect to the relative orientations of the two components (Hayward *et al.*, 1975). Inversion is aided by the presence of the *a* sequences (Davison and Wilkie, 1983; Poffenberger *et al.*, 1983).

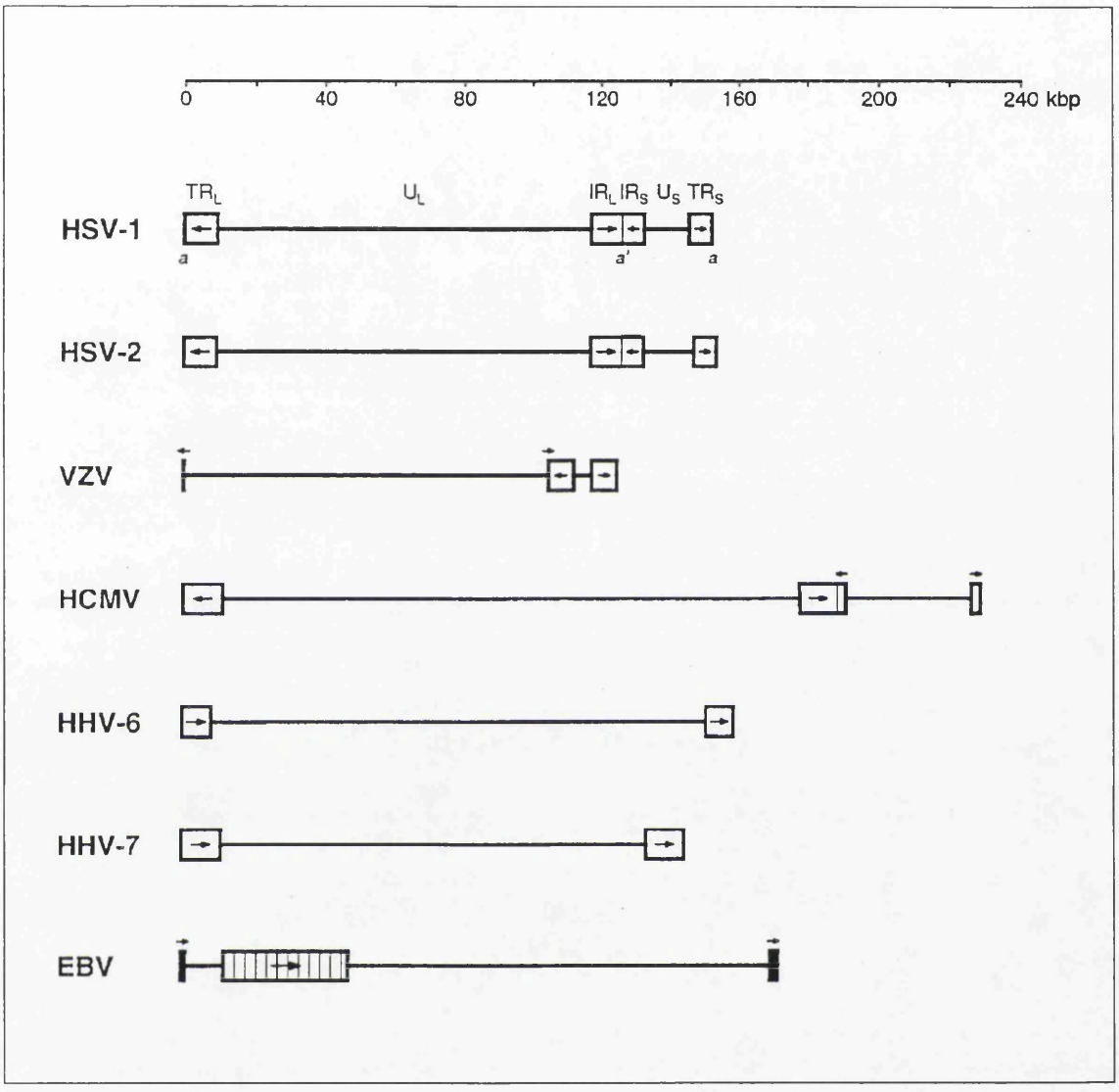


Fig. 4. Human herpesvirus genome structures

Sizes and structures of the human herpesvirus genomes are shown to scale. Unique and repeat regions are shown as horizontal lines and rectangles, respectively. The orientations of repeats are shown by arrows. The nomenclature used to designate regions of the HSV-1 genome is shown at the top of the figure. Reproduced from Davison (1993).

1.3.3. Genetic content

Seventy-one genes were predicted to be present in the HSV-1 genome by McGeoch *et al.* (1985, 1986, 1988). Their arrangement is shown in Fig. 5. This was acknowledged to be a conservative estimate since genes which are highly spliced, completely or almost completely contained within another gene or very small might have escaped identification. Indeed, an additional four genes have been identified: UL26.5, UL49A (or UL49.5), US8.5 and RL1. UL26.5 is contained completely within the UL26 ORF (Liu and Roizman, 1991a,b), and encodes a protein which corresponds to the C-terminal region of the UL26 protein. UL49A encodes a predicted protein translation product of only 91 residues (Barker and Roizman, 1992; Barnett *et al.*, 1992). US8.5 is also a small gene which partially overlaps US8 and US9 (Georgopoulou *et al.*, 1993; 1995). Despite a large number of sequence errors, Chou and Roizman (1990) proposed a gene ($\alpha_1$34.5, encoding ICP34.5) in R_L in HSV-1 strain F. The corresponding gene in HSV-1 strain 17, RL1, was later identified after correction of two errors in the published sequence (McGeoch and Barnett, 1991).

Thus, the genome is proposed at present to contain 58 genes in U_L, 13 in U_S, two in R_L and one in R_S; a total of 74 distinct genes. This number may rise further, as a number of novel proteins have been identified. Martinez *et al.* (1996a,b) proposed a second protein encoded within UL12 (UL12.5), which corresponds to the C-terminal portion of the UL12 protein. Lagunoff and Roizman (1994, 1995) identified a protein-coding region in R_L (ORF P) which contains 248 codons and is almost completely antisense to RL1 (ICP34.5). Ward *et al.* (1996) proposed a novel protein encoded antisense to UL43 (UL43.5). The status of these potential additional genes, however, remains uncertain for various reasons.

The majority of the HSV-1 genome is potentially protein-coding. Most genes are expressed as unspliced mRNAs whose expression is controlled by temporal class-specific promoters and which terminate at eukaryotic polyadenylation signals containing the canonical AATAAA motif. Partially overlapping transcripts sharing a common polyadenylation site are common: for example US5, US6 and US7. HSV-1 has four genes that are incontrovertibly expressed as spliced mRNAs, two (UL15 and RL2) with introns in protein-coding regions and two (US1 and US12) sharing a common spliced 5' non-coding leader in R_S (Wagner, 1994). There is evidence that some genes specify low-abundance spliced mRNAs in addition to the major unspliced transcripts, for example, minor variants of the UL44 and UL30 transcripts. In contrast to the four major spliced transcripts, however, the significance of these minor variants

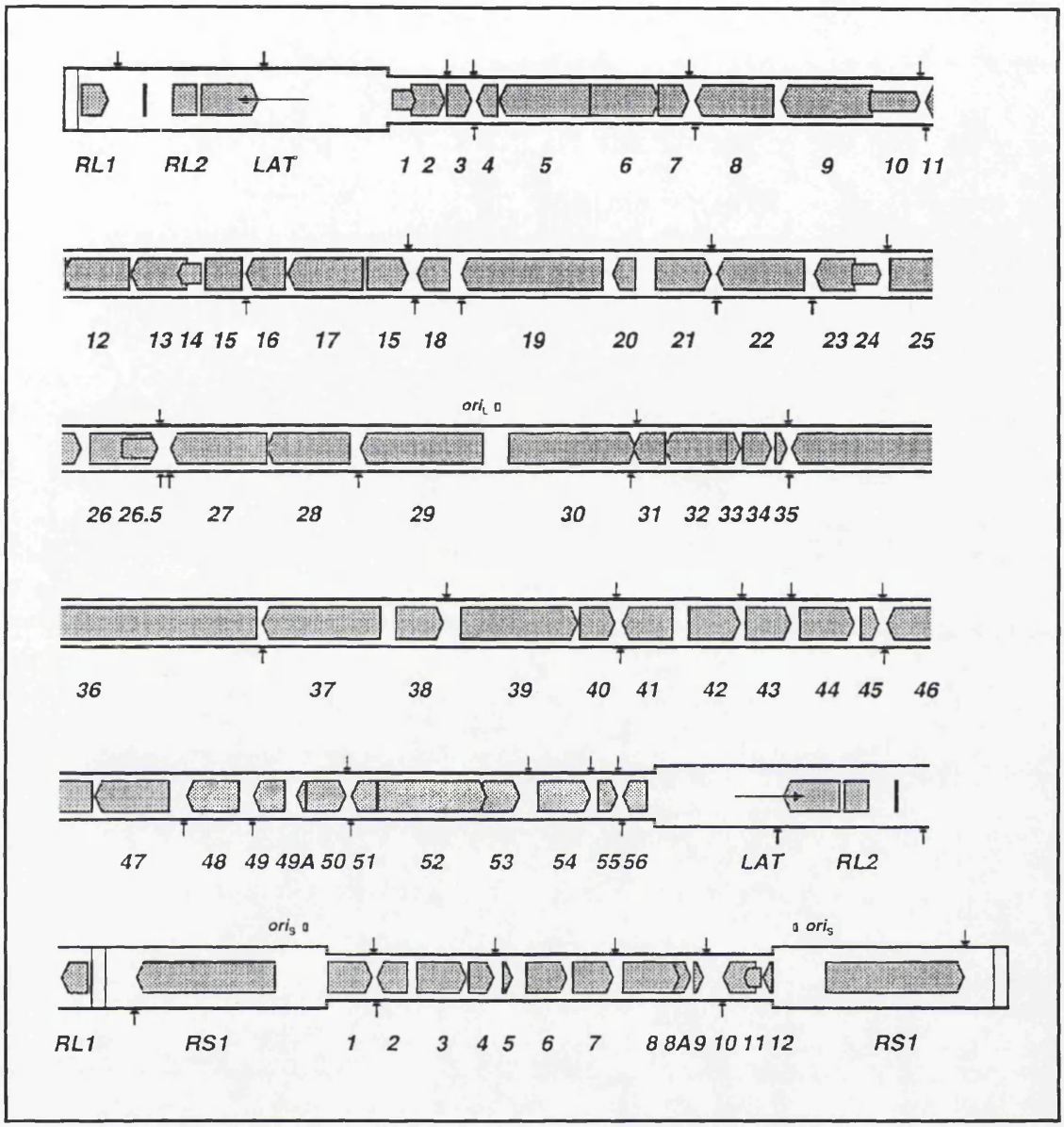


Fig. 5. Arrangement of genes in the HSV-1 genome

The genome is shown in six panels, each 25 kbp long except the last. Inverted repeats are denoted by the thicker parts of the genome outline and protein-coding regions are shown as shaded horizontal arrows. For the sake of clarity, the prefixes "UL" (58 genes) and "US" (13 genes) have been omitted from the gene nomenclature given below the genome; see Fig. 4 for the locations of these regions. The major LAT RNA, presumed to be derived from a stable intron generated from a larger transcript, is also indicated by a horizontal arrow. Genes RL2 and UL15 contain three and two exons, respectively. Possible polyadenylation sites for mRNAs are indicated by vertical arrows above and below the genome for right and left oriented genes respectively. The locations of ori_S and ori_L are shown by white rectangles above the genome. Reproduced from Davison (1990).

is not clear. In addition, the latency-associated transcripts (LATs), described in more detail in Section 1.10, are spliced.

The HSV-1 genome contains three origins of replication, one in U_L (ori_L), located between genes UL29 and UL30, and one in R_S (ori_S; two copies). There is considerable homology between the sequences of ori_S and ori_L, but whereas ori_L contains a perfect 144 bp palindrome the ori_S palindrome is less extensive (Weller *et al.*, 1985). Further details are given in Section 1.7.

1.3.4. Genetic variability

Differences in RE profiles between HSV-1 strains have two main causes: base substitution, which may add or eliminate restriction endonuclease cleavage sites; and variability in the number of repeated sequences, which results in size variability of restriction enzyme fragments.

Restriction endonuclease site polymorphism has been used in epidemiological studies of HSV transmission in the human population. For example, Sakoaka *et al.* (1994) compared the restriction patterns of HSV-1 strains from six countries and found that they vary from country to country and that HSV-1 strains from within the same ethnic group are more closely related. They also suggested that the virus co-evolves with its host and that the rate of mutation is 3.5×10^{-8} per site per year, a rate which corresponds to that proposed by McGeoch and Cook (1994) for much longer evolutionary periods (see Section 1.1.4.).

Several of the 19 sets of tandem reiterations which are present in the HSV-1 genome can vary in the copy number of repeat elements. The *a* sequence can also vary in copy number at the L-S junction and L terminus.

1.3.5. Gene functions

Table 5 contains information about the status in cell culture and proposed functions of HSV-1 genes. The functions of the majority of herpesvirus genes have been identified by direct genetic and biochemical experimentation, and computer-aided comparisons of predicted amino acid sequences with databases have also facilitated the identification of a few gene functions.

Table 5. Features of HSV-1 genes

Gene[a]	Status[b]	Protein function	References to mutants
RL1	NE	Neurovirulence factor (ICP34.5).	Bolovan *et al.*, 1994; Brown *et al.*, 1994; Perng *et al.*, 1993, 1995 a,b, 1996.
RL2	NE	IE protein; transcriptional regulator (ICP0, Vmw110).	Everett, 1989; Stow and Stow, 1986; Chen *et al.*, 1991.
LAT	NE	Latency-associated transcript; probably not protein coding.	Block *et al.*, 1990; Deshmane *et al.*, 1993, 1995; Perng *et al.*, 1994; Maggioncabla *et al.*, 1994.
UL1	E	Glycoprotein L; complexes with glycoprotein H (UL22).	Hutchison *et al.*, 1992a; Roop *et al.*, 1993.
UL2	NE	Uracil-DNA glycosylase.	Pyles and Thompson, 1994.
UL3	NE	Function unknown	Baines and Roizman, 1991.
UL4	NE	Function unknown.	Baines and Roizman, 1991.
UL5	E	Component of DNA helicase-primase complex; possesses helicase motifs.	Wu *et al.*, 1988, Zhu and Weller, 1992a, b; Bloom and Stevens, 1994.
UL6	E	Minor capsid protein.	Weller *et al.*, 1987; Patel *et al.*, 1996.
UL7	E?	Function unknown.	
UL8	E	Component of DNA helicase-primase complex.	Carmichael and Weller, 1989; Sherman *et al.*, 1992
UL9	E	*Ori*-binding protein essential for DNA replication.	Olivo *et al.*, 1988; Arbuckle and Stow, 1993; Abbots and Stow, 1993.
UL10	NE	Virion surface glycoprotein M.	McLean *et al.*, 1991, 1993; Baines and Roizman, 1991.
UL11	NE	Myristylated tegument protein; role in virion envelopment.	MacLean *et al.*, 1989; 1992; Baines and Roizman, 1992.
UL12	(E)	Deoxyribonuclease; role in maturation/packaging of DNA.	Weller *et al.*, 1990; Shao *et al.*, 1993
UL13	NE	Tegument protein; probable protein kinase.	Coulter *et al.*, 1993; Overton *et al.*, 1994.
UL14	?	Function unknown.	
UL15	E	Role in DNA packaging; putative terminase component.	Poon and Roizman, 1993; Baines *et al.*, 1994.
UL16	NE	Function unknown.	Baines and Roizman, 1991.
UL17	E?	Function unknown.	
UL18	E	Capsid protein (VP23); component of intercapsomeric triplex.	Desai *et al.*, 1993.
UL19	E	Major capsid protein (VP5); forms hexons and pentons.	Weller *et al.*, 1987; Desai *et al.*, 1993;
UL20	E/NE	Integral membrane protein; role in egress of nascent virions;host range phenotype; syn locus.	Baines *et al.*, 1991; MacLean *et al.*,1991.
UL21	NE	Tegument protein.	Baines *et al.*, 1994a.
UL22	E	Virion surface glycoprotein H; complexes with glycoprotein L (UL1); role in cell entry.	Desai *et al.*, 1988; Dargan and Subak-Sharp, 1991; Wilson *et al.*, 1994.
UL23	NE	Thymidine kinase.	Coen *et al.*, 1989; Hay *et al.*, 1995.

UL24	NE	Function unknown; syn locus.	Jacobson *et al.*, 1989a.
UL25	E	Capsid-associated tegument protein.	Addison *et al.*, 1984; Thopmson *et al.*, 1995; Ali *et al.*, 1996.
UL26	E	Protease, acts in virion maturation; N-terminal portion is capsid protein VP24.	Preston *et al.*, 1992, 1993; Register and Shafer, 1996; Godefray and Guenet , 1995; Desai *et al.*, 1994.
UL26.5	(E)	Internal protein of immature capsids (VP22a); processed by UL26 protease.	Preston *et al.*, 1992; Thomsen *et al.*, 1995; Kennard *et al.*, 1995.
UL27	E	Virion surface glycoprotein B; role in cell entry; syn locus.	Cai *et al.*, 1987; Highlander *et al.*, 1989; Yahasz and Stevens, 1993.
UL28	E	Role in DNA packaging.	Addison *et al.*, 1990; Tengelson *et al.*,1993.
UL29	E	Single-stranded DNA-binding protein (ICP8).	Gao *et al.*, 1989.
UL30	E	Catalytic subunit of replicative DNA polymerase; complexes with UL42.	Stow, 1993; Digard *et al.*, 1993.
UL31	E?	Function unknown.	
UL32	E?	Function unknown.	
UL33	E	Role in DNA packaging.	Al-Kabaisi *et al.*, 1991; Cunningham *et al.*, 1993.
UL34	E?	Membrane-associated phosphoprotein; substrate for US3 protein kinase.	
UL35	E?	Capsid protein (VP26); located on tips of hexons.	
UL36	E	Very large tegument protein.	Batterson *et al.*, 1983.
UL37	E?	Tegument protein.	
UL38	E	Capsid protein (VP19C) ; component of intercapsomeric triplex.	Flanagen *et al.*, 1991; Guzowski and Wagner, 1993.
UL39	E/NE	Ribonucleotide reductase large subunit (ICP6, Vmwl36, Rl).	Goldstein and Weller, 1988a, b; Jacobson *et al.*, 1989.
UL40	E/NE	Ribonucleotide reductase small subunit (Vmw38, R2).	Preston *et al.*, 1988a.
UL41	NE	Tegument protein; host shut-off factor.	Fenwick and Everett, 1990; Read *et al.*, 1993.
UL42	E	Subunit of replicative DNA polymerase which increases processivity and complexes with UL30 protein.	Gottleib *et al.*, 1990; Gottleib and Challberg, 1994.
UL43	NE	Function unknown; probable integral membrane protein.	McLean *et al.*, 1991.
UL44	NE	Virion surface glycoprotein C; role in cell entry.	Herold *et al.*, 1991; Dargan and Subak-Sharpe, 1991.
UL45	NE	Tegument/envelope protein.	Visalli and Brandt, 1991, 1993.
UL46	NE	Tegument protein; modulates IE gene transactivation by UL48 protein.	Barker and Roizman, 1990; Zhang and McKnight, 1991, 1993.
UL47	NE	Tegument protein; modulates IE gene transactivation by UL48 protein.	Barker and Roizman, 1990; McLean *et al.*, 1990; Zhang and McKnight, 1991, 1993.
UL48	E	Tegument protein; transactivates IE genes (VP16, Vmw65, α-TIF).	Ace *et al.*, 1989; Harris and Preston, 1991; Valyinagy *et al.*, 1991a.
UL49	?	Tegument protein.	
UL49A	NE?	Envelope protein disulphide-linked to tegument.	Romanelli *et al.*, 1992.

UL50	NE	Deoxyuridine triphosphatase.	Fisher and Preston, 1986; Pyles *et al.*, 1992.
UL51	(E)	Function unknown.	Barker and Roizman, 1990.
UL52	E	Component of DNA helicase-primase complex.	Goldstein and Weller, 1988c; Crute *et al.*, 1989; Klinedinst and Challberg, 1994.
UL53	(E)	Glycoprotein K.	Ramaswamy and Holland, 1992; Moyal *et al.*, 1992.
UL54	E	IE protein; post-translational regulator of gene expression (ICP27, Vmw63).	McCarthy *et al.*, 1989; Rice *et al.*, 1993.
UL55	NE	Function unknown.	Block *et al.*, 1991; Nash and Spivack, 1994.
UL56	NE	Function unknown.	Rosen-Wolf *et al.*, 1991; Nash and Spivack, 1994.
RS1	E	IE protein; transcriptional regulator (ICP4, Vmwl75).	Preston, 1979; Patterson *et al.*, 1990.
US1	E/NE	IE protein; function unknown (ICP22, Vmw68); host range phenotype.	Post and Roizman, 1981; Sears *et al.*, 1985; Poffenberger *et al.*, 1993.
US2	NE	Function unknown.	Longnecker and Roizman, 1987.
US3	NE	Protein kinase; phosphorylates UL34 protein.	Purves *et al.*, 1991; Nishiyama *et al.*, 1992.
US4	NE	Virion surface glycoprotein G.	Longnecker and Roizman, 1987; Balan *et al.*, 1994.
US5	NE	Proposed glycoprotein J.	Weber *et al.*, 1987; Balan *et al.*, 1994.
US6	E	Virion surface glycoprotein D; role in cell entry.	Ligas and Johnson, 1988; Johnson and Ligas, 1988.
US7	NE	Virion surface glycoprotein I; complexed with glycoprotein E (US8) in Fc receptor.	Johnson *et al.*, 1988; Balan *et al.*, 1994.
US8	NE	Virion surface glycoprotein E; complexed with glycoprotein I (US7) in Fc receptor.	Johnson *et al.*, 1988; Balan *et al.*, 1994.
US8A	NE	Function unknown.	Georgopoulou *et al.*, 1995.
US9	NE	Virion protein.	Umene, 1986; Frame, 1986.
US10	NE	Virion protein.	Longnecker and Roizman, 1987.
US11	NE	Virion protein; ribosome-associated in infected cell.	Roller and Roizman, 1992.
US12	NE	IE protein (ICP47, Vmwl2); role in preventing antigen presentation.	Longnecker and Roizman, 1986; Hill *et al.*, 1995; Johnson *et al.*, 1995.

[a] = Core genes are shown in bold type.

[b] = The status of each gene in cell culture is indicated: E = essential, NE = non-essential, E? = probably essential, NE? = probably non-essential, (E) = a mutant is viable, but very disabled, E/NE= non-essential under certain conditions, ? = unknown. E genes are those for which mutants have been constructed that require complementing cell lines or those for which attempts to generate insertion mutants using the cosmid system have yielded only *wt*, essential genes have also been identified by *ts* mutants. E? genes are those where attempts to derive a mutant by plasmid recombination have failed. NE? genes are those where data are available for alphaherpesviruses other than HSV-1.

Adapted from McGeoch *et al.* (1993).

The majority of HSV-1 genes can be considered in four main functional categories: control (see Section 1.6); DNA replication and packaging (see Section 1.7); structure and assembly (see Section 1.8); pathogenesis and latency (see Section 1.9 and 1.10). The HSV-1 "core" genes (i.e. genes which are found in all three herpesvirus families) are identified by bold type in Table 5, and most fall into the first three categories.

The status of HSV-1 genes (i.e. their importance in viral growth in cell culture) has been examined by the production of mutants, as described in Section 1.5. About half of the HSV-1 genes are individually dispensable for growth in cell culture, but most appear to be required for efficient viral replication and pathogenesis in animal models (Baines and Roizman 1991; McGeoch and Schaffer, 1992; McGeoch and Barnett, 1993).

1.4. THE INFECTIOUS CYCLE OF HSV-1

This section provides a thumbnail guide to the processes involved at each stage of the HSV-1 lifecycle (Roizman and Batterson, 1987; Everett, 1987b; Wagner, 1994; Campadelli-Fiume, 1994: Roizman and Sears, 1987, 1990, 1993; Beers *et al.*, 1994; Fraser *et al.*, 1991, 1992; Steiner and Kennedy, 1993; Rixon, 1993). Each process is discussed in more detail in later sections. Fig. 6 illustrates the life cycle of HSV-1.

Initial association of HSV-1 with the host cell is mediated by viral glycoproteins, which also have important roles in adsorption and penetration of the virus (see Section 1.8). The virus envelope fuses with the plasma membrane, the nucleocapsid is released into the cytoplasm and migrates to the nucleus where the DNA enters *via* the nuclear pores. At least some of the tegument proteins also enter the nucleus, although the process by which this occurs is not clear (see Section 1.8). Transcription, replication of viral DNA and capsid assembly take place in the nucleus.

Gene expression occurs in three main phases: immediate early (IE or α), early (E or β) and late (L or γ). The operational definition of these genes is based largely on inhibitor studies. An IE gene is one whose transcription is independent of viral and cellular protein synthesis. E genes are activated by IE gene products and are expressed before the onset of DNA replication. L genes are dependent on functional IE and E proteins

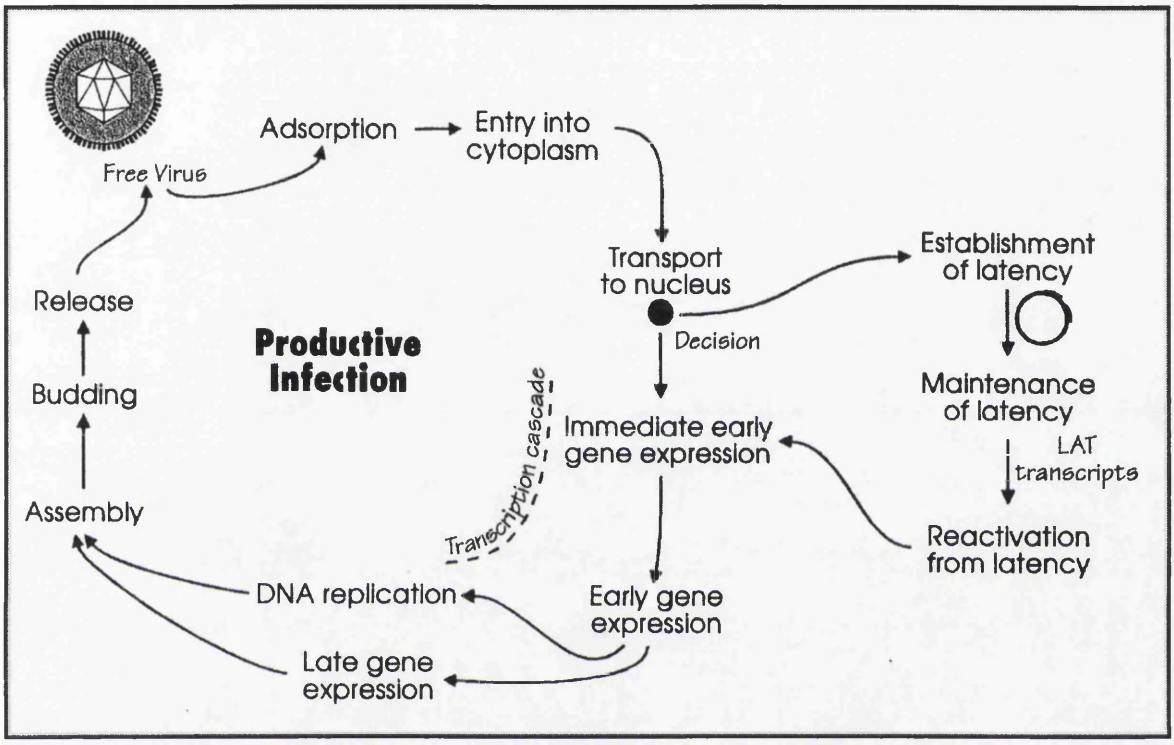


Fig. 6. Representation of the replicative cycle of HSV-1

Reproduced from Davison and Clements (1996).

and are only produced in quantity following the onset of DNA replication. They have been subdivided into "leaky late" (γ_1) and "true late" (γ_2). Expression of the former occurs at low levels before the onset of viral replication but becomes maximal afterwards. The latter are expressed only after DNA replication commences (see Section 1.6).

Three of the five IE genes regulate the expression of early or late genes directly or indirectly (Honess and Roizman, 1974). E gene products include enzymes involved in DNA replication, nucleotide metabolism and some glycoproteins, while L genes encode many of the virion proteins. The latter class includes the tegument protein Vmw65, which is the major transactivator of IE genes (O'Hare, 1993) (see Section 1.6).

Genomic DNA is circularised by direct ligation of the termini, and viral DNA synthesis is initiated in the nucleus from ori_S and ori_L to produce DNA in an endless conformation, probably as head-to-tail concatamers, by a rolling circle mechanism (Roizman, 1979; Jacob *et al.*, 1979). Replicated DNA is cleaved specifically into unit-length molecules at the *a* sequence and packaged into preformed capsids (see Section 1.7). The process by which the capsid acquires tegument and envelope is not fully understood; two proposed models are described in Section 1.8. The envelope is derived from altered host membranes containing viral glycoproteins, which are processed to their mature forms in the Golgi apparatus. Virions are released from the cell by exocytosis (see Section 1.8).

Productive infection leads to the induction of a number of viral and host proteins which specifically alter the metabolism of the cell to confer a replicative advantage to the virus. Upon entry into the cell, a tegument component, virion host shutoff protein (*vhs*, encoded by UL41) causes rapid inhibition of host macromolecular synthesis (see Section 1.6.6.). A late shutoff function also occurs which is dependent on viral RNA and protein synthesis (Fenwick, 1984), but the mechanism is poorly understood. The production of heat shock proteins and interferons is also induced in response to infection, but, at least in cell culture, the lytic lifecycle is not aborted and the cell is eventually destroyed (see Section 1.6).

In addition to the lytic cycle of infection, HSV-1 is able to establish a latent infection in dorsal root ganglia (DRG). The switch between the lytic and latent states depends on complex viral and cellular interactions. During latency, genomic DNA is retained in a circular (or concatemeric) state in association with nucleosomes, and transcription occurs only from a small region of the genome, producing the latency-associated

transcripts (LATs) from R_L (see Fig. 5). HSV-1 can remain in a latent state for the lifetime of the host and can be reactivated by appropriate stimuli resulting in further limited rounds of lytic infection which usually produce oro-facial lesions. Details of latency are given in Section 1.10.

1.5. HSV-1 MUTANTS

In earlier years, HSV-1 mutants were generated without detailed knowledge of the viral genes and were selected on the basis of a range of properties including plaque morphology, temperature sensitivity, host range properties and resistance to immune cytolysis or antiviral compounds. Sections 1.5.1-1.5.5 describe the production of mutants using these methods. Since the determination of the complete HSV-1 DNA sequence it has been possible to target any gene for specific mutation, including genes previously inaccessible to genetic analysis, using selectable or detectable marker genes. Plasmid-based methods (Section 1.5.6-1.5.9) allow mutations to be introduced easily at specifically targeted sites, and can also be used to generate revertants, thus allowing phenotypic changes to be associated directly with the mutation. However insertions can be quite large and may affect more than just the target gene. The major advantage of the cosmid-based system (Section 1.5.10) is that it allows for mutants to be isolated in the absence of *wt* virus. Also, since a marker gene is not required, mutations containing minimally disruptive changes (e.g. insertions or deletions of only a few base pairs) may be introduced. Revertants are not easily produced with the cosmid system, however, association between mutation and phenotype can be established by making several independent mutants in the same gene. Sections 1.5.6-1.5.10 describe the production of mutants with lesions in specific genes using these methods.

1.5.1. Plaque morphology

Ejercito *et al.* (1968) first described variants that induced cell fusion and resulted in the production of giant multinucleate cells or syncitia (*syn* plaques). They concluded that differences in plaque morphology invariably coincided with changes in the surface properties of the virions. Non-syncitial plaques consist of a localised area of rounded cells, and in *syn* plaques many cells are fused into a polykaryocyte (Timbury *et al.*, 1974). Syncitial mutants have been mapped to at least seven different loci in HSV-1

genome (Marsden, 1987), and at least five correspond to genes encoding glycoproteins or putative membrane proteins.

1.5.2. Temperature sensitivity

Temperature sensitive (*ts*) mutants are conditional lethal mutants whose replication is impaired at one temperature, the non-permissive temperature (NPT), but unimpaired at another temperature (usually lower), the permissive temperature (PT), while replication of *wt* virus is not significantly impaired at either temperature (Schaffer *et al.*, 1970). This type of mutant has formed the backbone of the majority of genetic investigations carried out on HSV-1. *Ts* mutants are still used today; indeed, a *ts* mutant of *UL15* was described recently by Poon and Roizman (1993).

In principle, any gene can be mutated to code for a *ts* gene product, but only *ts* mutations in genes which are indispensable to the virus lytic cycle are isolated by normal selection procedures. *Ts* mutations usually cause non-functionality because substitution of *wt* amino acids results in an alteration in protein conformation or stability at the NPT. Although spontaneous *ts* mutants have been reported (Manservigi, 1974), there are two principal methods for inducing mutants *in vitro*. In the first, a virus or viral DNA is treated with a substance that directly modifies the DNA: for example, nitroso-guanidine or UV light. The second and most widely used procedure involves treating infected cells with mutagens. Most *ts* mutants of HSV-1 have been isolated by treatment of cells with 5-bromodeoxyuridine (BUdR) (Subak-Sharpe, 1969) which is incorporated into DNA in place of thymidine, but base pairs with a guanine residue to cause replacement of the original T-A pairing by a G-C base pair in the next replication cycle (Freeze, 1963). These methods of making *ts* mutants are technically simple but have a number of disadvantages. The concentration of mutagen to be used must be accurately determined so that mutants are produced at a greater frequency than would occur spontaneously but without inducing multiple mutants. Also, the sites at which mutations are introduced cannot be selected.

One general problem with *ts* mutants, however produced, is their tendency to "leak" or revert. Leakage is the result of a small amount of functional protein being produced at the NPT. Reversion of a *ts* mutation, usually the result of a single base substitution, may give back the original *wt* sequence or another base which effectively replaces the *wt* base. Revertants can also occur by a change in another codon which counteracts the original change. Despite these problems, the use of *ts* mutants has been very important in investigating gene function.

1.5.3. Host range

Wt HSV-1 cannot replicate in dog kidney cells, although the virus absorbs and penetrates the cell. A host range mutant that could replicate in these cells was first described by Aurelian and Roizman (1964), and appeared to be able to overcome a block at the stage of nucleocapsid envelopment. This mutant was obtained without knowledge of the site or type of mutation and was of limited utility, but certain mutations introduced more recently into specific genes have been shown to result in a restriction to certain cell types. Thus, *US1* deletion mutants can only replicate in Vero cells, while *UL20* deletion mutants form small plaques in human *tk*$^-$ cells but fail to form plaques in Vero cells (Post and Roizman 1981; Baines *et al.*, 1991).

1.5.4. Immune cytolysis

Immune cytolysis mediated by antibody and complement is directed against viral glycoproteins present on the surface of infected cells; particularly glycoprotein B (gB), gC and gD. Machtiger *et al.* (1980) described mutants that contained lesions in surface glycoproteins and were resistant to immune cytolysis. Tests with a *ts* mutant of HSV-1 defective in glycoprotein synthesis at the NPT demonstrated that infected cells maintained at this temperature were not lysed when reacted with antibody and complement. In other studies, monoclonal antibodies specific for HSV-1 or HSV-2 glycoproteins have been used to select variants that are resistant to neutralization (Holland *et al.*, 1983).

1.5.5. Drug resistance

Many drug resistant mutants were isolated during evaluation of compounds as potential antiviral agents. The majority of drug-resistant mutants identified so far contain mutations in UL23 (*tk*) or UL30 (DNA polymerase). The main types of drug used to produce mutants are nucleoside analogues such as BUdR, which is incorporated in place of thymidine causing a base pair replacement (Jamieson *et al.*, 1974), arabinoside A which inhibits HSV-1 DNA polymerase (Coen *et al.*, 1982), and acyclovir which acts as a chain terminator during viral DNA synthesis. Pyrophosphate analogues such as phosphonoacetate inhibit viral DNA polymerase *via* the pyrophosphate binding site (Purifoy and Powell, 1977).

1.5.6. Selectable markers

The isolation of mutants is greatly facilitated by the introduction of selectable marker genes into the HSV-1 genome. Under selective pressure, only recombinants carrying the marker are able to grow. A number of markers are available; the most commonly used is HSV-1 *tk*. Specific deletions of viral sequences can then be made at the site where the marker is inserted.

TK^- virus can be selected using acyclovir or thymidine arabinoside treatment of infected TK^+ or TK^- cells (Mocarski *et al.*, 1980), or by BUdR treatment of TK^- cells. TK^+ virus can be isolated by overlaying infected TK^- infected cells with HAT medium (contains hypoxanthine, aminopterin, thymidine) (Compione-Piccardo *et al.*, 1979; Post *et al.*, 1981).

Post and Roizman (1981) described a general technique for introducing deletions into the genome using plasmid recombination. This method is an extension of the marker rescue technique and involves cotransfection of a plasmid containing a mutated gene into cells with *wt* viral DNA. Recombination between the *wt* and mutated DNA results in the production of a mutant virus. Recombinants are identified and isolated by a number of methods. Initially, *tk* is inserted at the target site in a genome containing a deletion in the natural *tk* gene, selecting for TK^+ progeny. Then the introduced *tk* gene and sequences flanking the insertion site are deleted by a second recombination step, selecting for TK^- virus.

Another selectable marker is the neomycin resistance gene (Neidhardt *et al.*, 1987). Mutant progeny are selected on the basis of their ability to grow in the presence of neomycin.

1.5.7. Detectable markers

The most widely used detectable marker is *E. coli lacZ* (Goldstein and Weller, 1988a). Insertion of this marker allows recombinants to be detected as blue plaques in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), against a background of clear *wt* plaques. This method is widely used, but has a few disadvantages. Since *lacZ* is large (~4 kbp), insertion into a gene may disrupt flanking genes, so that the resulting phenotype could be due in part to an effect on expression of a neighbouring gene rather than the target gene. Also, slow-growing mutants may be difficult to detect in the presence of a large *wt* background. Some advantages of the method include the ability to carry out a second step of mutagenesis so that the initial

lacZ insertion can be replaced by a deletion, and the production of revertants by replacing *lacZ* by *wt* sequences.

1.5.8. Tn5 transposon

Weber *et al.* (1987) reported a simple insertional mutagenesis system for inactivation of HSV-1 genes, but specific genes could not be targeted. The bacterial transposon Tn5 carrying a kanamycin resistance marker was inserted randomly in *E. coli* into plasmids. Cotransfection of plasmids with *wt* virus resulted in mutant progeny. Random insertion of Tn5 resulted in truncation of the corresponding gene product, owing to the presence of a stop codon in all three reading frames at the transposon termini.

1.5.9. Cre-*lox* system

Gage *et al.* (1992) described a cell-free recombination system for site-specific integration of multigenic shuttle plasmids into the herpesvirus genome, which takes advantage of the Cre-*lox* site-specific recombination machinery of bacteriophage P1. A plasmid containing the 34 bp *lox*P recombination site but lacking herpesvirus sequences was inserted at a *lox*P site previously engineered into PRV. Recombination occurred in a mixture containing viral DNA, plasmid, and the Cre recombinase. Transformation of the mixture into suitable eukaryotic cells allowed rescue of infectious recombinant viruses, which were identified on the basis of a blue plaque phenotype derived from expression of *lacZ* in the shuttle plasmid. The plasmid was stably maintained in the viral genome and, since the Cre recombination reaction is reversible, the inserted shuttle plasmid could be recovered from the viral genome producing rescued *wt* viruses. This method is useful only for mutation of a single locus of particular interest, not a means of obtaining mutants throughout the genome.

1.5.10. Cosmids

Van Zijl *et al.* (1988) reported the ability to generate PRV from subgenomic regions cloned as fragments of about 40 kbp into cosmids. Sets of four or five overlapping cosmid clones together comprising the complete viral sequence generated viral plaques after transfection of the inserts into pig kidney cells. Southern blot analysis revealed no differences between parental and reconstituted virus, and no differences were observed in the biological properties exhibited by the reconstituted virus as tested by infection of piglets. Cosmids can be manipulated *in vitro* in a similar manner to plasmids and

mutations transferred to viruses without the need for selection or extensive screening procedures since no *wt* background is generated. Even mutants with poor replicative capacities or carrying multiple gene alterations can be obtained in a single transfection step.

de Wind *et al.* (1990) described a method of linker insertion mutagenesis of single genes in PRV U_S. An oligonucleotide containing translational stop codons in all reading frames was inserted at quasi-random sites into a cosmid and viral mutants were then obtained. This technique takes advantage of the observation that digestion of circular DNA molecules in the presence of ethidium bromide by restriction enzymes with multiple recognition sites preferentially yields full length linear molecules (Parker, 1980).

Since the cosmid system was developed for PRV, similar systems have been described for other herpesviruses, including HSV-1 (Cunningham and Davison, 1993), VZV (Cohen and Seidel, 1993), EHV-1 (Dr E.A.R. Telford, unpublished data), BHV-1 (Ananvoranich *et al.*, 1995) and HCMV (Kemble *et al.*, 1995). Cunningham and Davison (1993) generated three sets of HSV-1 cosmids, each of which was capable of yielding plaques after digestion with *Pac*I to release the inserts and transfection into BHK cells. They constructed viral mutants containing frameshift mutations in either or both of genes *UL2* and *UL44* , which are not essential for growth in cell culture. They also produced a mutant with a frameshift mutation in *UL33*, an essential gene involved in DNA packaging, by transfecting a complementing cell line.

The use of modern methods for constructing mutants such as plasmid-based or cosmid-based systems allows for the production of mutants with lesions at specific sites. Genes of particular interest can be targeted, and this has lead to the generation of a battery of mutants with lesions in many of the HSV-1 genes. These have been used in many laboratories to identify and characterise gene functions.

1.6. CONTROL PROCESSES

1.6.1. Temporal regulation of gene expression

HSV-1 gene expression is temporally regulated, with three major classes of proteins expressed in a cascade (Honess and Roizman, 1974; Clements *et al.*, 1977). Immediate early (IE or α) proteins reach maximal levels of production at 3-4 h pi, and some are required for switching on the other classes of proteins (Honess and Roizman, 1975; Preston *et al.*, 1978). Early (E or β) proteins reach maximal levels of production at 5-7 h pi and require the presence of IE proteins for induction. These include non-structural components, for example, herpesvirus specified enzymes including thymidine kinase, DNA polymerase and DNase. Late (L or γ) proteins require the presence of IE and E proteins, are produced late in infection, and encode many of the major structural components. Some late proteins are made in small amounts prior to DNA replication, while others are strictly produced after replication (Honess and Roizman, 1974; Clements *et al.*, 1977; Jones and Roizman, 1979). Initiation of the transcription cycle is brought about by the action of the tegument component Vmw65, which is the major transactivator of IE genes; as such it begins the cascade of IE, E and L gene expression.

1.6.2. Vmw65 (VP16)

Vmw65 is encoded by UL48 and functions as both an essential structural component of the tegument and as a major transactivator of IE genes. There are between 500-1000 molecules of Vmw65 present in the virion which are transported to the nucleus after viral entry into cells (Spear *et al.*, 1972; Heine *et al.*, 1974).

The five IE genes are transactivated by Vmw65 *via* a specific target sequence (TAATGARAT) upstream of their promoters. To form stable complexes with these sites, Vmw65 requires at least two cellular factors, Oct1 and HCF (host cell factor). Fig. 7 illustrates the process of Vmw65 activation. In summary, the multiprotein complex responsible for IE gene induction is initiated through Oct1 binding to a region containing the TAAT sequence within the Vmw65 target sequence. The Vmw65/HCF complex binds to the Oct1/DNA complex and is stabilised by specific interactions between Vmw65 and the Oct1 POU-homeo domain and between Vmw65 and the GARAT sequences of the TAATGARAT site. HCF may act to stabilise Vmw65 in a conformation with the highest affinity for DNA binding or by interaction with Oct1 (O'Hare, 1993). The formation of the complex locates the acidic activation domain of Vmw65 near the IE gene TATA box, where it activates transcription possibly by promoting the assembly of an initiation complex.

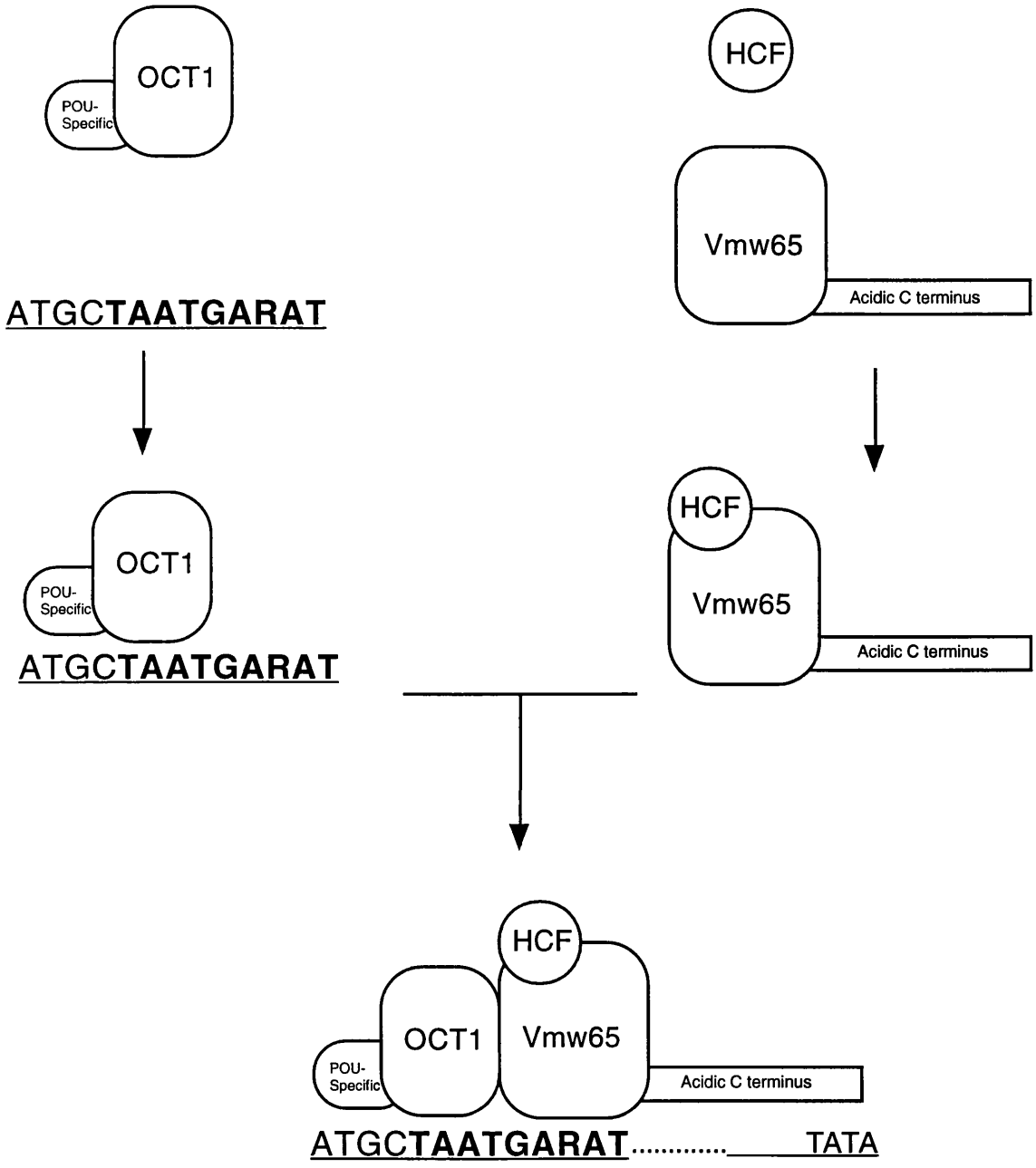


Fig. 7. Vmw65 transactivator complex formation

Cellular protein Oct1 binds to the TAAT region of the Vmw65 activation sequence. HCF binds to Vmw65, and this complex then binds to the Oct1/DNA complex at the GARAT sequence. The formation of the complex locates the acidic C terminus of Vmw65 in the region of the TATA box which promotes assembly of the initiation complex.

The C-terminus of Vmw65 is highly enriched in acidic amino acids; acidic domains have been implicated as transcriptional activators in other systems (Sadowski *et al.*, 1988). The acidic region is thought to promote assembly of an initiation complex containing the TATA box binding factor (TBF) topologically altered for productive initiation. The requirement for IE transactivation can be separated from complex assembly on the TAATGARAT site, since deletion of the acidic domain has no effect on assembly of the complex but abolishes transactivation (Greaves and O'Hare, 1989). The C-terminus of Vmw65 was shown to function as a transactivator independently of the complex when fused to the DNA-binding domain of the yeast transcription factor GAL4 (Sadowski *et al.*, 1988; Cousens, 1989).

Kristie and Roizman (1987) were first to demonstrate that a host cell protein binds to the IE upstream regions and suggest that it could be involved in Vmw65 transactivation. This protein was later identified as Oct1 (O'Hare *et al.*, 1988), a transcription factor implicated in the regulation of a number of cellular genes (Stern *et al.*, 1989). Oct1 is a member of a subclass of homeodomain proteins, the POU proteins, which share a highly related 150-160 residue POU domain (Ruvkun and Finney, 1991). The POU domain is divided into two regions, the POU-homeo domain, which includes three helices and a POU-specific domain. Helix 3 of the POU-homeo domain is the major DNA binding region which makes contact across the TAAT region of TAATGARAT, and the POU-specific domain contacts the ATGC sequence immediately upstream of the TAAT sequence (Herr, 1992). Mutations in GARAT have no effect on Oct1 binding but abolish formation of the Vmw65 complex. Vmw65 binds to Oct1 *via* helix 2 of the POU-homeo domain and to the GARAT sequence (Stern and Herr, 1991).

A direct interaction between Vmw65 and HCF was demonstrated in the absence of Oct1 or the TAATGARAT element. This suggests that the first step in the multi-protein complex assembly is the binding of HCF directly to Vmw65, an interaction which may take place in the cytoplasm. The Vmw65/HCF complex neither binds strongly to DNA nor detectably interacts with Oct1 alone, but does bind *via* the GARAT sequence following Oct1 binding to TAAT (O'Hare, 1993).

1.6.3. Immediate early genes

The five IE genes - RL2 (encoding Vmw110), RS1 (encoding Vmw175), US1 (encoding Vmw68), UL54 (encoding Vmw63) and US12 (encoding Vmw12) (see Table 6) - all have roles in control processes.

Table 6. Immediate early genes involved in control processes.

HSV-1 gene	Glasgow system		Chicago system		Size on SDS-PAGE (kDa)	Size from sequence (Da)	Role
	Gene	Product	Gene	Product			
RS1	IE1	Vmw110	α0	ICP0	110	78452	Non-specific transactivator of all classes of genes.
UL54	IE2	Vmw63	α27	ICP27	63	55376	Late gene expression. Post translational regulation.
RL2	IE3	Vmw175	α4	ICP4	175	132835	Major transactivator of IE,E and L genes.
US1	IE4	Vmw68	α22	ICP22	68	46521	Late gene expression. Host range phenotype.
US12	IE5	Vmw12	α47	ICP47	12	9791	Involved in inhibition of antigen presentation.

Adapted from Everett (1987).

Vmw175

Vmw175 is the major transcriptional regulatory protein and is essential for viral growth (Preston, 1979) and transcriptional activation of E and L genes. It exerts its repressing and activating actions through interactions with general transcription factors (TBP and TFIIB) as well as by binding to DNA (Smith, 1993). It was reported to be present in the tegument of purified HSV-1 virions (Yao and Courtney, 1989), but is actually predominantly found in L-particles (described in Section 1.8.3) (McLauchlan and Rixon, 1992).

The target sequence to which Vmw175 binds (ATCGTC) is present in the RS1 promoter (Faber and Wilcox 1986; 1988), facilitating autoregulation of Vmw175 expression (Roberts *et al.*, 1988; DeLuca and Schaffer, 1988). The region of Vmw175 which is involved in DNA binding, transactivation and repression has been located near the N-terminal end of the protein between amino acid residues 275-495, and mutations in this region showed a reduction in these functions (Patterson and Everett, 1988a; 1988b; Shepard *et al.*, 1989; Patterson *et al.*, 1990). Vmw175 has five domains, two of which (1-314 residues and 1225-1298 residues) are not essential for function. Another two (351-484 residues and 485-496 residues) are essential for transcriptional activation and repression. DNA binding occurs *via* one of these and the other contains nuclear localisation sites. Another domain (497-1224 residues) also contains nuclear localisation sites

Vmw110

RL2 contains three exons and the spliced transcript encodes Vmw110 (Perry *et al.*, 1986), a potent activator of the expression of all three classes of viral genes, as well as a number of cellular genes (Everett, 1984b; Cai and Schaffer, 1989, 1992). Vmw110 has the ability to enhance the expression of any gene that exhibits a basal level of transcription, and can act by itself or synergistically with Vmw175 (Everett, 1984b, 1986, 1988a). It acts without apparent DNA sequence specificity and is itself regulated by Vmw175 (Resnick *et al.*, 1989).

Vmw110 is not essential for viral growth in cell culture but at low moi deletion mutants show a growth defect which is overcome at high moi (Stow and Stow, 1986). The major determinant of transactivation maps to a cysteine-rich region encoded in the second exon (Chen *et al.*, 1991) which is conserved in Vmw110 counterparts in other alphaherpesviruses. Two regions of Vmw110, are essential for function: residues 105-222 and residues 638-775. Residues 105-222 contain unique zinc binding motifs.

Vmw175 acts in concert with and may physically interact with Vmw110 to stimulate transcription (Everett, 1984b; Knipe and Smith, 1988; Yoa and Schaffer, 1994). Vmw110 alone is not sufficient to transactivate early gene transcription and it is thought to mediate transactivation by interacting with a cellular factor, the interacting domain encoded by the first two exons (Weber and Wigdahl, 1992).

Vmw110 appears to play a major role in reactivation from latency. Mutants lacking Vmw110 are unable to stimulate latent viral genomes to reactivate in an *in vitro* latency system (Russell *et al.*, 1987a), and expression of Vmw110 from a heterologous vector facilitates reactivation (Harris *et al.*, 1989) (see Section 1.10.8). Also, Vmw110 mutants are impaired in their ability to reactivate from latency in animal models (Clements and Stow, 1989; Cai *et al.*, 1993). Davido and Leib (1996) showed that Vmw110 promoter elements which are important for Vmw110 expression and virulence are dispensible for the establishment of and reactivation from latency.

Vmw63

Vmw63 affects late gene expression through a post-transcriptional mechanism (Smith *et al.*, 1992; Sandri-Goldi and Mendoza, 1992). This mechanism is poorly understood, but may involve processing at the 3' poly A signal of late transcripts (McLauchlan *et al.*, 1992), or inhibition of splicing (Schroder *et al.*, 1989; Sandri-Goldin and Mendoza, 1992; Hardwich and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994). Two regulatory regions have been identified, an activator region (residues 240-434) and a repressor region within the C-terminal 78 residues which contains a putative zinc-binding domain (Vaughn *et al.*, 1992; Rice *et al.*, 1993). Vmw63 has been shown to bind RNA directly (Ingram *et al.*, 1996).

A *ts* mutant in Vmw63 over-expressed E gene products but did not synthesize detectable amounts of L mRNAs (Sacks *et al.*, 1985). An analysis of deletion mutants showed that Vmw63 is necessary for L gene expression and the down-regulation of IE genes (Sacks *et al.*, 1985; McCarthy *et al.*, 1989; Curtin and Knipe, 1993). There is also evidence from plasmid transfection studies that, in the presence of Vmw110 and Vmw175, Vmw63 can repress E promoters and enhance L promoters (Everett, 1986; Rice and Knipe, 1988; Su and Knipe, 1989; McMahan and Schaffer, 1990). Vmw63 can act synergistically with Vmw175 and Vmw110 to stimulate or repress gene expression.

Vmw63 may contribute to the overall shutoff of host gene expression by virtue of its ability to inhibit pre-mRNA splicing. Since splicing is widespread in the host cell genome and rare in HSV-1, Vmw63 might enable selective expression of viral mRNA (Sandri-Goldin and Mendoza, 1992). Infection of cells with *wt* virus but not Vmw63 mutants leads to the redistribution of components of the spliceosome termed small nuclear ribonucleoprotein particles (snRNPs) from their diffuse speckled pattern in the nuclei of uninfected cells to discrete clusters on the nuclear periphery (Martin *et al.*, 1987; Phelan *et al.*, 1993). Since Vmw63 is required for inhibition of host cell splicing and is localised in the redistributed snRNP-containing nuclear structures (Phelan *et al.*, 1993; Hardy and Sandri-Goldin, 1994), it was proposed that redistribution of snRNPs by Vmw63 is related to the inhibition of host cell splicing. However, a Vmw63 *ts* mutant can redistribute snRNPs without inhibiting splicing (Sandri-Goldin *et al.*, 1995).

Vmw68

Vmw68 has a role in determining host range and in L gene regulation (Sears *et al.*, 1985). Vmw68 mutants grow normally on Vero cells (Post and Roizman, 1981), but grew poorly in cell lines such as HFLs (Sears *et al.*, 1985; Poffenberger *et al.*, 1993). Expression of E proteins is delayed and the expression of L proteins is delayed and substantially reduced.

Vmw12

Vmw12 is a cytoplasmic protein (Marsden *et al.*, 1982), which is non-essential for growth in cell culture (Longnecker and Roizman, 1986; Umere, 1986; Brown and Harland, 1987). A role for Vmw12 in preventing antigen presentation to $CD8^+$ T-lymphocytes has been described (York *et al.*, 1994; Johnson *et al.*, 1995). Hill *et al.* (1995) have shown that Vmw12 binds to TAP (transporter associated with antigen processing) and prevents peptide translocation into the endoplasmic reticulum. It binds to a site which includes the peptide binding domain of TAP and remains bound to this site in a stable fashion, thus preventing antigen presentation by competitive binding (Tomazin *et al.*, 1996; Ahn *et al.*, 1996).

1.6.4. Early genes

Following expression of IE proteins a more complex population of viral mRNAs becomes abundant prior to viral DNA replication, specified by genes which map throughout the genome (Wagner, 1985). They do not contain an obvious conserved class-specific sequence analogous to TAATGARAT in IE genes, although a number of regulatory elements have been identified in promoters of early genes such as UL23 (*tk* gene) and US6 (gD gene) (see Fig. 8), including a TATA box upstream of the transcriptional start site, GA- and GC- rich regions and a CAAT box (McKnight *et al.*, 1985; Everett, 1983, 1984a; Eisenberg *et al.*, 1985; El-Kareh *et al.*, 1985; Jones *et al.*, 1985; Graves *et al.*, 1986).

E genes exhibit a variation in their kinetics of expression and as a result have been divided into two subgroups, early (E or β_1) and delayed early (DE or β_2). For example, gD is expressed as an early protein but it is not synthesised maximally until after the onset of viral DNA replication (Gibson and Spear, 1983), and thus may be viewed as a DE gene (Roizman and Batterson, 1985; Wagner, 1985). This property may simply reflect an increased template copy number.

The *tk* and gD promoters have been shown to contain Vmw175 binding sites (Imbalzano *et al.*, 1990; Tedder *et al.*, 1989), but binding of Vmw175 to the *tk* promoter is not necessary for transactivation (Imbalzano *et al.*, 1990), and removal of three binding sites in the gD promoter does not affect the efficiency of gD transcription during a normal HSV-1 infection (Smiley *et al.*, 1992). Mutational analysis of many E promoters and promoter regulatory regions has failed to define specific sequences involved in transactivation by Vmw110 or Vmw175. Transactivation of E gene expression by IE proteins may be mediated indirectly through their interactions with certain cellular transcription factors (Everett, 1986; Eisenberg *et al.*, 1985; Coen *et al.*, 1986).

1.6.5. Late genes

Most L genes encode structural proteins of the virion. Some L proteins, termed leaky late or γ_1, are detectable in small amounts prior to viral DNA replication and then in larger amounts after DNA replication: for example the major capsid protein encoded by UL19 (Costa *et al.*, 1985). Others, termed true late or γ_2 are not detected prior to DNA replication (Roizman and Batterson, 1985; Wagner, 1985; Johnson *et al.*, 1986): for example, the capsid protein encoded by UL38 (Flanagan *et al.*, 1991) and the US11 protein (Johnson *et al.*, 1986).

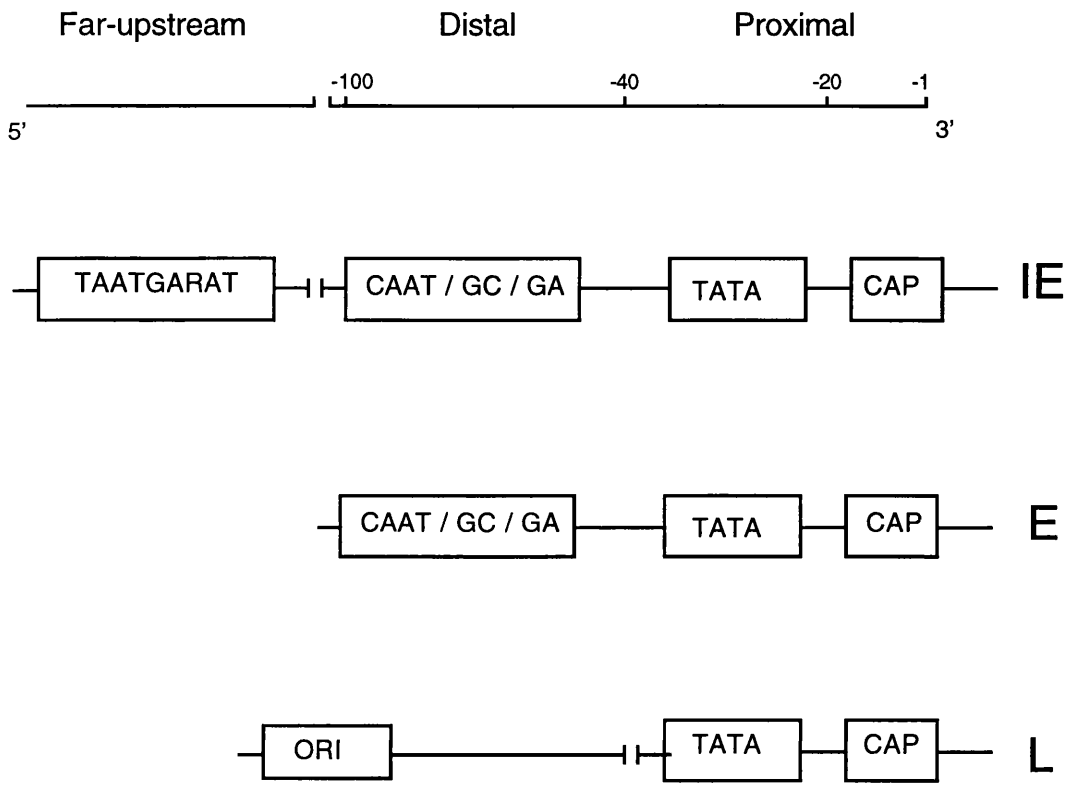


Fig. 8. Differences between IE, E and L promoters

The scale in residues upstream of the RNA cap site is given as a guide to the position of the various elements. IE promoters contain a cap site, a TATA box, distal promoter sequences within 100 bases of the cap site and a far upstream regulatory element. E promoters include only the distal and proximal promoter sequences while L promoters may consist simply of a TATA box and cap site on a replicating (i.e. *ori*-containing) template. Adapted from Everett (1987a).

L promoters do not have any obvious conserved class-specific sequence and seem to have a simpler structure than E promoters (Everett, 1984a,b) (see Fig. 8). Mutational analysis of the promoters of US11 and UL44 (gC) demonstrated that the presence of a TATA box and a cap site, in combination with a *cis*-acting *ori*, were sufficient for full expression (Johnson and Everett, 1986a; Homa *et al.*, 1986). Deletion of the regulatory regions of the US6 gene (gD) to leave a TATA box and cap site resulted in conversion of the gene from E to L regulation (Johnson and Everett, 1986a,b).

Experiments with DNA synthesis inhibitors and DNA-negative *ts* mutants demonstrated that L gene expression is dependent on viral DNA synthesis (Honess and Roizman, 1974; Holland *et al.*, 1980; Conley *et al.*, 1981; Pederson *et al.*, 1981) in addition to presence of E and IE proteins. The role of viral DNA replication is unclear, but the increase in template copy number is likely to contribute towards an increase in L gene expression. L gene expression may also be activated as a result of structural changes occurring during the replication process (Johnson and Everett, 1986a; Mauromara-Nazos and Roizman, 1987).

1.6.6. Other aspects of control

Viral proteins

Several tegument proteins appear to play important roles in enhancing viral infectivity. One major component of the tegument is the protein encoded by UL48, the major transactivator of IE genes, Vmw65 (see Section 1.6.2.). In addition, virion host shut-off protein (*vhs*), encoded by UL41, is a tegument protein which acts to shut off host macromolecular synthesis (Roizman and Roane, 1964). *Vhs* is a phosphoprotein which accumulates late in infection (McLauchlan *et al.*, 1992) and is not required for virion assembly (Smibert and Smiley, 1990). *Vhs* inhibits cellular protein synthesis and glycosylation of host proteins, decreases ribosomal RNA production and reduces the stability of cellular mRNAs (Read and Frenkel, 1983; Wagner and Roizman, 1969; Spears *et al.*, 1970). The latter effect is not limited to pre-existing cellular mRNAs, as *vhs* also induces destabilisation and degradation of viral IE, E and L mRNAs (Fenwick and Everett, 1990a,b). *Vhs* may act as a nuclease, cause dissociation of ribosomes from RNA leaving it more vulnerable to attack by preexisting ribonucleases or activate a cellular nuclease (Fenwick and Everett, 1990a,b). Also, *vhs* can bind to Vmw65 (Weinheimer *et al.*, 1992) and may prevent Vmw65 from entering the multiprotein complex on the IE TAATGARAT element (Smibert *et al.*, 1994).

A well as *vhs*-induced host shutoff it appears that an uncharacterised independent late shut off function also functions during infection (Fenwick and Clark, 1982; Fenwick *et al.*, 1984).

Protein kinases may act on proteins which have a role in the initiation of viral infection and in this way exact a measure of control. The potential protein kinase encoded by UL13 was identified as a virion component by Cunningham *et al.* (1992). This protein contributes to mRNA instability in infected cells (Overton *et al.*, 1994) although the ability to induce mRNA instability may not require phosphorylation of *vhs* since phosphorylation was unaffected in virions from a UL13 mutant (Overton *et al.*, 1994). The protein kinase encoded by US3 phosphorylates the UL34 protein and may also exert some control should it also phosphorylate any viral proteins involved in control.

Other viral proteins may also influence control processes. These include Vmw63, which contributes to the shut-off of host gene expression by inhibiting RNA splicing (see Section 1.6.3) (Hardwick and Sandri-Goldin, 1995). The C-terminus of RL1 is required to prevent the premature shut-off of protein synthesis in infected cells (Chou and Roizman, 1994). This protein may act to prevent the stress response in infected cells which would lead to inhibition of protein synthesis (see Section 1.9). Also, the tegument proteins encoded by UL46 and UL47 modulate IE gene transactivation by Vmw65 (UL48).

Host proteins

Cells under stress produce heat shock proteins (*hsps*), and the synthesis of other cell proteins is down-regulated. The high degree of amino acid sequence homology among *hsps* and the fact that they are normally present in organisms that are not under stress conditions suggests that they function in normal cellular operations, but that they are required to a greater extent under stress conditions. Two *hsps*, *hsp*70 and *hsp*90, act as molecular chaperones mediating the correct assembly of other polypeptides (Ellis, 1990). It has been proposed that *hsps* not only mediate protein assembly but also promote disassembly of proteins that have been damaged as a result of stress (Pelham, 1986).

Hsp 70 alone is transiently induced by HSV-1 infection (Philips *et al.*, 1991). Notarianni and Preston (1982) demonstrated that the over-expression of IE polypeptides during infection with the mutant *ts*K (which contains a temperature-sensitive lesion in RS1) at the NPT led to increased synthesis of *hsp* 70, and later it was

shown that overproduction of mutant Vmw175 was responsible for the induced stress response (Russell *et al.*, 1987a). Later, Russell *et al.* (1987b) suggested that accumulation of *hsp*70 was important in eliminating non-functional forms of Vmw175 or neutralising their damaging effects, rather than merely resulting from viral IE protein accumulation.

The interferons are a group of proteins that are not normally expressed but are produced in response to a range of stimuli, including viral infection. They are released from virus-infected cells and bind to interferon receptors on neighbouring cell membranes. There are two types of interferon receptors: one for IFNα and IFNβ and the other for IFNγ (Branca and Baglioni, 1981). Binding of IFN activates transcription of a defined set of cellular genes, many of which are involved in the establishment of an antiviral state (Lengyel, 1982; Fellous *et al.*, 1982; Samuel, 1991; Sen and Lengyel, 1992).

IFN-α has been shown to inhibit HSV-1 replication at the level of IE gene transcription (Oberman and Panet, 1988; DeStasio and Taylor, 1990), while cellular genes are insensitive (Mittnacht *et al.*, 1988; Oberman and Panet, 1989; DeStasio and Taylor, 1990). Researchers have consistently found that adsorption, penetration and uncoating of HSV-1 is unaffected in cells pre-treated with IFN (Oberman and Panet, 1988; DeStasio and Taylor, 1990). The reduction in HSV-1 IE mRNA levels occurs as a result of an inhibition of transcription initiation rather than of transcript elongation or mRNA degradation (Mittnacht *et al.*, 1988; DeStasio and Taylor, 1990).

1.7. DNA METABOLISM

1.7.1. Nucleotide metabolism and DNA repair

HSV-1 specifies a number of enzymes which have roles in nucleic acid metabolism and DNA repair and as such are important in DNA replication. Many of these enzymes are not required for viral growth in cell culture but have been shown to be important in neurovirulence and latency (see Section 1.9).

The alkaline nuclease (encoded by UL12) is involved in the cleavage of DNA concatemers and in resolving replicative intermediates produced during DNA

replication. It complexes with the UL29 gene product (Weller *et al.*, 1990; Thomas *et al.*, 1992). It is required for efficient virus production in cell culture and has 5'-3' exonuclease activity (Strobel-Fidler and Francke, 1980; Chou and Roizman, 1989). Uracil-DNA glycosylase (encoded by UL2) is involved in DNA repair and proof reading (Caradonna *et al.*, 1987; Mullaney *et al.*, 1989; Pyles *et al.*, 1994). It removes uracil residues from DNA arising from misincorporation or from spontaneous deamination of cytosine residues. Ribonucleotide reductase (RR) is a tetramer of two molecules of the UL39 protein and two molecules of the UL40 protein, and converts ribonucleotides to the corresponding deoxyribonucleotides, thus supplementing the pool of substrates for DNA synthesis (Goldstein and Weller, 1988; Jacobson *et al.*, 1989; Paradis *et al.*, 1989; Idowu *et al.*, 1992). Thymidine kinase (encoded by UL23) phosphorylates thymidine and deoxycytidine to TMP and dCMP (Jamieson and Subak-Sharpe, 1974; Efstathiou *et al.*, 1989; Coen *et al.*, 1989), which are then converted to the triphosphates by cellular enzymes. Deoxyuridine triphosphatase (dUTPase) (encoded by UL50) hydrolyses dUTP to dUMP and pyrophosphate, thus reducing misincorporation of uracil residues into DNA and supplementing the pool of dUMP which can be converted to dTMP by cellular thymidylate synthase (Caradonna and Cheung, 1981; Fisher and Preston, 1986; Pyles *et al.*, 1992).

HSV-1 was thought to specify a DNA ligase (Sparelari, 1976), which could join the Okazaki fragments produced by discontinuous synthesis on the lagging strand, and a topoisomerase (Muller *et al.*, 1985). No viral genes, however, have been shown to encode these functions.

1.7.2. DNA replication

HSV-1 DNA replication is first detected in cells at 3 h pi, peaks at about 9-11 h and is virtually completed by 16 h (Wilkie, 1973). Replication initially occurs at discrete sites within the infected cell nucleus called "replication compartments", and then at later times the nucleus becomes filled with replicating DNA (Quinlan *et al.*, 1984). The current model of DNA replication proposes that following entry into the nucleus, the DNA circularises (Jacob and Roizman, 1977) by ligation of the terminal *a* sequences (Davison and Wilkie, 1983), is replicated by a rolling circle mechanism involving continuous production of one strand of DNA and discontinuous synthesis of the other (Roizman, 1979) to generate multiple head-to-tail concatemers (Jacob *et al.*, 1979). The concatemeric DNA is then cleaved into unit-length molecules and packaged into preformed capsids (see Section 1.7.5).

1.7.3. Origins of DNA replication

The HSV-1 origins of replication were identified in a number of ways, including electron microscopic studies, which provided evidence for the existence of two origins of DNA synthesis in the HSV-1 genome, one near the center of U_L (*ori*$_L$) and the other in R_S (*ori*$_S$) (Friedmann *et al.*, 1977) and by analysis of defective interfering genomes which are spontaneous deletion mutants of *wt* genomes containing origins of replication and packaging signals (Frenkel *et al.*, 1976; Schroder *et al.*, 1976). The origins have been defined as those sequences that must be present in a fragment of HSV DNA for it to be amplified in permissive cells transfected with the fragment and transfected or infected with a helper virus (Vlazny *et al.*, 1981).

It is now known that there are three origins of replication in a single HSV-1 DNA molecule (see Section 1.4) (see Fig. 5): *ori*$_L$ (one copy) and *ori*$_S$ (two copies). Both *ori*$_S$ and *ori*$_L$ contain A+T rich palindromes and share considerable homology. *Ori*$_L$ is a perfect 144 bp palindrome, but *ori*$_S$ palindrome is less extensive (Weller *et al.*, 1985). *Ori*$_S$ is divided into a core region which is 90 bp in size and contains an almost perfect 45 bp palindrome and an 18 bp region containing A and T residues (Stow and McMonagle, 1983) and surrounding sequences which enhance DNA replication (UL9 binding sites) (Stow, 1992).

The significance of three origins of replication within the HSV-1 genome is unclear since mutational analysis has confirmed that all three origins together are not required for viral DNA replication in cell culture. *Ori*$_L$ (Polvino-Bodnar *et al.*, 1987) and at least one copy of *ori*$_S$ (Longnecker and Roizman, 1986) but not both copies (Smith *et al.*, 1989) are dispensable and *ori*$_L$ is not required for establishment and reactivation from latency in mice (Polvino-Bodnar *et al.*, 1987). The origins appear to be functionally equivalent (Lockshon and Galloway, 1988) and it is likely that any two of the three is sufficient *in vivo*. However, Igarashi *et al.* (1993) successfully deleted both *ori*$_S$ sequences with little effect on viral yields or viral DNA acummulation in infected cells, and thus none of the three may be uniquely required.

1.7.4. Gene requirements for DNA replication

Seven HSV-1 genes are essential for viral DNA replication: UL5, UL8, UL9, UL29, UL30, UL42 and UL52 (Schaffer *et al.*, 1987; Wu *et al.*, 1988). The function of each is described in Table 7. Fig. 9 illustrates the current model of events occurring at the replication fork. These proteins form complexes which are required for efficient replication.

Table 7. HSV-1 DNA replication genes

Function	Gene	Details	Reference
Origin-binding protein.	UL9	Binds to *ori via* the sequence CGTTCGCACTT. Possible helicase activity and role in assembly of the pre-initiation complex which leads to the initiation of DNA synthesis.	Abbots and Stow, 1995; Olivio, 1988; Elias *et al.*, 1990; Koff *et al.*, 1991; Gorbalenya *et al.*, 1989; Fierer and Challberg, 1992; Elias *et al.*, 1990.
DNA polymerase. (1:1 complex)	UL30	Functions as the the replication subunit and also has proof reading 3'-5' exonuclease activity and 5'-3' exonuclease and RNase activity. Complexed to the UL42 protein *via* the C-terminus.	Chartrand *et al.*, 1980; Ostrander and Cheng, 1980; Crute and Lehman, 1989; Gallo *et al.*, 1988; Digard *et al.*, 1993; Marsden *et al.*, 1983; Gottlieb *et al.*, 1990; Mace and Alberts, 1984.
	UL42	Has DNA binding activity and is thought to stabilise the binding of the UL30 protein to the template, leading to the production of longer DNA products.	
Helicase and primase function. (1:1:1 complex)	UL5	ATPase and helicase activities are probably associated with UL5 since it shares motifs with a superfamily of DNA and RNA helicases. UL5 does not act as a helicase in the absence of UL52.	Crute *et al.*, 1988; 1989; Wu *et al.*, 1988; Hodgman, 1988; Dodson *et al.*, 1989; Calder and Stow, 1990; Calder *et al.*, 1992; Sherman *et al.*, 1992.
	UL52	UL52 and UL5 form a complex with enzyme activity indistinguishable from that of the three protein complex.	
	UL8	Is not required for enzyme activity, but may play a role in efficient nuclear uptake and stabilise the association between primer RNA and template DNA.	
Single-stranded DNA-binding protein (ssb).	UL29	Binds to and stabilises regions of ssDNA at the replication fork. The C-terminal region contains DNA-binding sites.	Bayliss *et al.*, 1975; Powell *et al.*, 1981; Gallo *et al.*, 1989.

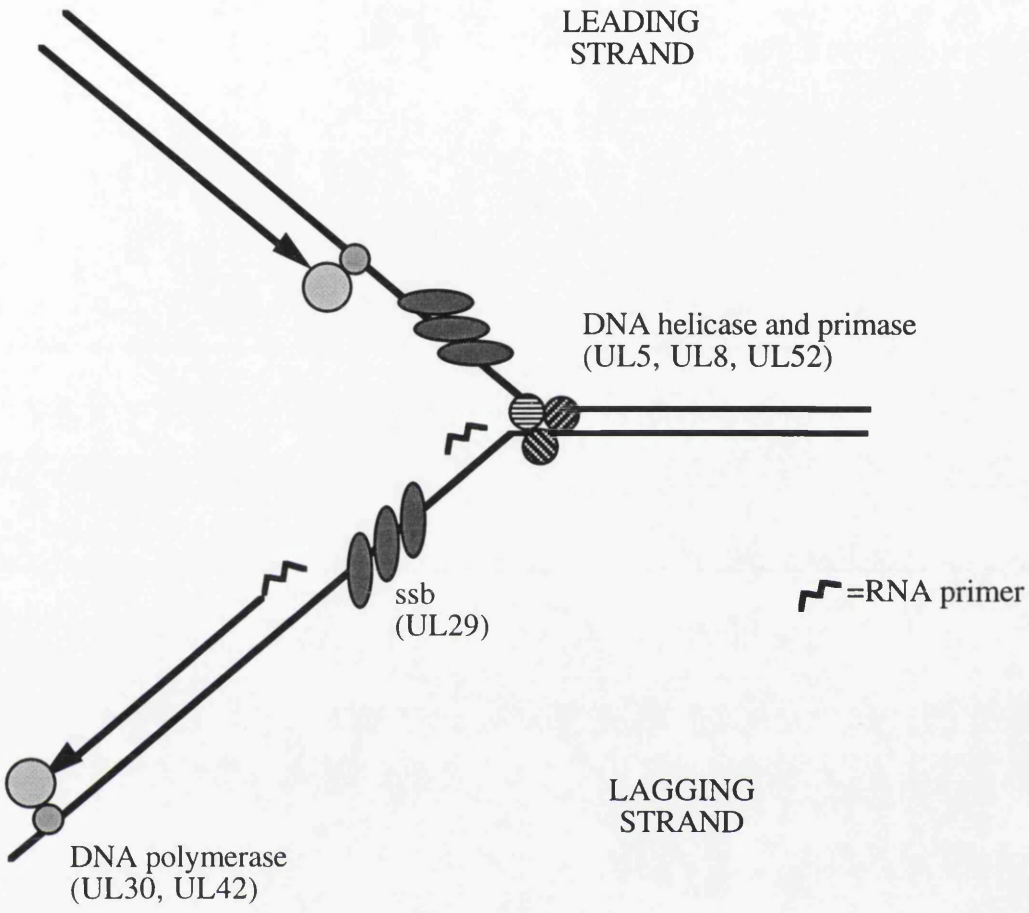


Fig. 9. Functions involved at the DNA replication fork

Adapted from a model provided by ND Stow.

1.7.5. Cleavage and packaging of replicated DNA

Replication produces "endless" DNA which must be cleaved into unit-length molecules for packaging. The signal for cleavage has been determined as the *a* sequence. Tandem reiterations of the *a* sequence are found at the L-terminus and the L-S joint region of the genome, but only a single copy is found at the S terminus (Wilkie, 1976; Wagner and Summers, 1978). The functions of the *a* sequence include circularisation of the genome following infection, site-specific recombination and cleavage/packaging of the DNA (Davison and Wilkie, 1983; Poffenberger and Roizman, 1985). Although the sequence of the *a* sequence varies between strains (Davison and Wilkie, 1981; Mocarski and Roizman, 1981; 1982), two conserved regions (pac1 and pac2) located about 30 bp from each end of linear genomes appear to be essential for the cleaving and packaging process (Varmuza and Smiley, 1985; Deiss *et al*, 1986).

Replicated DNA is packaged into preformed capsids, since mutants defective in packaging still assemble capsids (Al-Kobaisi *et al.*, 1991; Addison *et al.*, 1984; 1990). Packaging mutants also fail to cleave replicated DNA into unit lengths, indicating that cleavage and packaging are closely linked (Preston *et al*, 1983). The scaffolding proteins VP22a and VP21 which are present in immature capsids are lost at the time of DNA packaging (Gibson and Roizman, 1972) (see Section 1.8.2). Deiss *et al.* (1986) proposed that a protein binds to an *a* sequence and in turn binds to a site on the capsid. The DNA is then injected into the capsid until a second *a* sequence in the same orientation as the first is reached, whereupon cleavage occurs. The model suggests that the region of DNA packaged is related to the distance between two direct repeats of the *a* sequence (Deiss *et al.*, 1986; Roizman and Sears, 1993). The internal *a* sequences can be deleted without fully inhibiting packaging (Poffenberger and Roizman, 1985; Jenkins and Roizman, 1986).

Two viral gene products, Vmw273 (encoded by UL36) and a protein encoded by an as yet unidentified gene, have been shown to interact with the *a* sequence (Chou and Roizman, 1989), and it is possible that these function in the cleavage and packaging of DNA. There are also a number of other viral genes that play a role in cleavage and packaging since viruses with mutations in these genes produce of capsids but fail to package viral DNA. These include, UL6 (Weller *et al.*, 1987; Patel *et al.*, 1996), UL12 (Shao *et al.*, 1993; Weller *et al.*, 1990), UL15 (Poon and Roizman, 1993; Baines *et al.*, 1994), UL25 (Addison *et al.*, 1984), UL26 (Preston *et al.*, 1983), UL28 (Addison *et al*, 1990; Tengelson *et al.*, 1993), UL32 (Coen *et al.*, 1984) and UL33 (Al-Kobaisi *et al.*, 1991) (see Table 5 for details).

1.8. VIRION STRUCTURE

1.8.1. Core

Cryoelectron microscopy has shown that the core contains the viral genome in a tightly packed structure envisaged as a "ball of yarn" (Booy *et al.*, 1991). Early observations suggesting that the DNA is present in a toroidal manner around a central protein core (Furlong *et al.*, 1972) were probably artifactual.

1.8.2. Capsid

The core is surrounded by an icosadeltahedral capsid which is 125 nm in diameter and is composed of five major proteins (Schrag *et al.*, 1989). This shell is made up of 162 capsomers: 150 hexavalent (hexons) and 12 pentavalent (pentons) capsomers arranged in an icosahedral lattice with the pentons located at the vertices and the hexons at the capsid faces and edges (Rixon, 1993). Cryoelectron microscopy has shown that all of the capsomers are cylindrical with central channels extending from the outside to the inside of the capsid. The hexons have six-fold symmetry and pentons have five-fold symmetry, and all of the capsomers are connected in groups of three by trigonal nodules or triplexes on the capsid surface (Schrag *et al.*, 1989; Booy *et al.*, 1991).

Three types of capsid may be isolated from virus infected cells (Gibson and Roizman, 1972; Rixon, 1993): A (empty), B (intermediate) and C (full) (see Table 8). C capsids eventually form mature virions. A capsids contain the five capsid proteins of C capsids but lack DNA and are thought to be the end products of abortive DNA packaging (Newcomb and Brown, 1991). B capsids also lack DNA and contain the five proteins present in A capsids plus two additional scaffolding proteins which are lost during DNA packaging. There are two types of B capsid, small cored and large cored. It is thought that the large cored capsids become small cored and are then able to have DNA packaged into them (Rixon, 1993). C capsids may be derived from B capsids.

Capsid proteins assume the icosahedral structure spontaneously when a critical concentration of viral structural proteins is obtained in the nucleus (Pertuiset *et al.*, 1989). Thomsen *et al.* (1994) and Tatman *et al.* (1994) investigated the assembly of HSV-1 capsids using a set of recombinant baculoviruses, each expressing one of the seven capsid proteins. Co-expression of six genes, UL18 (VP23), UL19 (VP5), UL35 (VP26), UL38 (VP19C), UL26 (VP21, VP24) and UL26.5 (VP22a), resulted in formation of capsids in insect cells that were indistinguishable in appearance and

Table 8. Capsid components

Protein	Gene	Role	A capsid	B capsid	C capsid	Copy number[a]
VP5	UL19	Makes up the hexons and pentons.	+[b]	+	+	960
VP19C	UL38	Component of the intercapsomeric triplex.	+	+	+	320
VP23	UL18	Component of the intercapsomeric triplex.	+	+	+	640
VP24	UL26 (5' portion)	Protease.	+	+	+	147 ±67
VP21	UL26 (3' portion)	Scaffolding protein.	-[c]	+	-	87 ±42
VP22a	UL26.5	Scaffolding protein.	-	+	-	1153 ±169
VP26	UL35	Located on the tips of the hexons.	+	+	+	900
DNA			-	-	+	1

[a] Copy numbers are empirical (±SD) or precise (from a combination of empirical and theoretical data) (Newcomb *et al.*, 1993).

[b] Present.

[c] Not present.

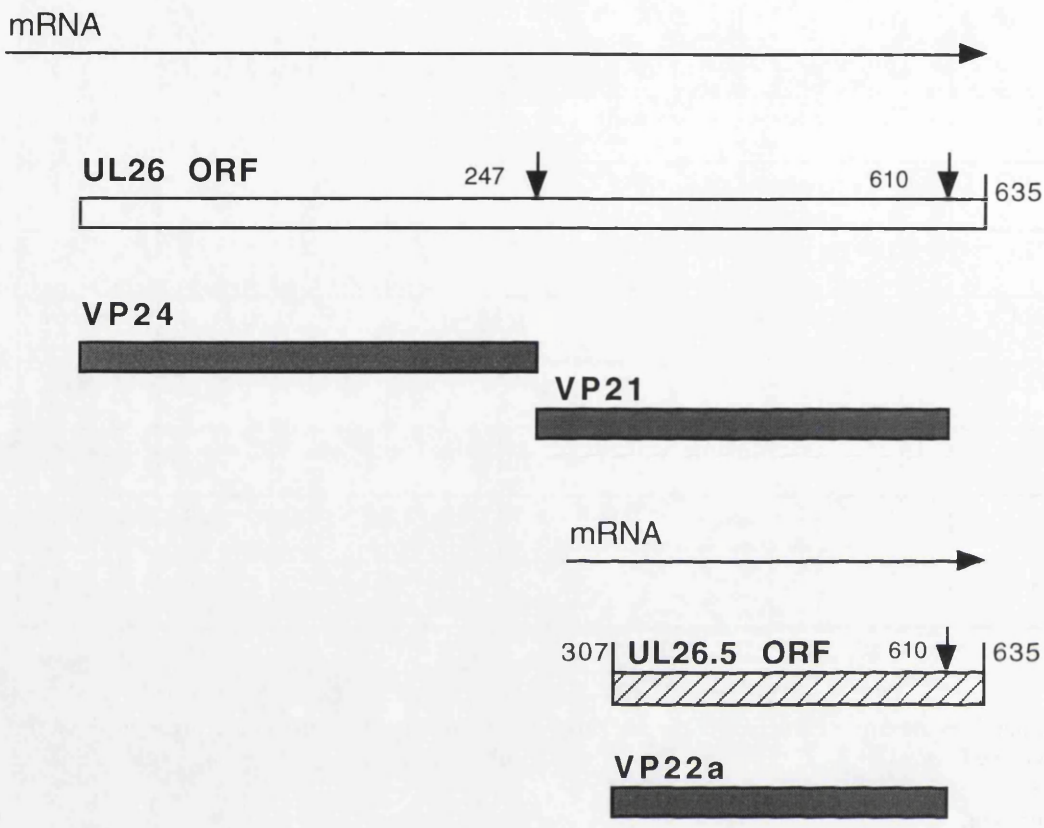


Fig. 10. Organisation of UL26 and UL26.5 and derivation of VP21, VP22a and VP24

The UL26 ORF is denoted as an open rectangle and encodes proteins VP24 and VP21 (shaded rectangles), which are produced following cleavage at the sites indicated by arrows. The UL26.5 ORF (hatched rectangle) is contained entirely within UL26. Cleavage by the protease (VP24) at the site indicated produces VP22a. The numbers refer to amino acid residues in UL26. Adapted from Preston *et al.* (1994).

protein composition from those made during HSV-1 infection. Capsid assembly did not take place in the absence of UL18, UL19 or UL38, but absence of UL35 had no effect. Assembly in the absence of the UL26 gene products resulted in a large core phenotype and some intact capsid shells were formed in the absence of the major scaffolding protein VP22a. Omission of both UL26 and UL26.5 resulted in the formation of large numbers of partial or deformed capsid shells.

Genetic and structural studies suggest that the UL26 and UL26.5 proteins are important in the early stages of capsid assembly. UL26 encodes a protease (VP24) which cleaves within the UL26 protein to produce another scaffolding protein (VP21). UL26.5 is contained entirely within the UL26 ORF. The encoded protein is cleaved by VP24 to produces the scaffolding protein VP22a (see Fig 10). VP22a, the most abundant protein in B capsids, is thought to form a scaffold around which the capsid assembles (Preston *et al.*, 1994). Insertion of the DNA molecule results in loss of VP21 and VP22a, and subsequent tegumentation and envelopment results in the formation of mature virions (Rixon, 1993).

There are thought to be minor capsid proteins which have yet to be identified. The protein encoded by UL6 has recently been shown to be a capsid component (Patel and MacLean, 1995). It is is essential in cell culture (Patel *et al.*, 1996), and is required for DNA cleavage and packaging, but it is not known whether this is due to a direct role in these processes or as an indirect result of its possible structural role.

1.8.3. Tegument

The tegument contains the proteins encoded by UL11, UL13, UL21, UL25, UL36, UL37, UL41, UL45, UL46, UL47, UL48 and UL49 (see Table 5) in addition to several others whose genetic origins are yet to be identified.

The observation that some tegument proteins have roles in control processes, including Vmw65 (Section 1.6.2.) and *vhs* (Section 1.6.6.), suggests that others may have roles which enhance the viral life cycle when introduced into the cell by virus particles. It is possible, for example, that a subset of tegument components contributes towards maintaining structure while others are carried into the cell in order to function in control processes.

Following adsorption and penetration of the virus (see Section 1.8.4.), the nucleocapsid is released into the cytoplasm and transported to the nucleus (Batterson *et al.*, 1983) *via*

host cell microfilaments (Lycke *et al.,* 1984). At the nuclear membrane, DNA release precedes disassociation of the capsid. The DNA enters the nucleus *via* the nuclear pores by a process that is not dependent on the presence of viral proteins (Hummeler *et al.,* 1969). *De novo* RNA and protein synthesis are not required for uncoating, and this process may depend on a structural component of the virus or a cell enzyme (Hochberg and Becker, 1968). It has been shown that the tegument protein Vmw273 (MacNabb and Courtney, 1992), encoded by UL36 is essential for uncoating (Batterson *et al.,* 1983).

L-particles are virion-like enveloped structures that lack the capsid and core but contain most of the proteins normally found in virions, in addition to a few proteins absent from virions (Szilagyi and Cunningham, 1991; Rixon *et al.,* 1992; McLauchlan and Rixon, 1992). They are released in equal amounts with virions from infected cells in cell culture. The assembly of L-particles occurs independently of virion maturation (Rixon *et al.,* 1992), indicating that tegument formation is not dependent on capsid assembly (McLauchlan and Rixon, 1992).

The role of L-particles in the viral life-cycle is not known, but they have been shown to be as efficient as virions at delivering functional tegument proteins into cells (McLauchlan *et al.,* 1992). This suggests that they could influence the infectious process by increasing the supply of tegument-derived helper functions. Vmw175 is present in L-particles in significant amounts and its transactivating function could be significant at early stages of infection (McLauchlan and Rixon, 1992). Vmw65 in L-particles could also produce added transactivating activity. The role of L-particles *in vivo* has not been determined.

Yang and Courtney (1995) suggested that the host cell type influences the amount of Vmw175 and Vmw110 associated with the tegument of virions and L-particles. Virions released from Vero and Hep2 cells contain high levels of Vmw175 and Vmw110, wheras low levels of these proteins were produced in BHK C13 cells. Also, Vmw175 levels were high only in L-particles from BHK C13-infected cells. Thus the availability of transactivating proteins in the L-particles may only be important in some cell types.

1.8.4. Envelope

The viral envelope has a trilaminar appearance (Epstein, 1962), and the presence of lipid was demonstrated by the sensitivity of virions to lipid solvents and detergents (Asher *et al.*, 1969; Spear and Roizman, 1972). It is derived from patches of altered cellular membranes (Morgan *et al.*, 1959) as the capsid and tegument buds through the nuclear or Golgi apparatus membrane (Torrisi *et al.*, 1992).

So far HSV-1 has been shown to encode 10 glycoproteins (gJ has not yet been identified) (see Table 9). Their functions include adsorption, penetration, membrane fusion, envelopment, prevention of superinfection and egress. Glycoproteins are also involved in the immune response, and gE and gI together produce the IgG Fc binding component which may prevent recognition of the virus and infected cells by the host immune response. At least two non-glycosylated proteins are present in the envelope: UL49A (Barnett *et al.*, 1992; R. Adams, personal communication) and the UL11 protein which resides in the tegument but is also associated with the envelope *via* a myristoyl moiety (MacLean *et al.*, 1992).

Entry of HSV-1 into a cell involves a number of events at the cell surface. Initial association with the host cell is mediated by the association of gC with heparin sulphate proteoglycans on the cell surface (WuDunn and Spear, 1989; Kaner, 1990; Shieh *et al.*, 1992). gC appears to be principally involved in adsorption of the virus to the cell surface (Kuhn *et al.*, 1990; Herold *et al.*, 1991), but gD and gB may also play a role since monoclonal antibodies against these can inhibit binding to the cell surface (Kuhn *et al.*, 1990). However, there must also be an alternative pathway to gC binding, since mutants lacking gC can still attach with reduced efficiency (Herold *et al.*, 1991). Adsorption *via* fibroblast growth factor receptors (Kaner *et al.*, 1990) is restricted to a minority of cell types (Shieh and Spear, 1991; Muggeridge *et al.*, 1992).

Penetration occurs by fusion of the virion envelope with the plasma membrane of the cell, and at least five glycoproteins, gB, gD, gH, gK and gL, are involved (Cai *et al.*, 1987; Ligas and Johnson, 1988; Desai *et al.*, 1988; Fuller and Spear, 1987; Fuller *et al.*, 1989; Hutchison *et al.*, 1992a; Roop *et al.*, 1993). gB, gD, and gH are essential in cell culture. gK plays a role in controlling virus induced cell fusion and mutants are not viable (MacLean *et al.*, 1991). gL plays a role in the production of the functional gH (Hutchison *et al.*, 1992b). Although there is no definite molecular model for the fusion event, several results have indicated that gD may interact with a cellular receptor after initial binding of gC to heparan sulphate, since the resistance of cell lines to infection expressing large amounts of gD suggests that gD sequesters a receptor (Campadelli-

Table 9. Properties of HSV-1 glycoproteins

Property	HSV-1 glycoprotein										
	gB	**gC**	**gD**	**gE**	**gG**	**gH**	**gI**	**gJ**	**gK**	**gL**	**gM**
Encoded by gene	UL27	UL44	US6	US8	US4	UL22	US7	US5	UL53	UL1	UL10
Present in α-, β- and γ-herpesviruses	+[a]	–[b]	–	–	–	+	–	–	–	+	+
Essential for replication	+	–	+	–	–	+	–	–	+	+	–
Present in virions	+	+	+	+	+	+	+	?[c]	–	+	+
Mediates adsorption	+	+	+	–	?	–	–	?	–	–	?
Essential for penetration	+	–	+	–	–	+	–	–	?	+	–
Primary role in cell fusion	+	–	–	–	?	?	?	?	+	?	?
Neutralization	+	+	+	+	+	+	+	?	?	?	?
Cell-mediated immunity	+	+	+	+	+	?	?	?	?	?	?

[a] Yes
[b] No
[c] Not known

Adapted from Davison and Clements (1996).

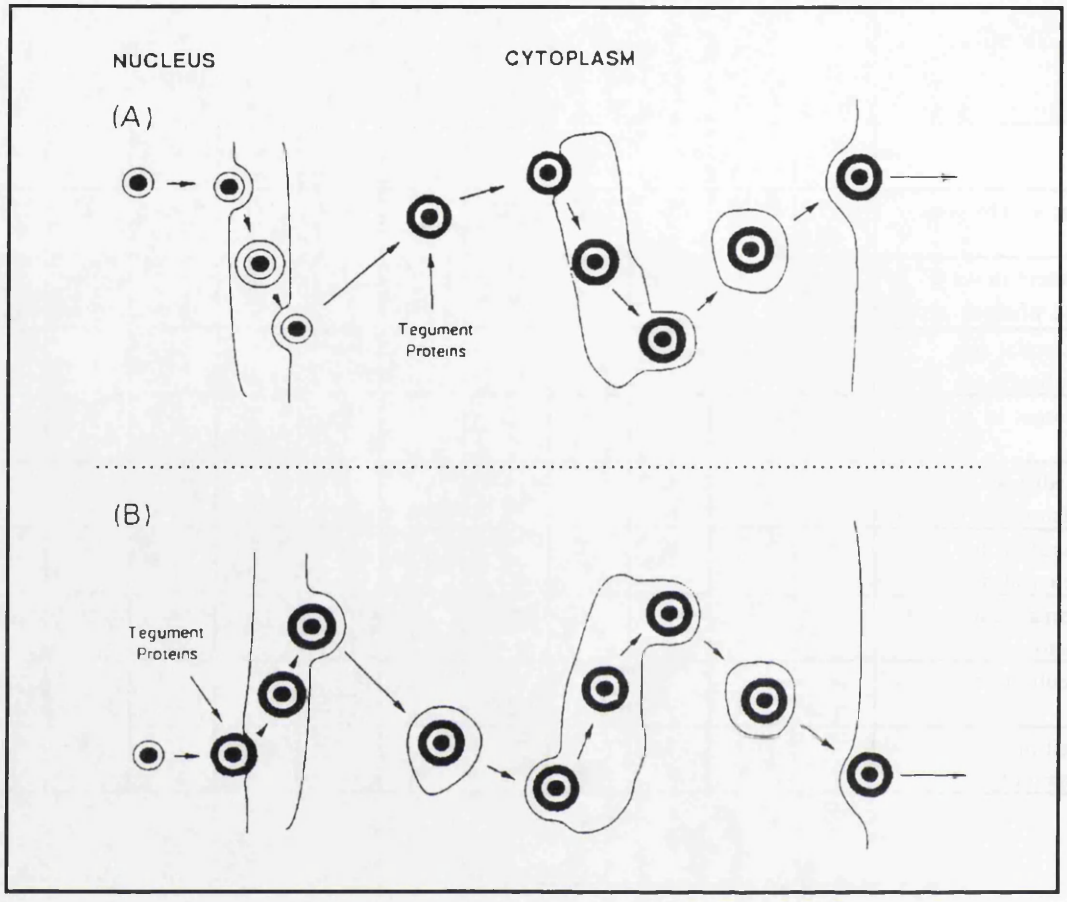


Fig. 11. Models for viral tegumentation, envelopment and egress.

Two models, (A) and (B), are proposed. In (A), assembled capsids bud into the inner nuclear membrane and then out into the cytoplasm, where they acquire the tegument and then the envelope by budding through a cytoplasmic membrane (probably the Golgi), or (B), they gain the tegument before budding through the inner nuclear membrane, and gain the membrane from here. The virion is then released from the cell by exocytosis. Reproduced from Rixon (1993).

Fiume *et al.*, 1988; Johnson and Spear, 1989). Also, infection of cells can be prevented by prior exposure to UV-inactivated *wt* virus but not a gD^- mutant (Ligas and Johnson, 1988). The interaction of gD with a receptor is required for virus entry but not for adsorption which occurs whether or not the gD receptors are blocked (Ligas and Johnson, 1988; Johnson and Ligas, 1988). The precise roles of gD, gB and gH in viral penetration are not known. Moreover, it is possible that tegument proteins may participate in the molecular interactions required for penetration, since a *ts* mutant of UL25, which encodes a tegument protein, is deficient in penetration at the NPT (Addison *et al.*, 1984).

1.8.5. Virus assembly

Assembly of the tegument can be uncoupled from capsid assembly (McLauchlan and Rixon, 1992), but the mechanisms by which tegument proteins come together is unknown. The preformed tegument probably joins to the capsid and then undergoes envelopment. The envelope is derived from altered host cell membranes containing viral glycoproteins which may require further processing to reach their mature state (Spear, 1985). Two models of viral egress have been proposed (Fig. 11). Assembled capsids may acquire the tegument in the cytoplasm and then the envelope by budding through a cytoplasmic membrane (probably the Golgi), or they may gain the tegument in the nucleus and gain the membrane by budding from here. The virion is then released from the cell by exocytosis (Rixon, 1993). Recently, Browne *et al.* (1996) presented evidence that viruses loose their initial membrane by fusion at the outer nuclear membrane endoplasmic reticulum and acquire their final membrane at the Golgi or post-Golgi compartment, as in model A (Fig. 11).

1.9. HSV-1 PATHOGENESIS

1.9.1. Animal models

HSV-1 causes a variety of diseases ranging from superficial cutaneous lesions to encephalitis in humans (Whitley, 1985). Various animal models have been used to study pathogenicity, including mice, rabbits and guinea pigs. Genes involved in pathogenesis *in vivo* have been analysed in these models by inoculation of *wt* virus or mutants at various sites: most commonly, the footpad, ear, eye, peritoneum or directly

into the cranium. The pathogenicity of mutants is usually expressed as the titre of virus required to kill 50% of animals. Severity of disease can also be assessed, by measuring weight loss, determining the physical appearance of the animal, and by quantification of the production of visible lesions. In this way a number of host factors important in pathogenesis have also been identified, including the immune response (McKendall *et al.*, 1979; Nash *et al.*, 1985), interferons (Lopez *et al.*, 1985), the condition of skin and mucous membranes (Sprecher and Becker, 1982), the age of the animal (Ben-Hur *et al.*, 1983) and the route of inoculation (Dix *et al.*, 1983).

1.9.2. Viral determinants

Many viral gene products affect pathogenicity, including glycoproteins, enzymes involved in DNA replication and repair, and the protein products of genes UL56, US1, RL2, UL48, UL41, UL46, UL47, US3 and RL1. The animal models used to date to identify the genes involved in pathogenesis and to determine their roles are detailed in Table 10 A-C. There is no doubt that there are more genes important in pathogenesis still to be identified. It should be noted that the avirulence of many of these mutants may be due to their poor replication ability.

Table 10 A-C summarises the studies that have been carried out using mutants to identify virulence determinants. All of the studies carried out for each gene have been included except for those where a large amount of data is available and two to four main studies have been discussed (UL23, UL39/40, RL1, RL2 and UL48). Most studies have been done using only a single mutant or variant, in 4-15 mice per dilution of virus, and results were usually reported for a single experiment. Many of the reports used intertypic recombinants between HSV-1 and HSV-2. These are of limited value since precise genotypes were not determined, and other mutations may have contributed towards the virulence phenotype. Other mutants tested contained insertions or deletions generated specifically, but the link between genotype and phenotype was not established by the use of revertants (i.e. mutants rescued *in vitro* to *wt*).

In Table 10 A-C it can be seen that a wide range of experimental procedures have been used to investigate the roles of genes in pathogenesis. Systems differ in the type of animal used, the site of inoculation, the strain of virus, the type of mutant and the titres tested. The age and general state of health of the animals is also an important variable. Many workers did not produce revertants, and thus the observed effect cannot be confidently linked to the interrupted gene. Also, in the latency experiments, many workers did not test the genotype of the reactivated virus and so cannot be sure that the

Table 10 A. Glycoprotein genes involved in pathogenesis

Gene	Type of mutant[a]	HSV-1 strain[b]	Animal[c]	Site[d]	Inoculum pfu/animal	Effect	Revertant[e]	References
US7 (gI)	*lacZ* in	SC16	M	ear	2×10^6	Virus hardly detectable in sensory ganglia or CNS. Severe attenuation.	+	Balan *et al.*, 1994
US8 (gE)	*lacZ* in	SC16	M	ear	2×10^6	Virus hardly detectable in sensory ganglia or CNS. Severe attenuation.	+	Balan *et al.*, 1994
UL10 (gM)	del	17+	M	IC FP	10^2-10^4 10^4-10^6	Established latent infection but impaired for growth at the periphery and for spread to or growth in neurons.	+	MacLean *et al.*, 1991; 1992; 1993
US6 (gD)	rec	ANG/KOS	M	FP	2×10^3	Avirulent. One residue change compared to virulent strain.	+	Kaerner *et al.*, 1983
UL27 (gB)	rec	KOS/ANG	M	FP	Not stated	Avirulent. One residue change compared to virulent strain.	+	Yuhasz and Stevens, 1993
US4 (gG)	del	F	M	IC; eye	Not stated	Reduced neurovirulence and reactivation from latency.	-	Meignier *et al.*, 1988.
UL53 (gK)	ir	F/*HFEM*	M	IC	1.35×10^5	Virus replicated in the brain but was not pathogenic. Three single residue changes compared to virulent strain.	-	Moyal *et al.*, 1992; Ramaswamy and Holland, 1992.

[a] HSV-2 strain is shown in italics where appropriate.

[b] *lacZ* in=insertion of *lacZ* gene into a gene; del=deletion; rec= intratypic recombinant (i.e. HSV-1:HSV-1); ir=intertypic recombinant (i.e. HSV-1:HSV-2).

[c] M=mouse

[d] FP=foot pad; IC=intracranial.

[e] +=revertant produced and behaved as *wt* unless stated; -=revertant not produced.

Table 10 B. DNA replication genes involved in pathogenesis

Gene	Type of mutant[a]	HSV-1 strain[b]	Model[c]	Site[d]	Inoculum pfu/animal	Effect	Revertant[e]	References
UL30 (DNA polymerase)	1 ir 2 dr	1 17+/*186* 2 SC16	M	1 eye 2 ear; flank	1 3 x 10^5 2 Not stated	1 *Wt* HSV-1 grows 100 fold better than *wt* HSV-2 in TG following eye inoculation. Recombinant grows as well as HSV-1. 2 Mutant killed 50% fewer animals than *wt*.	1 - 2 + (virulence not fully recovered)	1 Oakes *et al.*, 1986; Day *et al.*, 1987 a, b 2 Larder *et al.*, 1986
UL5 (component of helicase /primase complex)	ir	17+/*HG52*	M	IC	1 Not stated 2 5 x 10^3	1 10^4-fold less neurovirulent than either parental virus. Replicated to normal titres in mouse brain. 2 Avirulent, restricted replication in neurons but established latency efficiently	1 + 2 +	1 Javier *et al.*, 1988 2 Bloom and Stevens, 1994
UL23 (TK)	1 del 2 del; dr 3 dr	1 SC16 2 KOS 3 KOS,17+, B2006,C1101, Pat+	M	1 ear 2 eye 3 eye	1 5 x 10^4 2 2 x 10^6 3 Not stated	1 Not essential for establishment of latency but failed to reactivate. Avirulent. 2 Failed to reactivate. High titre in eye but low titre in TG. 3 No replication in TG. 100-fold reduced titre in ganglion tissue.	1 - 2 + 3 -	1 Efstathiou *et al.*, 1989 2 Coen *et al.*, 1989 3 Tenser *et al.*, 1981
UL39/UL40 (Ribonucleotide reductase)	1 *ts* (39;40) 2 del (39) 3 del; (39) *lacZ* in (39)	1 17+ 2 KOS 3 KOS	1 M 2 M 3 GP	1 IP; IC 2 eye 3 ID	1 4 x 10^7 - 5x 10^8 2 2 x 10^6 3 3 x 10^6	1 10^6-fold reduction in virulence. 2 Poor replication in the eye and TG. Failed to establish reactivatable latent infection. 3 Unimpaired neurovirulence.	1 + (virulence not fully recovered) 2 + 3 -	1 Cameron *et al.*, 1988 2 Goldstein and Weller, 1988a; Jacobson *et al.*, 1989 3 Turk *et al.*, 1989
UL50 (dUTPase)	del	17+	M	IC FP	Not stated 10^8	10-fold (IC) or 1000 fold (FP) reduced virulence. Established latent infection but failed to reactivate efficiently.	+ (virulence not fully recovered)	Pyles *et al.*, 1992
UL2 (uracil-DNA glycosylase)	in	17+	M	IC; eye	10^6	Replicated well at the periphery and in the CNS. Established latent infection but showed reduced reactivation frequency. 10^5-fold reduced neurovirulence.	+	Pyles *et al.*, 1994

[a] HSV-2 strain is shown in italics where appropriate.

[b] in=insertion; *lacZ* in=insertion of *lacZ* gene into a gene; del=deletion; ir=intertypic recombinant (i.e. HSV-1:HSV-2); dr=drug resistant; *ts*=temperature sensitive.

[c] M=mouse; GP=guinea pig.

[d] FP=foot pad; IC=intracranial; IP=intraperitoneal; ID=intradermal.

[e] +=revertant produced and behaved as *wt* unless stated; -=revertant not produced.

Table 10 C. Other genes involved in pathogenesis

Gene	Type of mutant[a]	HSV-1 strain[b]	Model[c]	Site[d]	Inoculum pfu/animal	Effect	Revertant[e]	References
UL56	1 rec 2 del	1 HFEM/F 2 F	1 M; TS 2 M	1 IP 2 IP	1 Not stated 2 10^6	1 Recombinant containing the UL56 from the virulent strain was virulent in IP inoculations in both models. 2 Not required for virulence or establishment of latency	1 + 2 -	1 Rosen *et al.*, 1986; Becker *et al.*, 1986; Rosen *et al.*, 1985 2 Nash and Spivack, 1994
US1 (Vmw68)	del *lacZ* in	F	M; GP	IC; IP; eye; IV	10^7; 1.6 x 10^8; 5 x 10^8	Contributes to virulence and to the ability to replicate at the site of infection and invade and establish reactivatable latent infection.	-	Poffenberger *et al.*, 1994
RL2 (Vmw110)	1 del 2 del 3 del	1 17+ 2 KOS; 17+ 3 KOS	M	1 IC; FP 2 eye 3 eye; IP	1 1-5 x 10^4 2 2-2 x 10^7 3 Not stated	1 Reduced virulence compared to *wt*. Able to establish and reactivate from latency (reduced efficiency). 2 Replicates in eye and ganglia. Able to establish latency but no spontaneous reactivation after explantation. Induced to reactivate by DMSO treatment. 3 Less virulent than *wt* but can establish and reactivate from latency as efficiently as *wt*.	1 + 2 - 3 +	1 Clements and Stow, 1989 2 Leib *et al.*, 1989 3 Davido and Leib, 1996
UL48 (Vmw65)	1 in 2 del	17+	M	1 IC; IP 2 eye	1 Not stated 2 1 x 10^5	1 Much less virulent that *wt* (100-1000 fold reduction). 2 Mutant does not replicate in the eye but does form latent infection in TG and reactivates as rapidly as *wt*. Reduced virulence.	1 + 2 +	1 Ace *et al.*, 1989 2 Steiner *et al.*, 1990
UL41 (*vhs*)	1 term 2 del	1 Not stated 2 KOS	M	1 eye; IC 2 eye	1 Not stated 2 2 x 10^6	1 Poor growth in TG and brain and low reactivation frequency. 2 Reduced replication in corneas and reduced reactivation.	1 + 2 -	1 Strewlow and Leib, 1994 2 Strewlow and Leib, 1996
UL46 or 47	del	F	M; R	eye; IC	Not stated	Ocular pathogenicity the same as *wt*. Less neurovirulent.	-	Romanowski *et al.*, 1991

US3 (protein kinase)	*lacZ* in	*186*	M	FP; IP; IC;eye	Not stated	Route-dependent avirulence (FP, IP). Establishes and reactivates from latent infection as efficiently as *wt* virus.	+ (virulence not fully recovered)	Nishiyama *et al.*, 1992
RL1 (ICP34.5)	**1** in; del **2** del; in; term **3** ir **4** del	**1** 17+ **2** F **3** *HG52* /17+ **4** 17+	M	**1** eye; FP **2** IC **3** IC, FP **4** FP; IC	**1** 10^6-3 x 10^7 **2** 10^6 **3** 10^8; 10^7-10^{-1} **4** 10^1-10^7	**1** Low titre in eye, no virus in TG. Limited replication and reactivatable infection in DRG. **2** Reduced virulence. **3** Non-neurovirulent; grew as well as *wt* at the periphery but not in the brain. Established latent infection. **4** Avirulent due to the inability to replicate in the brain. Can establish and reactivate from latency.	**1** - **2** + **3** + **4** +	**1** Spivack *et al.*, 1995 **2** Chou *et al.*, 1990 **3** Thompson *et al.*, 1989; Thompson and Stevens, 1983 **4** MacLean *et al.*, 1991

[a] HSV-2 strain is shown in italics where appropriate.

[b] in=insertion; *lacZ* in=insertion of *lacZ* gene into a gene; del=deletion; del *lacZ* in=insertion of *lacZ* gene in place of deleted HSV sequence; rec= intratypic recombinant (i.e. HSV-1:HSV-1); ir=intertypic recombinant (i.e. HSV-1:HSV-2); term= causes truncation of protein.

[c] M=mouse; TS=tree shrew; GP=guinea pig; R=rabbit.

[d] FP=foot pad; IC=intracranial; IP=intraperitoneal; IV=intravaginal.

[e] +=revertant produced and behaved as *wt* unless stated; -=revertant not produced.

mutant and not a revertant arising *in vivo* is reactivating. Thus care is needed when interpreting data from the current literature since the investigations may not have been sufficiently thorough. A few good studies have been reported, but many suffer from the failings described. A summary of the data (discussed in greater detail in Chapter 5) is described below.

1.9.2.1. Glycoproteins

Glycoproteins play an important role in the early stages of the virus life cycle, including adsorption and penetration (see Table 9). All of those investigated except gC (UL44) are required for growth in the CNS (Johnston *et al.*, 1986; Dix *et al.*, 1983; Sunstrum *et al.*, 1988), although only three have been shown to be essential for virus growth in cell culture (gB, gD and gH) (see Table 10A). Because of the role of glycoproteins in adsorption and penetration, the failure of mutants to cause pathology in mice may be due to the failure of cell-to-cell spread, so that mutants may be able to replicate at the periphery but are unable to spread to the CNS.

1.9.2.2. Proteins involved in DNA replication

The DNA polymerase (UL30), the UL5 protein (part of the helicase/primase activity), TK (UL23), RR (UL39/UL40), dUTPase (UL50) and the UL2 protein have roles in pathogenicity (see Table 10B). UL30 variants are less neurovirulent than *wt*, UL5 mutants are also avirulent but establish latency efficiently. TK is not essential for the establishment of latency but mutants fail to reactivate. RR mutants are less virulent and cannot form reactivatable latent infections in mice, but neurovirulence is not impaired in the guinea pig. A dUTPase mutant has a reduced ability to establish and reactivate from latency and is less virulent, but it has been established that the mutation also affected the UL49A promoter. A UL2 mutant is avirulent, can establish latency but reactivates poorly.

1.9.2.3. Others

Table 10 C lists other genes involved in virulence and latency.

UL56

Analysis of an intertypic recombinant showed that UL56 is involved in neurovirulence, but an insertion mutant was later found to be as virulent as *wt* by IP inoculation and

could establish and reactivate from latency normally. Further investigation is required to determine the true role of the UL56 protein *in vivo*.

US1

Use of a Vmw68 mutant indicated that US1 contributes to virulence, to the ability of the virus to replicate at the site of infection and to the ability to establish and reactivate from latent infection.

RL2

Vmw110 mutants show reduced virulence compared to *wt*. They can establish latency but Vmw110 is thought to be important for reactivation. Several groups have come to different conclusions, one Vmw110 mutant failed to reactivate without induction, another reactivated but at a reduced efficiency while a third reactivated the same as *wt*. In an *in vitro* latency system (see Section 1.10) reactivation only occurs when Vmw110 is available.

UL48

Investigations using the Vmw65 mutant *in*1814 (see Section 1.10) have revealed that the mutant can establish latency and reactivate normally. The virulence is greatly reduced compared to *wt*.

UL41

Vhs mutants did not grow as well as *wt* in the brains of mice, and established and reactivated from latent infection at a reduced level.

UL46 or UL47

The use of mutants with a deletion in UL46 or UL47 revealed that although these genes are non-essential in cell culture, they are required for neurovirulence. The UL46 and UL47 proteins are involved in the modulation of Vmw65 activity, and the effect may be linked to this role since Vmw65 mutants are avirulent. The mutants have no effect on ocular pathogenicity.

US3

A *lacZ* insertion mutant of HSV-2 has shown route-dependent avirulence, being virulent *via* the eye or IC but avirulent *via* FP or IP inoculation. The mutant can establish and reactivate from latency as efficiently as *wt*. An HSV-1 mutant has yet to be analysed *in vivo*.

RL1 (ICP34.5)

Thompson *et al.* (1983) located a determinant of neurovirulence in HSV-1 R_L. The gene involved, RL1 ($\gamma_1$34.5) encodes a protein of 284 residues in strain F (Chou and Roizman, 1990) and 263 residues in strain 17^+ (Dolan *et al.*, 1992).

In cell culture, RL1 mutants replicate as *wt* viruses on some cell lines (e.g. Vero cells) whereas on others, including the human neuroblastoma cell line SK-N-SH, host and viral protein synthesis is inhibited early in infection and all protein synthesis ceases several hours before normal completion of the replicative cycle. Also, growth of mutants is impaired on mouse embryo fibroblast 3T6 cells (Chou and Roizman, 1994).

The RL1 protein (ICP34.5) is expressed early in infection, although the use of inhibitors of DNA synthesis reduced production slightly. The C-terminal domain possesses striking homology to a mouse protein MyD116 induced during cell differentiation (McGeoch and Barnett, 1991), a Chinese hamster protein, GAAD34, which is induced by growth arrest and DNA damage (Chou and Roizman, 1994) and an African swine fever virus protein which is thought to have a role in host range determination (Sussman *et al.*, 1992). These homologous genes may have a role in blocking apoptosis in terminally differentiated cells.

ICP34.5 is a major neurovirulence determinant that seems to work by limiting the response of the host cell to infection in preventing the stress response which normally leads to total shut down of protein synthesis and programmed cell death (Chou and Roizman, 1992). Recently He *et al.* (1996) have shown that the C-terminus of MyD116 can substitute for the corresponding domain of ICP34.5 and successfully preclude premature shut-off of total protein synthesis.

In the absence of ICP34.5, HSV-1 fails to multiply in the CNS of mice following intracerebral inoculation, although limited replication was detected in the urogenital tract after intravaginal inoculation (Spivack *et al.*, 1995). Mutants also fail to replicate in the neurons of the peripheral nervous system although they are capable of establishing and reactivating from latency (Robertson *et al.*, 1992). Spivack *et al.* (1995) investigated the behaviour of ICP34.5 mutants in a mouse model of latency, and concluded that ICP34.5 may have a role in reactivation that is independent from LAT. Perng *et al.* (1995) reported the involvement of ICP34.5 in virulence using the rabbit eye model. A deletion mutant appeared to establish and maintain latency but with much lower efficiency than *wt* virus, and ICP34.5 was not essential for spontaneous reactivation.

The situation concerning RL1 is complicated by the recent identification of a novel expressed open reading frame, ORF P, on the opposing strand, which has 248 codons and is almost completely antisense to RL1 (Lagunoff and Roizman, 1994). Mutations in RL1 will also interrupt ORF P and so the effects observed with RL1 mutants may not be due to disruption of RL1 alone.

1.10. HSV-1 LATENCY

All herpesviruses examined to date have the ability to establish and maintain latent infections (Roizman and Sears, 1987) and can exist for extended periods of time in infected individuals. The basic concept of HSV-1 latency is the maintenance of circular or concatameric, extrachromosomal viral DNA in the nuclei of neuronal cells and limited transcription from these genomes. Latency can be divided into several stages: viral replication at the peripheral site of infection, transport of the viral particles to the nervous system, establishment of latent infection, maintenance of the latent state and reactivation. In true latent infection, virus cannot be detected until reactivation and replication take place, whereas persistent infection produces a low level of virus throughout infection, often with asymptomatic shedding.

1.10.1. Animal models

Several animal models have facilitated studies of HSV-1 latency, the most frequently used of which are mice, rabbits and guinea pigs. It is important to note, however, that no single model can mimic the situation in humans.

Following inoculation of the animal with HSV-1, latency is established in tissues enervating the site of inoculation. Latent virus can then be reactivated by exposing the animal to various stimuli, including UV light or heat shock, or by explantation of the ganglia and cocultivation with permissive cells. The models of latency described in Table 11 (also see Table 10A-C) have been valuable for examining latency and host-virus interactions and have enabled genes with important roles in viral pathogenesis and host responses to be investigated.

Table 11. Animal models of latency

Reference	Animal[a]	Site[b]	Reactivation factors	Features of system
Hill *et al.*, 1975; 1978.	M	Ear	Some spontaneous reactivation, increased by UV light, ice or tape stripping of the skin of the ear.	Latent infection established in cervical ganglia and DRG. Reactivation characterised by erythma or vesicles at inoculation site.
Sawtell and Thompson, 1992a.	M	eye, FP, S	Hyperthermic stress at 43°.	Latent infection in lumbosacral DRG and TG. Reactivation in only a small proportion of neurons.
Fawl and Roizman, 1993.	M	eye	Cadmium administration.	Only $CdSO_4$ and no other metallothioneins induced reactivation.
Shimeld *et al.*, 1989.	M	eye	Iontophorosis with cyclophosphamide and dexamethasone.	Treatment causes ocular shedding of virus in 50% of animals.
Cook *et al.*, 1991a, b; Kwon *et al.*, 1981; Haruta *et al.*, 1989.	R	eye	Iontophorosis as above, also with adrenaline.	Small percentage of spontaneous ocular shedding and induced shedding with treatment. Latent infection in cornea cells and TG.
Stanberry *et al.*, 1985a,b; 1992.	GP	IV, IU	No treatment required.	Self-limiting primary infection. Latent infection established in lumbosacral DRG. Spontaneous reactivation.

[a] M=mouse; R=rabbit; GP=guinea pig
[b] FP=foot pad; S=snout; IV=intravaginal; IU=intraurethral

1.10.2. Sites of latency

Goodpasture (1929), suggested that HSV-1 remains in the ganglia in a latent state after the local lesion has healed. Later, HSV-1 was isolated from human peripheral sensory ganglia (PSG) (Stevens and Cook, 1971), the trigeminal ganglia (TG) and spinal ganglia, the autonomic nervous system (Price *et al.*, 1975), the brainstem (Deatly *et al.*, 1988) and the cerebral hemispheres (Fraser *et al.*, 1981). The possibility of extraneuronal latency has been investigated in experimental animals, and HSV-1 was demonstrated in the skin (Clements and Subak-Sharp, 1988), cornea (Abgharis and Stulting, 1988) and adrenal medulla (Stevens, 1978). However, disorders caused by reactivation usually result from reactivation from nervous tissue.

Following peripheral inoculation of virus, HSV-1 DNA can be detected in the relevant PSG. Viral replication at the periphery is not essential for this process, since replication-deficient mutants can reach the PSG and establish latent infection in ganglia (Steiner *et al.*, 1990). Once the virus reaches the PSG it can resume replication and infect neurons as well as satellite cells in the PSG. Following a full replication cycle in PSG neurons, infectious particles can leave the neuron to infect satellite cells within the ganglion and proceed by retrograde axonal transport to the central nervous system (CNS) where the same pattern of replication takes place. In the CNS the virus can also spread transneurally *via* synapses. After a period of 10-14 days following primary infection, no replicating or infectious virus can be detected in the nervous system of the infected animal and the virus is deemed to have entered the latent state.

Replication is thought to be initially dependent on the presence of sufficient amounts of Vmw65 which enables initiation of the replication cascade. Studies with a mutant, *in*1814, which contains a 12 bp insertion in Vmw65 rendering it unable to form the IE transactivation complex and stimulate IE transcription but not impairing the structural role of the protein (Ace *et al.*, 1989), suggest that replication occurs in the nucleus only when sufficient amounts of Vmw65 are available in the cell body, a process which results in cell destruction. The lack of Vmw65 aborts replication at a very early stage of infection (Valyi-Nagy *et al.*, 1991a,b) and enables the infected cell and thus the latent HSV-1 genome to survive.

1.10.3. State of the latent genome

The entire genome of HSV-1 has been detected in the ganglia of latently infected mice by Southern blotting (Rock and Fraser, 1983, 1985). The latent DNA is maintained as a circular molecule or in a concatemeric form (Rock and Fraser, 1983, 1985; Efstathiou

et al, 1986) and is not integrated into the host genome (Mellerick and Fraser, 1987). Partial micrococcal nuclease digestion has demonstrated that latent DNA is associated with nucleosomes in a pattern similar to that of the host nuclear chromatin structure (Deshmane and Fraser, 1989). It has been suggested that the nucleosomes may be necessary for the long term stability of the genome and may have some involvement in silencing of the genome during latency.

Estimates of the number of copies of the viral genome per latently infected cell range from 0.01-1, but these are probably underestimates. The importance of copy number is not known, but it has been suggested that a low number is associated with viral inability to initiate replication within the nucleus, whereas a high number may facilitate reactivation (Roizman and Sears, 1987; 1990; Fraser *et al.,* 1991). *In vitro*, a Vmw65 mutant (Ace *et al.,* 1989) and a Vmw68 mutant (Sears *et al.,* 1985) are both replication-deficient, a condition which can be overcome by increasing the moi in cell culture.

1.10.4. Establishment and maintenance of latency

The virus reaches the neuronal cell body by travelling along the axon from the periphery or after replication in an adjacent cell. The lytic viral replication cycle will eventually shut off host cellular protein synthesis and result in cell death, and so latency cannot be established in cells in which the virus has completed a full replication cycle (Steiner and Kennedy, 1991). Viral or cellular factors that interfere with viral replication will therefore favour the establishment of latency. During transport to the nucleus (Lycke *et al.,* 1984; 1988) the amount of Vmw65 that is lost may determine the fate of the infection. If sufficient remains, lytic replication and cell destruction will occur, but if there are insufficient amounts, the lytic replication cascade will not begin and a latent infection will be formed.

Latchman *et al.* (1989) suggested that cellular factors have an important role in facilitating the establishment of latency. For example, a cellular promoter may compete for some limiting factor required for transactivation by Vmw65. The role of Vmw65 in the virus lifecycle is to direct the limited amount of Oct1 in the infected cell away from cellular genes and towards TAATGARAT-containing genes, allowing transcriptional activation of the IE genes to occur. The failure of IE gene expression in neuronal cells is likely to be caused by differences in the nature of cellular transcription factors. The factor which has been identified as the lymphocyte- and neuron-specific octamer binding protein (Oct2), is present in DRG neurons, the natural site of HSV-1 latency *in vivo* (Lillycrop *et al.,* 1991, 1994). Experimentally, raised levels of Oct2 can

repress the IE promoter by preventing binding of transactivating Oct1 protein to TAATGARAT (Perry *et al.*, 1986), or by virtue of the fact that Oct2 cannot form a complex with Vmw65 (Gerster and Roeder, 1988). It is not known which viral functions are required for the establishment of latency or if any viral genes are expressed at this stage.

Neither the TK gene (Efstathiou *et al.*, 1989; Coen *et al.*, 1989) or the IE proteins Vmw110, Vmw175 or Vmw63 (Leib *et al.*, 1989a,b; Clements and Stow, 1989) are required for the establishment of latency, but the deficient replication of such mutants may render the reactivation inefficient. There are a number of complex viral and cellular interactions which likely to be involved in the establishment and maintenance of latency, but they are not yet fully understood.

1.10.5. Gene expression during latency

The only region of the genome which is transcribed during latency is located largely within R_L (Stevens *et al.*, 1987, 1988) and specifies RNAs which accumulate during latency (Rock *et al.*, 1987). The LATs are transcribed in the opposite direction from RL2 (encoding Vmw110), overlaping the RL2 mRNA at its 3' end by approximately 700 bp (see Fig. 12B). The LATs have been shown to consist of several RNA species. A large 8 kb RNA, which has been detected in latently infected TG (Zwaagstra *et al.*, 1990), is considered to be the primary transcript from which the other LATs are derived (Fig. 12D) (Deatly *et al.*, 1987; 1988). Three collinear LATs of 2, 1.5 and 1.45 kbp have been detected by Northern blot analysis of RNA isolated from latently infected ganglia (Spivack and Fraser, 1987). Only the 2 kbp LAT has been detected during viral replication in cell culture and at much lower levels than are present in latency (Krause et al, 1988). This LAT is thought to be a stable intron formed by splicing of the 8 kbp primary transcript (Farrel *et al.*, 1991; Devi-Rao *et al.*, 1991), and the 1.5 and 1.45 kb LATs are thought to be spliced products from the 2 kb transcript (Wagner, 1988b). However, if the 2 kb LAT is an intron then this situation is unusual, since introns are not usually further processed and the 6 kb LAT RNA which would result from splicing of the 8 kb LAT to remove the 2 kb intron has not been identified.

The LAT promoter (see Fig. 12 C) found upstream of the 5' end of the 2 kb LAT (Batchelor and O'Hare, 1990) controls the entire region transcribed during latency, since mutants with lesions within the promoter do not have any detectable LATs produced (Steiner *et al.*, 1989). The region contains binding sites including a TATA box, CAAT and CREB sites (Batchelor and O'Hare, 1990), and there are potential

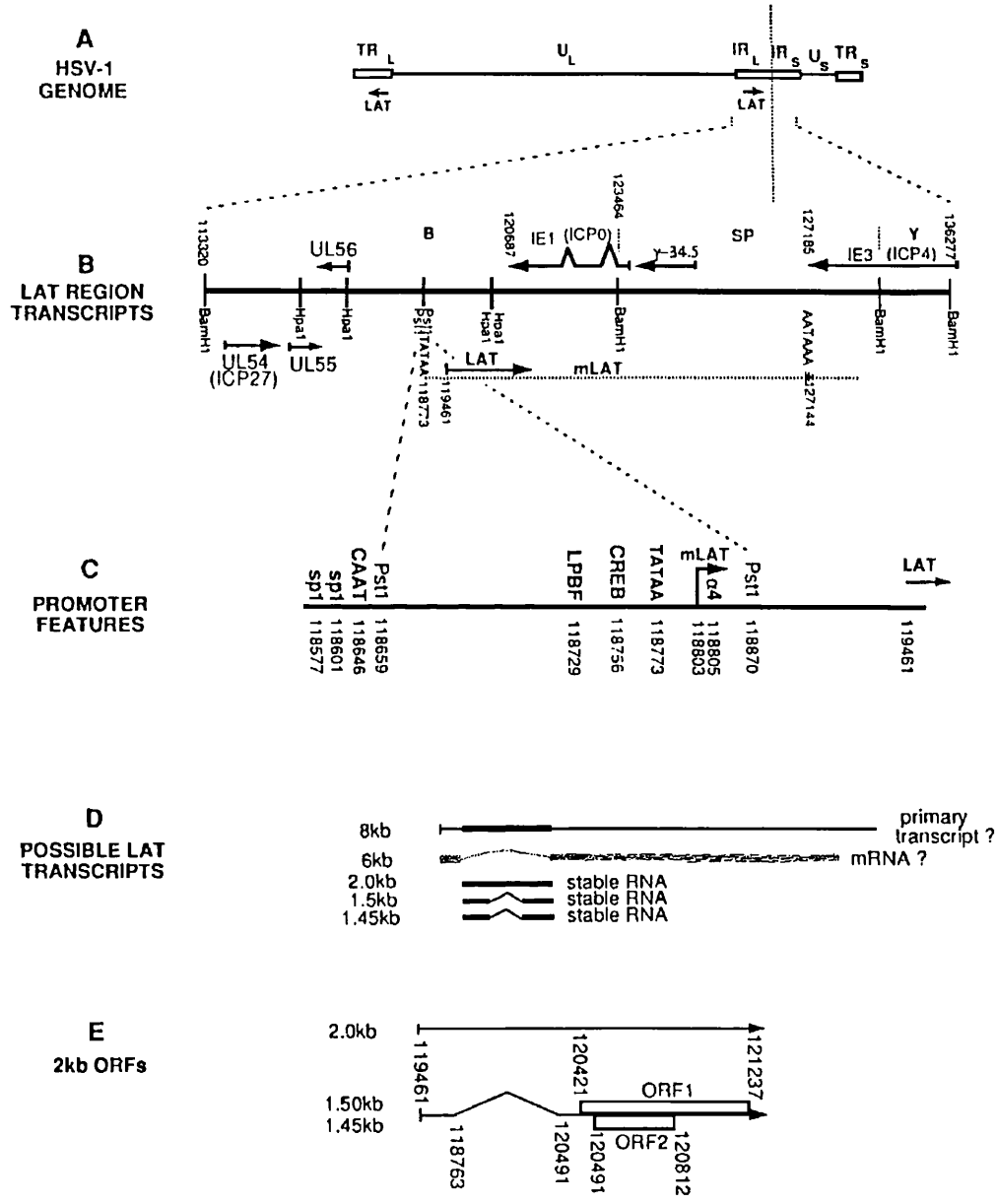


Fig. 12. Transcripts expressed during latency.

A shows the HSV-1 genomic structure, B, the transcripts in the LAT region in more detail, and C, the LAT promotor elements. D shows the possible LAT transcripts, and E, the possible open reading frames within the LAT transcripts although no protein has been reliably detected. Reproduced from Fraser *et al.* (1992).

binding sites for Vmw175 and Vmw65. The activity of the promoter is repressed by Vmw175 but there is little effect by Vmw65 (Batchelor and O'Hare, 1990).

1.10.6. Function of the LATs

The initial hypothesis about the function of the LATs was that that they are antisense inhibitors of RL2 (Stevens *et al.*, 1987). Thus, they could inhibit the expression of Vmw110, shut off viral replication and promote maintenance of latency. This hypothesis implied that LAT$^-$ mutants would replicate better, express higher levels of Vmw110, fail to establish latent infections or reactivate more efficiently than *wt* virus. LAT mutants, however, are not impaired in their ability to establish and maintain latency (Deshmane *et al.*, 1993), replicate normally *in vivo* and in cell culture, express normal levels of Vmw110 (Leib *et al.*, 1989b; Steiner *et al.*, 1989) and are able to reactivate from latency normally or somewhat less well than *wt* viruses (Steiner *et al.*, 1989; Block *et al.*, 1990). Therefore, the role of LATs as antisense RNAs has been dismissed.

A second possibility is that the LATs could act *via* a translation product (see Fig. 12E). Two ORFs are located at the 3' end of the LATs, beyond the proposed intron and splicing site of the 2 kb LAT. They have been shown to encode 30 kDa and 12 kDa polypeptides by *in vitro* transcription-translation (Spivack *et al.*, 1991), but these proteins have not been detected during HSV-1 replication or latency. In an *in vitro* latency model, an 80 kDa protein from the region including part of ORF 1 was identified (Doerig *et al.*, 1991a), but the size is not consistent with the sequence data. Also, these ORFs are not conserved between HSV-1 and HSV-2 (McGeoch *et al.*, 1991). It remains to be seen if this or any other protein is expressed during latency *in vivo*.

Thus, the function of the LATs has not yet been determined.

1.10.7. Reactivation of latent infection

Reactivation of HSV-1 and HSV-2 in humans results in cold sores or genital lesions respectively, and can be triggered by a number of stimuli including injury to the tissue innervated by the neuron harbouring the latent infection, exposure to UV light, or certain stress situations (Hill *et al.*, 1975). However, reactivation is not associated with significant damage or destruction of latently infected neurons and so must differ from the mass cell destruction observed in cell culture during HSV-1 lytic infection. The

main question posed by the phenomenon of reactivation concerns how replication begins in the absence of Vmw65 which normally initiates IE gene expression. It has been shown that lack of functional Vmw65 prevents reactivation in cell culture (Ace *et al.*, 1989), but explant reactivation of the mutant *in*1814 from latently infected mouse TG occurs similarly to *wt* (Steiner *et al.*, 1990). It is possible that an increase in the number of HSV-1 genomes per latently infected cell overcomes the lack of Vmw65 in the same way that the mutant can replicate in cell culture at high moi (Roizman and Sears, 1990). The observation that compounds such as HMBA and DMSO can enhance the reactivation of HSV-1 (Leib *et al.*, 1989a) and improve the replication of *in*1814 in cell culture (McFarlane *et al.*, 1992) suggests that they can replace the transactivation function of Vmw65. Thus, it is possible that viral or cellular factors act similarly.

The LAT promoter contains a cAMP-responsive element, and cAMP has been shown to increase reactivation of *wt* HSV-1 viruses (Leib *et al.*, 1991), indicating that reactivation might occur *via* second messenger signal transduction. In one *in vitro* system, nerve growth factor (NGF) is required to maintain the latent state, and removal of the compound results in reactivation (Wilcox and Johnston, 1987, 1988). Also, NGF mRNA has been detected in the peripheral sensory ganglia (PSG) of latently infected mouse ganglia post explantation, at the same time as reactivation was occurring (Valyi-Nagy *et al.*, 1991b). Thus, NGF and cAMP may be implicated in reactivation, but their role, if any, requires clarification.

1.10.8. *In vitro* models

There are two main features required in an *in vitro* latency system if it is to mimic the situation *in vivo*: non-integrated viral DNA in an endless conformation and expression of the LATs. Many of the systems employed involve infection of cells in culture with HSV-1 under unnatural conditions, including, the use of *ts* mutants at the NPT or addition of agents such acyclovir which prevents viral DNA replication and cyclohexamide which prevents protein synthesis (Wigdahl *et al.*, 1982; Russell and Preston, 1986; Cook and Brown, 1987; Wilcox and Johnson, 1987). Owing to these features, results from such systems need to be considered cautiously. None of the *in vitro* systems described in Table 12 mimics the *in vivo* situation exactly, but the ability to reactivate latent genomes simultaneously is useful in characterising the establishment, maintenance and reactivation of latency at the molecular level.

Table 12. *In vitro* models of latency

References	Cell type	Inhibitors which induce/maintain the latent state	Induction of reactivation	Features of the system
Wilcox and Johnson, 1987; 1988; Wilcox *et al.*, 1990; Doerig *et al.*, 1991a,b.	Sensory neurons from DRG of rats, monkeys or humans.	Acyclovir.	Removal of NGF. Activation of phosphokinase C or cAMP pathways.	100% of neurons reactivate and the 2 kb LAT is expressed. Structure of latent genome is unknown. Unconfirmed report of a LAT-encoded protein.
Nilheden *et al.*, 1985 a, b.	C1300 mouse neuroblastoma cells.	Treat cells with neutralising HSV-1 antibody.	Superinfection with HSV-2.	Genome is maintained in an endless state.
Wigdahl *et al.*, 1982; 1983; 1984.	Human embryo lung fibroblasts or rat foetal neurons.	(E)-5-(2-bromovinyl)-2'-deoxyuridine plus IFNα, incubation at 40.5°.	Remove inhibitor and incubate at 37°, or superinfect with HCMV.	Genome is non-integrated and linear. LATs are not expressed.
Russell and Preston, 1986; Harris *et al.*, 1989.	Human foetal lung cells (HFL).	Low moi infection, incubation at 42°.	Superinfection with an HSV-1 *ts* mutant or *wt* HCMV.	Genome is non-linear. No inhibitors are required.
Harris and Preston, 1991.	HFL.	Use of the Vmw65 mutant *in*1814 at low moi plus AraC teatment. Incubate at 37° or 42°.	Superinfection with a virus expressing Vmw110.	Genome is non-linear. LATs are not expressed.
Jamieson *et al.*, 1995; (CM Preston and LH Robinson, unpublished data).	HFL.	Use of the Vmw65/Vmw110 mutant *in*1820 at high moi, plus IFNα and AraC treatment.	Superinfection with a virus expressing Vmw110.	Genome is non-linear, and associated with nucleosomes. LATs are not expressed.

Despite the fact that the latency systems of Preston and co-workers do not express LATs, they do have several advantages over the inhibitor-induced systems. Incidentally, the absolute requirement for the expression of LATs in latency has not been determined, and it is thought that many latently infected neurons do not express LATs (Ramakrishwan, 1994; Mehta *et al.*, 1995). In the first system, the use of high temperatures avoided the use of inhibitors, the latent state was stable, a high proportion of cells harboured a reactivatable infection, the latent genomes were non-linear and Vmw110 was the sole requirement for reactivation (Russell and Preston, 1986). Later, the use of the Vmw65 mutant *in*1814 (Ace *et al.*, 1989) which establishes latency in HFL cells (Harris and Preston, 1991) and a mutant (*in*1820) which has the Vmw65 insertion and also behaves like a Vmw110 mutant in HFL cells have been useful models (Jamieson *et al.*, 1995). These represent useful tools in the study of the molecular aspects of latency and reactivation without the added complications of host responses to infection *in vivo*.

1.11. AIMS OF THE THESIS

1.11.1 Background

Genes

HSV-1 UL14, UL15, UL16 and UL17 are part of a block of genes (UL10 to UL19) which is conserved among the alpha-, beta- and gammaherpesviruses (Davison, 1993). They are expressed as late mRNAs (McGeoch and Schaffer, 1992).

UL15 is the only spliced gene in U_L (McGeoch *et al.*, 1988). UL15 homologues in the three herpesvirus subfamilies have an equivalent structure with two exons, and a variable number of genes (2-4) in the intron (Dolan *et al.*, 1991). In contrast, the proposed counterpart in CCV has three exons, and the boundary between the first and second exons corresponds precisely to that in other herpesviruses (Davison, 1992). Construction of an HSV-1 mutant expressing UL15 without the intron has shown that separation of the two exons is not essential for replication of HSV-1 in cell culture (Baines and Roizman, 1992b). The effect of the absence of the intron in an animal model has not been reported.

The UL15 protein contains potential ATP-binding motifs (Dolan *et al.*, 1991), and a possible function was suggested by Davison (1992) who noted that the UL15 protein may be related to a protein encoded by T4 bacteriophage gene 17. This protein is part of the phage terminase, or DNA packaging protein complex, and mutations result in the arrest of phage production during DNA packaging, leading to an accumulation of empty proheads and uncleaved concatemeric DNA (Bhattachorgga and Rao, 1993; Powell *et al.*, 1990; Carrascose and Kellenberge, 1978).

During the course of this work, Poon and Roizman (1993) described a *ts* mutant with a lesion in UL15. The mutant produced viral DNA at the NPT, but failed to package it into capsids. In a subsequent paper, Baines *et al.* (1994) showed that the UL15 gene encodes two proteins (one is a truncated form of the full length UL15 protein, similar to the situation with UL26 and UL26.5, see Section 1.8.2). The full length protein is necessary for cleavage and packaging of viral DNA. The published data on UL15 are therefore consistent with the view that UL15 is a subunit of the herpesvirus terminase.

No mutants have been described for UL14 or UL17, and the roles of the protein products of these genes are not known. The status of both genes in cell culture is also unknown, although Baines and Roizman (1991) suggested from their inability to obtain a mutant that UL17 may be essential.

The function of the UL16 protein is unknown although it has been shown to be non-essential in cell culture (Baines and Roizman, 1991). Growth curves of a mutant lacking 988 bp of UL16 (1119 bp) indicated that yields of UL16$^-$ virus in Vero or BHK cells were reduced from those of *wt* (10- or 3-fold respectively) (Baines and Roizman, 1991).

Cosmids

The manipulation of large fragments of HSV-1 DNA is difficult, but in principle the cosmid system should permit easier manipulation and production of mutants. Mutants produced using a mutant cosmid set can be isolated in the absence of a background of *wt* virus, and even viruses which have multiple mutations or are at a severe growth disadvantage to *wt* can be isolated. A set of 5 cosmids, comprising cos32, cos24 (which contains genes UL14-17 in a region which does not overlap adjacent cosmids), cos 46 (which contains a proportion of ori_L^+ molecules), cos51 and cos48, which together contain the entire HSV-1 sequence were utilised (see Fig 13) to generate frameshift mutations in genes UL14-17 (further details in Section 4.1).

1.11.2 Aims

The aims of this thesis were firstly to modify an available cosmid set to enable the production of mutations in HSV-1 genes UL14 to UL17, and to produce mutant cosmids with 4 bp insertions in UL14, UL15 and UL17. The second stage was to ascertain whether UL14, UL15 or UL17 are essential by attempting to generate virus mutants on non-complementing cell lines, and to produce virus mutants in these genes on potential complementing cell lines where necessary. The third stage was to produce UL16$^-$ mutants and characterise their phenotypic features *in vitro* and *in vivo*. The last stage was to attempt to detect the UL14, UL16 and UL17 proteins.

Chapter 2

Materials

2.1. Cells and viruses

Wild type HSV-1 strain 17^+ (Brown *et al.*, 1973) and mutants derived therefrom were grown in baby hamster kidney clone C13 (BHK C13) cells (MacPherson and Stoker, 1962) or MeWo cells (Bean *et al.*, 1975). A number of other cell lines were used in the *in vitro* analysis of $UL16^-$ viruses, details are given in Table 17 Section 4.1.

2.2. Cell culture growth media

Components of media and some complete media were produced in house by the media department or were supplied by Gibco-BRL.

Eagles A:	0.23 g/l $CaCl_2.2H_2O$
	0.23 g/l $MgSO_4.7H_2O$
	0.1 ml/l conc HCl
Eagles B:	50% (v/v) salts/plus
	40% (v/v) amino acids/plus
	3.2% (v/v) vitamins
salts/plus:	10.24 g/l NaCl
	0.64 g/l KCl
	0.24 g/l $NaH_2PO_4.2H_2O$
	7.2 g/l glucose
	0.00016% (v/v) $Fe_2(NO_3)_3$
	0.468 g/l L-glutamine
	0.016% (v/v) penicillin
	0.16 g/l streptomycin
	0.00032% (v/v) amphotericin B (0.025%)
amino acids/plus:	0.84 g/l arginine mono HCl
	0.48 g/l cystine
	0.384 g/l histidine mono HCl
	1.048 g/l isoleucine
	1.048 g/l leucine
	1.462 g/l lycine mono HCl
	0.66 g/l phenylalanine
	0.952 g/l threonine
	0.16 g/l tryptophan

	0.724 g/l tyrosine 0.936 g/l valine 0.3 g/l methionine 0.07 g/l inositol 0.03% (v/v) phenol red 55 g/l $NaHCO_3$
vitamins:	0.05 g/l choline chloride 0.05 g/l folic acid 0.05 g/l nicotinamide 0.05 g/l pantothenic acid, Ca salt 0.05 g/l pyridoxal HCl 0.05 g/l thiamine HCl 0.005 g/l riboflavine
ETC10:	70% (v/v) Eagles A 10% (v/v) Eagles B 10% (v/v) tryptose phosphate 10% (v/v) newborn calf serum (NBCS)
ETF10:	As above with NBCS replaced by foetal calf serum (FCS)
ETC5:	As for ETC10 but with 5% (v/v) NBCS
EC2:	85.75% (v/v) Eagles A 12.25% (v/v) Eagles B 2% (v/v) NBCS
Methyl cellulose overlay:	39% (v/v) carboxymethylcellulose 1.56% (v/v) tryptose phosphate 3.9% (v/v) NBCS 24.3% (v/v) $NaCO_3$ (7.5%) 1.56% (v/v) penicillin/streptomycin (10000 IU/ml) 0.8% (v/v) L-glutamine (200mM) 0.08% (v/v) amphotericin B

	7% (v/v) 10 x Glasgow's modified medium
Emet$^-$:	7:1 (v/v) Eagles A:Eagles B lacking methionine
Emet$^+$:	Emet$^-$ containing: 20% (v/v) 7:1 (v/v) Eagles A:Eagles B 2% (v/v) NBCS
Ecys$^-$:	7:1 (v/v) Eagles A:Eagles B lacking cystine
Ecys$^+$:	Ecys$^-$ containing: 20% (v/v) 7:1 (v/v) Eagles A:Eagles B 2% (v/v) NBCS
Dulbecco's medium:	500 ml Dulbecco's modified Eagle medium 9% (v/v) FCS 1% (v/v) penicillin/streptomycin 1% (v/v) L-glutamine 1% (v/v) non-essential amino acids
Cell storage medium:	55.6% (v/v) Eagles A 7.2% (v/v) Eagles B 7.2% (v/v) tryptose phosphate 25% (v/v) FCS 10% (v/v) glycerol

2.3. Bacterial strains

Escherichia coli (*E. coli*) XL1-Blue (Stratagene), MAX efficiency DH5α and DH5α F'IQ competent cells were used (Gibco-BRL). The genotype of each is as follows: XL1-Blue - *recA, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac,* [F' *proAB*, *lacI*^{q}ZΔ*M15*, *Tn10*(*ter*^{r})]. DH5α - F$^-$, φ80d*lacZ*ΔM15, Δ(*lacZYA-arg*F), U169, *deoR*, *recA*1, *end* A1, *hsd*R17(r_K-, m_K-), *sup*E44, λ$^-$, *thi*-1, *gyr* A96, *rel*A1. DH5αF'IQ - F',

φ80d*lacZ*ΔM15, Δ(*lacZ*YA-*arg*F), U169, *deoR*, *recA*1, *end* A1, *hsd*R17(r_K-, m_K-), *sup*E44, λ-, *thi*-1, *gyr* A96, *rel*A1/F', *pro*AB+, *lac*I^{q}ZΔM15, *zzf*::Tn5[Km^r].

2.4. Bacterial growth media

2YT broth:	85 mM NaCl 1% (w/v) bactopeptone 1% (w/v) yeast extract
L-broth:	177 mM NaCl 1% (w/v) bactopeptone
L-broth agar:	1.5% (w/v) agar in L-broth
Top agar:	0.6% (w/v) bacto-agar in H_2O
SOC medium:	2% (w/v) bactopeptone 0.5% (w/v) yeast extract 10 mM NaCl 25 mM KCl 10 mM $MgCO_3$ 10 mM $MgSO_4$ 20 mM glucose
TSB:	L-broth containing: 10% (w/v) PEG 3350 10 mM $MgCl_2$ 10 mM $MgSO_4$
TSB/DMSO	TSB containing 5% (v/v) DMSO
TSB/glucose:	TSB containing 20 mM glucose

2.5. Radiochemicals

These were obtained from the Radiochemical Centre, Amersham.

Hexaprimer and nonaprimer kits for radiolabelling DNA probes were obtained from Appligene (details of the labelling process is given in Section 3.14).

2.6. Enzymes

All restriction endonucleases and buffers were obtained from Bethesda Research Laboratories (BRL), New England Biolabs or Boehringer Mannheim.

Lysozyme (3 x crystallized, dialysed and lyophilized) and ribonuclease A (type II-A; DNAse free) were obtained from Sigma Chemical Co.

Trypsin (modified, sequencing grade) was obtained from Promega or Boehringer Mannheim.

2.7. Chemicals

The chemicals used were of analytical grade and most of these were supplied by BDH Chemicals or Sigma Chemical Co.

The exceptions were:

Geneticin G-418 sulphate: Gibco BRL.

Ampicillin (Penbritin): Beecham Research.

Ammonium persulphate (APS) and N,N,N',N'-tetramethylethylenediamine (TEMED): Bio-Rad Laboratories Ltd.

Sulforhodamine B: Kodak.

Coomassie brilliant blue: Bio Rad Laboratories.

100 mM dGTP, dATP, dTTP, dCTP, 5 mM deaza-dGTP, ddGTP, ddATP, ddTTP, ddCTP: Pharmacia.

ECL Western blotting detection reagents: Amersham Life Sciences.

Ecoscint A: National Diagnostics.

En^{3}Hance: DuPont.

2.8. Plasmids

The plasmid vector used to construct p8A and p14, p15 and p17 was pUC19gD/neo^r, which was provided by Dr A Patel. pUC19gD/*neo*^{r} consists of pUC19, the neomycin resistance gene driven by the SV40 late promoter and the HSV-1 US6 (gD) promoter upstream from two unique restriction sites (*Kpn*I, *Bgl*II). In some constructs the gD promoter was used to drive inserted genes (p15, Section 4.1.4) in others, inserted genes were inserted under the control of their own promoters. Plasmids pGEM-1 and pGEM-2 were obtained from Promega and were used for the construction of plasmids to be used in transcription and translation assays. Plasmid pMJ511 was produced by Dr A.J. Davison and modified by Dr V.G. Preston. The cosmids used are described in Section 4.1. They are derived from the SuperCos1 vector (Stratagene) which contains a bacterial origin of replication, dual cos sites, the ampicillin resistance gene for prokaryotic selection and the neomycin marker under the control of the SV40 promoter for expression in eukaryotic cells.

2.9. Protein blotting for mass spectrometric analysis

10 x PVDF buffer:	0.5 M Trizma base 0.5 M glycine
Transfer buffer:	1 x PVDF buffer 20% (v/v) methanol 0.01% (w/v) SDS
Matrix:	10 mg/ml α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile plus 0.1% (v/v) trifluoroacetic acid
Sulforhodamine stain:	50 mg/l sulforhodamine 30% (v/v) methanol 0.2% (v/v) acetic acid
Formic acid/ethanol:	1:1 (v/v) formic acid:ethanol

2.10. Protein gels

(i) Single concentration acrylamide/NN'-methylenebisacrylamide gels

Acrylamide solution:	37.5 g acrylamide and 1 g NN'methylenebisacrylamide in 100 ml H_2O
Stacking gel buffer:	0.5 M Tris-HCl pH 6.8
Resolving gel buffer:	1.5 M Tris-HCl pH 8.8
Electrophoresis buffer:	6 g/l Trizma base 57.6 g/l glycine 0.2% (w/v)SDS
Destain solution:	30% (v/v) methanol 10% (v/v) acetic acid

Stain solution:	Destain solution containing 0.1% (w/v) Coomassie brilliant blue
Boiling buffer:	16% (v/v) stacking buffer 5% (w/v) SDS 10% (v/v) glycerol 0.5 mM dithiothreitol (DTT) 1% (w/v) bromophenol blue

(ii) Single concentration acrylamide/DATD gels

Acrylamide solution:	30 g acrylamide and 0.5 g diallyltartardiamide (DATD) in 100 ml H_2O
Resolving gel buffer	185 g/l Tris-HCl 4 g/l SDS to pH 8.8
Stacking gel buffer:	59 g/l Tris-HCl 4 g/l SDS to pH 6.8
Electrophoresis buffer:	63.2 g/l Tris-HCl 4 g/l glycine 1 g/l SDS
Stain solution:	0.25 g/l Coomassie brilliant blue 45% methanol 9% acetic acid
Destain solution:	17% (v/v) acetic acid 44% (v/v) ethanol

(iii) Gradient acrylamide/NN'-methylenebisacrylamide gels

Electrophoresis buffer:	63.2 g/l Tris 4.0 g/l glycine 1.0 g/l SDS

Acrylamide solution:	95 g acrylamide and 5 g NN'-methylenebisacrylamide in 100 ml H_2O
Fix solution:	50% (v/v) methanol 7% (v/v) acetic acid
Stain solution:	0.2% (w/v) Coomassie brilliant blue in fix solution
Destain solution:	5% (v/v) methanol 7% (v/v) acetic acid
Boiling buffer:	30% (v/v) stacking gel buffer (Section 2.10 (ii)) 6% (w/v) SDS 30% (v/v) glycerol 15% (v/v) β-mercaptoethanol 1% (w/v) bromophenol blue

2.11. BHK C13 transfections

HeBS buffer:	8 g/l NaCl 0.37 g/l KCl 0.1 g/l anhydrous Na_2HPO_4 1 g/l D-glucose 5 g/l Hepes to pH 6.9
HeBS/DMSO:	20% (v/v) DMSO in HeBS buffer

Optimem medium and lipofectamine for transfecting cells by the lipofection method were obtained from Gibco BRL.

2.12. Agarose gel electrophoresis

10 x TBE:	109 g/l Tris 55 g/l boric acid 9.3 g/l EDTA
DF dyes:	37.2 g/l EDTA 100 g/l Ficoll 400 50% 10 x TBE 1% (w/v) bromophenol blue

2.13. Small scale DNA preparation

TE:	10 mM Tris-HCl pH 8 1 mM EDTA
GTE:	2.5 mM D-glucose 6.25 mM Tris-HCl pH 8 10 mM EDTA
GTE/lysozyme:	4 mg/ml lysozyme in GTE
SDS/NaOH:	1% (v/v) SDS 5% (v/v) 10M NaOH in GTE (prepared fresh)
STET buffer:	5% (v/v) Triton X-100 50 mM EDTA 0.5 M Tris-HCl pH8 8% (w/v) sucrose
STET/lysozyme:	4 mg/ml lysozyme in STET buffer

2.14. Southern blotting

20 x SSC:	174 g/l NaCl 88.2 g/l sodium citrate

50 x Denhardt's solution:	1% (v/v) Ficoll 4000 1% (w/v) polyvinylpyrrolidone (PVP) 1% (w/v) bovine serum albumin 3 x SSC
Hybridisation buffer:	3 x SSC 5 x Denhardt's solution 0.1 g/l calf thymus DNA 0.1% (w/v) SDS 20 mM Tris-HCl pH 7.5
Wash buffer:	2 x SSC 0.1% (w/v) SDS 20 mM Tris-HCl pH 7.5
Gel soak I:	175 g/l NaCl 40 g/l NaOH
Gel soak II:	121.1 g/l Tris 35 g/l NaCl to pH 8 with HCl
TCA:	5% (w/v) trichloroacetic acid

Rapid-hyb buffer, used for prehybridisation and hybridisation in some experiments, was obtained from Amersham Life Sciences.

2.15. Western blotting

Western blot transfer buffer:	3 g/l Tris 14.4 g/l glycine 80% (v/v) methanol to pH 8.3 with HCl

PBS A:	170 mM NaCl 35 mM KCl 1 mM Na_2HPO_4 2 mM KH_2PO_4
Complete PBS:	PBS A 6.8 mM $CaCl_2$ 4.9 mM $MgCl_2$
PBS A/BSA:	0.05% (w/v) BSA in PBS A
PBS A/Tween 20/BSA:	0.1% (v/v) Tween 20 1% (w/v) BSA in PBS A
PBS A/Marvel/BSA:	0.05% (w/v) BSA 5% (w/v) Marvel (low fat milk substitute) in PBS A

2.16. DNA Sequencing

PEG/NaCl:	20% (w/v) PEG 6000 2.5 M NaCl
40% acrylamide solution:	40 g acrylamide 5 g NN'-methylenebisacrylamide in 100 ml H_2O
Top gel mix:	460 g/l urea 0.5 x TBE 6% (v/v) acrylamide solution
Bottom gel mix:	460 g/l urea 50 g/l sucrose 50 mg/l bromophenol blue 2.5 x TBE 6% (v/v) acrylamide solution

Formamide dyes:	1 g/l xylene cyanol FF 1 g/l bromophenol blue 10 mM EDTA in deionised formamide
X-gal:	40mg/ml 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside in dimethyl formamide
IPTG:	30mg/ml isopropyl β-D-thiogalactopyranoside
2 mM dNTPs:	2 mM each of dGTP, dATP, dTTP, dCTP
Chase solution:	0.125 mM each of dGTP, dATP, dTTP and dCTP

dNTP/ddNTP solutions: volumes in µl

	G	A	T	C
TE	2000	1000	2000	2000
0.5 mM 7-deaza-dGTP	50	1000	1000	1000
0.5 mM dTTP	1000	1000	50	1000
0.5 mM dCTP	1000	1000	1000	50
5 mM ddGTP	20	-	-	-
5 mM ddATP	-	2	-	-
5 mM ddTTP	-	-	50	-
5 mM ddCTP	-	-	-	7

2.16. Other buffers

Low pH citrate buffer:	0.4 M citric acid 0.1 M KCl 0.135 M NaCl to pH 3 with HCl

5 x ligase buffer:	3 M Tris-HCl pH 7.5 30 mM $MgCl_2$ 30 mM DTT 1.45% (w/v) PEG 6000
Ficoll gradient solutions:	5% (w/v) Ficoll in Eagles A plus Eagles B without phenol red; 15% (w/v) Ficoll in Eagles A plus Eagles B without phenol red
Cell lysis buffer:	0.6% (w/v) SDS 10 mM Tris-HCl pH 7.5 1 mM EDTA 5 mg/l protease
Stripping buffer:	1% (v/v) NP40 in PBS A
PBS/calf:	14% (v/v) NBCS in PBS A
PBS A/SDS:	1% (w/v) SDS in PBS A
Versene:	0.6 mM EDTA 0.0002% (w/v) phenol red in PBS A
Trypsin:	0.25% (w/v) trypsin dissolved in Tris-saline
Tris-saline:	140 mM NaCl 30 mM KCl 280 mM Na_2HPO_4 25 mM Tris-HCl pH 8 1 mg/ml glucose 0.1 mg/ml streptomycin 100 units/ml penicillin 0.0015% (w/v) phenol red
Trypsin/versene:	1:1 (v/v) trypsin:versene

2.17. Immune precipitation

Zweig's buffer:	0.1 M Tris-HCl pH 8 10% (v/v) glycerol 0.5% (v/v) NP40 0.5% (w/v) sodium deoxycholate 0.2 mM phenylmethylsulphonyl fluoride
RIPA buffer:	1% (v/v) NP40 1% (w/v) sodium deoxycholate 0.1% (w/v) SDS 1 mM DTT in PBS A

2.18. HSV-1 DNA preparation

RSB:	10 mM Tris-HCl pH 7.4 10 mM KCl 15 mM $MgCl_2$
RSB/NP40:	0.5% (v/v) NP40 in RSB
NTE:	10 mM Tris-HCl pH 7.4 100 mM NaCl 1 mM EDTA

2.19. Miscellaneous

The following items were used in addition to standard laboratory materials and equipment:

Spin-X centrifugation filter tubes: Costar.

Sephaglas BandPrep kits: Pharmacia LKB Biotechnology.

Hybond-N nylon membrane: Amersham Life Sciences.

Immobilon-P polyvinylidene difluoride microporous membrane: Millipore Corporation.

TNT™ SP6 and T7 coupled reticulate lysate system: Promega.

Kodak X-OMAT XS-1 and duplicating film: Kodak.

Antibodies potentially directed against the UL16 and UL17 proteins were supplied by Charles Cunningham.

Electroporator II and electroporation cuvettes 0.1 cm: Invitrogen.

3MM chromatography paper: Whatman.

UV Stratalinker 1800: Stratagene.

Chapter 3

Methods

3.1. Cell culture

BHK C13 cells and MeWo cells were grown in ETC10 or Dulbecco's medium at 37° in a humidified atmosphere comprising 95% (v/v) air and 5% (v/v) CO_2. The cells were harvested from plastic roller bottles by pouring off the medium and washing the monolayer with 20 ml versene. They were then washed twice with 20 ml trypsin/versene to remove the monolayer and resuspended in fresh medium at a concentration of approximately 10^7 cells/ml. For experimental use, the cells were incubated in 24 x 5 mm or 6 x 35 mm well tissue culture plates as required.

3.2. Virus stocks

BHK C13 cells were grown in roller bottles until 90% confluent in Dulbecco's medium, and then infected. The medium was replaced with 50 ml of Dulbecco's medium, and the appropriate virus dilution was added in 500 µl of Dulbecco's medium. The roller bottles were gassed with CO_2 to 5% (v/v) and returned to 37°. The infected cells were incubated at 37° and harvested when the cpe was maximal (usually 2-4 days pi). Infected cells were shaken into the medium and pelleted by centrifugation at 2000 rpm using a Sorvall RC-5B refrigerated Superspeed centrifuge for 5 min. The supernatant was decanted and the cell pellet was resuspended in 1-2 ml of complete PBS, to give the cell-associated virus stock. The supernatant was centrifuged again at 12,000 rpm for 1 h and the pellet was resuspended in 1-2 ml of complete PBS to give the cell-released virus stock. Both stocks were disrupted in a bath ultrasonicator at 4°, frozen on dry ice and stored at -70°. Virus stocks were titrated before use.

3.3. Titration of virus stocks

Virus stocks were titrated at 37° on 80-90% confluent monolayers of BHK C13 cells on 35 mm 6-well trays in Dulbecco's medium. Aliquots of 200 µl of virus dilutions (usually 10^{-3} to 10^{-8}) were added to drained monolayers and the plates were returned to the incubator for 1 h before being overlayed with 3 ml methyl cellulose overlay. After 2-4 days the overlay was removed and the plates were stained with Giemsa stain for 30 min at room temperature. The plaques were counted under a dissection microscope and the virus titre established.

3.4. Picking plaques

Plaques were picked using sterile 20 µl pipette tips, placed into 1 ml of Dulbecco's medium, sonicated and stored at -70°.

3.5. Particle counts

Equal volumes of the virus stock, latex beads (at a known concentration) and stain (1% phosphotungstic acid pH 7.0) were mixed and adsorbed onto a parlodion-coated copper grid. Excess liquid was drained off and the numbers of virus particles and latex beads present in the field of view of the electron microscope were counted. The concentration of the virus particles was calculated by comparison with the number of latex beads. The particle counts were carried out by Mr J. Aitken.

3.6. Virus absorption

(a) At 4°

90% confluent BHK C13 cells in 35 mm 6-well plates were precooled to 4° for 30-60 min, infected with 200 pfu virus/plate in 500 µl of Dulbecco's medium and maintained at 4°. Non-adsorbed virus was removed by washing the monolayers twice with PBS A at 0, 15, 30, 45, 60, 90, 120 or 240 min pi. Plates were overlayed with 3 ml methyl cellulose overlay, incubated at 37° for 2-3 days and then stained with Giemsa stain by removing the overlay, replacing it with stain for 30 min at RT, washing the plate with water and air drying. The number of plaques on each plate was counted and the number at each time point was calculated as a percentage of the virus adsorbed at 240 min. The percentage of virus bound at each time point was calculated as the mean of the plaque count from three plates.

(b) At 37°

The experiment was carried out as described above except that the cells were maintained at 37° for times of 0, 5, 10, 15, 20, 30, 45, 60, 90 and 120 min pi.

3.7. Virus penetration

90% confluent BHK C13 cells in 35 mm 6-well plates were precooled to 4° and infected with 200 pfu virus/plate in 500 µl of Dulbecco's medium. Virus was adsorbed at 4° for 10 min and non-adsorbed virus was removed by washing twice with 2 ml of cold PBS A. Low pH citrate buffer was added to three wells of one 6 well plate and PBS A was added to the other three. The plate was incubated at 4° for 5 min before being washed twice with 2 ml of PBS A and overlayed with methyl cellulose overlay and placed at 37° C. The low pH citrate buffer wash removes unpenetrated and unbound virus and the PBS A wash removes unbound virus only. The remaining plates were taken to a 37°C room, overlayed with preheated Dulbecco's medium at 37° and incubated at 37° for 5, 10, 15, 20, 30, 45 or 60 min. The medium was then replaced by 1 ml of low pH citrate

buffer or PBS A. After 5 min at 37°, the plates were washed twice with PBS A, overlayed with methyl cellulose overlay and incubated at 37° for 2 days. The plates were stained with Giemsa stain and plaques were counted. The percentage of virus bound at each time point represents the mean of the plaque count from three plates and was calculated as a percentage of the virus that penetrated at the same time point when the cells were washed with PBS A.

3.8. One-step growth curve

Confluent monolayers of BHK C13 cells on 35 mm 6-well plates were infected at a moi of 5 pfu/cell in Dulbecco's medium and incubated at 37° for 1 h. The cells were washed once with low pH citric acid buffer, twice with PBS A, and overlayed with 2 ml Dulbecco's medium. The zero time point sample was harvested immediately after washing and further samples were harvested at 3, 6, 9, 12, 16, 20, 24, and 36 h pi by scraping the cells into the medium with a rubber policeman. The samples were sonicated using a waterbath and titrated using confluent BHK C13 cells as described previously in Section 3.3. The virus yield at each time point was calculated in pfu/ml.

3.9. Multi-step growth curve

This was carried out as described for the one-step growth curve (Section 3.8), except that BHK C13 cells were infected at 0.01 pfu/cell in Dulbecco's medium and harvested at 0, 6, 12, 24, 36, 48, 60, 72 and 96 h pi.

3.10. Radiolabelling of virus particles

Confluent roller bottles of MeWo or BHK C13 cells were infected with the appropriate virus in Dulbecco's medium at low moi for 12-24 h. The monolayers were washed in low methionine medium (Emet$^+$) and then incubated in this medium for 4 h at 37°. [^{35}S]-methionine was then added (500 µCi/roller bottle) in 20-40 ml Emet$^+$ and incubation was continued for 3-4 days. Virions and L-particles were prepared, resuspended in 100-200 µl of Dulbecco's medium and stored at -70° (see Section 3.18).

3.11. Radiolabelling infected cells

(a) Long term labelling

35 mm 6-well plates of 80-90% confluent BHK C13 cells were infected with virus in Dulbecco's medium at 2.5 x 10^7 pfu/plate at 37° for 1 h. The plates were washed twice

with Emet$^+$ or Ecys$^+$ medium and then maintained in 1 ml of the appropriate medium. At 4 h pi, 50 µCi of [^{35}S]-methionine or [^{35}S]-cystine was added directly to the plate. At 20-24 h pi, the sample was harvested by washing twice in PBS A, adding 250 µl of boiling solution and heating the plate at 80° for 5 min. The lysed cells were transferred to a vial, heated at 100° for 5 min and stored at -70°.

(b) Pulse labelling

35 mm 6-well plates of 80-90% confluent BHK C13 cells were washed with Emet$^+$ or Ecys$^+$ medium and incubated in 2 ml of the appropriate medium for 1 h at 37°. Cells were infected with virus diluted in the appropriate medium at 2.5 x 10^7 pfu/plate for 1 h at 37°, washed and maintained in Emet$^+$ or Ecys$^+$ medium. At appropriate times the monolayers were washed in Emet$^-$ or Ecys$^-$, 50 µCi of [^{35}S]-methionine or [^{35}S]-cystine were added and the incubation continued at 37° for 2 h. The cells were washed twice in PBS A and lysed by the addition of 250 µl of boiling solution. The labelling times were 0-2 h, 2-4 h, 4-6 h, 6-8 h, 8-10 h, 10-12 h, in addition to 4-24 h long term labelling.

The harvested samples were electrophoresed on a 5-12.5% gradient polyacrylamide gel and a 12% single concentration gel (see Section 3.16). The gels were fixed for 1 h, destained for a minimum of 1.5 h with three changes of destain buffer, treated with En^{3}Hance, dried for 1.5 h at 80°C on a gel drier, and autoradiographed at -70°.

3.12. Binding of virus to cells

[^{35}S]-methionine labelled virions were prepared as described previously (Section 3.11). They were titrated on BHK C13 cells, the radioactivity was counted and particle counts were obtained. Confluent monolayers of BHK C13 cells were infected with approximately 100,000 particles/cell (approximately 10-100,000 cpm) in 200 µl of Dulbecco's medium. Infected cells were maintained at 37° for 0, 5, 10, 15, 20, 30, 45, 60, 90 or 120 min, then the supernatant was transferred into a scintillation vial for counting. The monolayers were washed three times with 1 ml PBS A, the washes were collected together and 300 µl from the 3 ml final volume were prepared for scintillation counting. 200 µl of PBS A/1% SDS was added to the monolayers for 5 min and the lysed cells were scraped into a vial for scintillation counting. 4 ml of Ecoscint A was added to each vial and the radioactivity of the samples was counted in a scintillation counter for 4 min, using a program for detecting ^{35}S. The percentage binding of viral particles was determined by the equation: (a/a+b+10c) x 100, where a= radioactivity of cells, b= radioactivity of supernatant and c= radioactivity of the washes.

3.13. Infected cell DNA

Confluent monolayers of BHK C13 cells in 24-well trays were infected with virus diluted in 500 µl of Dulbecco's medium for 1 h at 37°. The virus aliquot was replaced by 1 ml of medium and the trays were incubated at 37° until complete cpe was observed. 400 µl of cell lysis buffer was added and the cells were incubated at 37° for 1-4 h. The lysed cells were transferred to a vial containing 30 µl of 5 M NaCl and extracted with 400 µl of phenol:chloroform (see Section 3.23). The DNA was ethanol precipitated by mixing the DNA sample with two volumes of ethanol and incubating at -20° for up to 1 h, then centrifuged at 12,000 rpm for 10 min and resuspended in 40 µl 5 µg/ml RNase A (see Section 3.23).

3.14. Southern blotting

(a) Labelling of probe

A hexa- or nona-primer kit from Amersham was used to label and purify probes. Usually 1 µg of plasmid DNA containing the appropriate HSV-1 fragment to be used as a probe was denatured at 100° for 10 min, quenched on ice for 5 min and then centrifuged briefly at 12,000 rpm. 4 µl of nonaprimer, 1 µl each of dATP, dTTP, dCTP and 5 µl of [^{32}P]-dGTP were added to the vial. 1 µl of this mixture was spotted onto two Whatman disc filters (A and B), then 1 µl of the Klenow fragment of *E. coli* DNA polymerase I was added and the vial incubated at 37° for 1 h. After incubation, 1 µl was spotted onto a third filter (C). To purify the probe, 60 µl of absorb solution and 2 µl of resin were added and the mixture was vortexed and centrifuged briefly. The pellet was resuspended in 100 µl of wash buffer, centrifuged at 12,000 rpm briefly and resuspended in 100 µl of elute solution. The vial was placed at 65° for 5 min and then centrifuged at 12,000 rpm briefly. The supernatant was heated at 100° for 2 min and then stored at -20°. 50 µl of purified probe was used for each membrane hybridisation.

The filters (A, B and C) were used to estimate the percentage incorporation of the probe. Filters B and C were washed three times for 5 min in cold 0.5% trichloroacetic acid then twice in ethanol. Filter A was not washed. Dried filters were placed in vials containing 5 ml Ecoscint A and counted in a scintillation counter. The percentage incorporation was calculated from the equation [(C-B) / A] x 100. Usually 75-95% incorporation was obtained.

(b) Transfer of DNA

DNA samples were electrophoresed on an agarose gel and denatured by two 20 min washes in gel soak I, followed by two 20 min washes in gel soak II at RT. Hybond-N

membrane was cut to size (approximately 10.5 x 13.5 cm) and soaked in 20 x SSC before use. The gel was placed on a platform with filter paper bridging a reservoir of 20 x SSC buffer. The membrane was placed on top and care was taken to remove air bubbles. Filters and paper towels which were cut to the correct size (approximately 9.5 x 12.5 cm) were placed on top and then a weight was added. Blotting occurred at room temperature overnight. The membrane was removed, dried at RT for 15 min and UV cross linked using a UV Stratalinker 1800.

(c) Hybridisation

The membrane was wrapped in a nylon sheet and placed into a hybridisation bottle in 50 ml of hybridisation buffer. The membrane was incubated in a hybridisation oven at 65° for 3-4 h and then the probe was added (50µl, see Section 3.14(a)). Incubation was continued overnight at 65°, then the membrane was washed with 50 ml of blot wash buffer. The membrane was rinsed with blot wash buffer and then incubated at 60° with blot wash buffer a further six times for 15 min each. The membrane was soaked in 25% (v/v) cold blot wash buffer, air dried and placed in a plastic bag for autoradiography at RT or -70°.

3.15. Blotting by centrifugation and rapid hybridisation

Samples were electrophoresed using a small agarose gel kit, and the gels were treated in the same way as described in Section 3.14b, except for the inclusion of an initial 5 min wash step using 0.2 M HCl. The gel and membrane were placed together in a sandwich of 3MM filter paper and centrifuged at 1000 rpm for 1 h. The membrane was air dried, UV cross-linked and pre-hybridised in a bottle containing 15 ml of Rapid-hyb buffer at 65° for 15 to 60 min. The probe was added and hydridisation continued at 65° for a further 2 h. The membrane was washed four times for 10 min with blot wash at 65°, rinsed in 50% (v/v) cold blot wash buffer, air dried and autoradiographed.

3.16. Polyacrylamide gel electrophoresis

Single concentration gels

(a) Stocks of acrylamide/NN'-methylenebisacrylamide were prepared and dialysed using Amberlite beads. Polymerisation was initiated by addition of 1.125ml of 1.5% (w/v) APS and 108 µl of TEMED per 45 ml of resolving gel solution and 1.4 ml of APS and 48 µl of TEMED per 20 ml of stacking gel.

(b) Single concentration 30% (v/v) acrylamide/0.5% (v/v) DATD gels were also used. Polymerisation was initiated by addition of 240 µl of 10% (v/v) APS and 35 µl of TEMED per 40 ml of resolving gel solution and 100 µl of APS and 10 µl of TEMED per 15 ml of stacking gel solution.

The resolving gel solution was poured between two well-washed glass plates separated by 1.5 mm spacers and sealed with Teflon tape. Butan-2-ol was layered on top of the resolving gel solution until it polymerised, then removed prior to adding the stacking gel solution, into which was placed a Teflon well-forming comb. Before electrophoresis, protein samples were boiled for 5 min in boiling solution to denature them and applied to the gel which was electrophoresed overnight at 10 mA at RT.

Gradient gels

From a stock of acrylamide/NN'-methylenebisacrylamide a 30% stock of acrylamide solution containing 19:1 acrylamide/NN'-methylenebisacrylamide was made. From this stock, a 5% acrylamide solution was polymerised by the addition of 450 µl of 10% (v/v) APS and 30 µl of TEMED per 72 ml and a 12.5% (v/v) solution was polymerised using 198 µl of 10% (v/v) APS and 30 µl of TEMED per 72 ml.

The gel was poured using a gradient mixer between two well-washed glass plates separated by 1.5 mm spacers and sealed with tubing. Butan-2-ol was layered on top of the gel solution until the latter polymerised and was replaced by a 5% (v/v) acrylamide stacking gel, into which was placed a Teflon well-forming comb. The samples were electrophoresed at 10 mA overnight at RT.

3.17. Staining gels with Coomassie brilliant blue

Following electrophoresis, proteins were fixed for 30 min in fix solution and then stained in the appropriate stain solution. This was followed by destaining in the appropriate destain solution for at least 1 h or (usually) overnight.

3.18. Preparation of virions and L-particles

Ten roller bottles of BHK C13 or MeWo cells were infected at a low moi with the appropriate virus diluted in Dulbecco's medium. After incubation at 37° for 3-4 days (BHK C13 cells) or at 31°C for 4-5 days (MeWo cells), the infected cells were shaken off into the medium and centrifuged at 2000 rpm for 5 min. The supernatant was decanted and centrifuged at 12,000 rpm for 2 h. The pellet was resuspended in 1-2 ml

complete PBS and carefully layered on top of a 5-15% (w/v) Ficoll gradient. The gradient was centrifuged in a Sorvall ultracentrifuge (Du Pont) at 12,000 rpm for 2 h at 4°. Virions and L-particles were removed from the gradient *via* needle puncture, diluted to 30 ml in complete PBS and pelleted by centrifugation at 20,000 rpm for at least 40 min. The pellets were carefully resuspended in 100 µl of complete PBS, frozen on dry ice and stored at -70°.

3.19. Fractionation of virions and L-particles

Five µl of 10% NP40 in PBS A was added to 45µl of a virion or L-particle preparation and incubated on ice for 20 mins, mixing occasionally. The sample was centrifuged at 12,000 rpm for 10 min at 4°. The supernatant containing the envelope fraction was transferred to a fresh vial. The capsid-tegument pellet was resuspended in 500 µl of 1% NP40 in PBS A, sonicated and pelleted by centrifugation at 12,000 rpm for 10 min at 4°, and the pellet resuspended in 50 µl of complete PBS. To each sample of the envelope and capsid-tegument fraction, an equal volume of boiling solution was added, and the samples were incubated at 100° for 5 min, frozen on dry ice and stored at -70°.

3.20. Blotting polyacrylamide gels for mass spectrometric analysis

The electrophoresed gel was cut to size and soaked in transfer buffer for at least 5 min. The membrane was prepared by soaking in methanol and then in transfer buffer for 5 min. The membrane was applied to the gel and they were sandwiched between two filter papers and electroblotted at 100 V for 1 h at RT. The membrane was removed, washed extensively in distilled water and dried *in vacuo* overnight. The membrane was stained with sulforhodamine solution for 1 min, washed in water and air dried.

3.21. Mass spectrometric analysis of virion proteins

A photograph of the stained membrane was taken using a photo-imager (Appligene). Appropriate bans were excised from the membrane and placed into 0.5 ml tubes, 3 µl of trypsin (sequencing grade) in 50 mM ammonium bicarbonate containing 1% (w/v) n-octyl β-D-glucopyranoside (Sigma) was added and the tubes were incubated at RT overnight. 6 µl formic acid:ethanol (1:1 v/v) was added for 1 h at RT to elute the peptides, then 0.5 µl of each sample was placed onto the mass spectrometer loading strip and air dried. 0.5µl of matrix solution containing insulin B chain as a size marker was added to each sample and air dried before insertion into the mass spectrometer (Finnigan MAT Lasermat). The sample was subjected to laser desorption mass spectrometric

analysis (Pappin *et al.*, 1993, 1994), producing a mass spectrum characteristic of the protein analysed. The protein was identified using the Massmap software (Finnigan MAT), where the peptide profile produced is compared to the theoretical tryptic peptide profiles of proteins stored in a database.

3.22. Transformation of bacterial cells with plasmid or cosmid DNA

(a) Heat shock of *E. coli* DH5α cells

10 µl of competent DH5α cells were added to pre-cooled 15 ml Falcon tubes, 1-2 µl of ligated DNA (usually cosmid DNA) was then added. Tubes were maintained on ice for 30 min, placed at 42° for 45 sec and transferred to ice for 2 min. 300 µl of SOC medium was added to each sample and the tubes were shaken at 37° for 1 h. Transformed cells were spread onto L-broth agar plates containing 100 µg/ml ampicillin, and incubated at 37° overnight.

(b) DMSO shock of *E. coli* XL-1 cells

A single colony of XL-1 cells from a streaked L-broth agar plate was added to 10 ml of 2YT broth and shaken at 37° overnight. 100-200 µl from this culture was added to 10 ml of 2YT broth and shaken at 37° for 3 h (until the optical density of the culture at 540 nm was 0.3). The culture was centrifuged at 2000 rpm for 10 min and the pellet was resuspended in 1 ml of TSB/DMSO and maintained on ice for 10 min. 100 µl of these cells was added to precooled 15 ml Falcon tubes, 1-4 µl of ligated DNA was added and the mixture was incubated on ice for 20 min. Each tube then received 300 µl of TSB/glucose or SOC medium and was shaken at 37° for 1 h. The samples were spread onto L-broth agar plates containing 100 µg/ml ampicillin and incubated at 37° overnight.

(c) Electroporation

Competent XL-1 cells were produced from an overnight culture of XL-1 cells grown in 10 ml 2YT. One l of 2YT was inoculated with the overnight culture and incubated for 3-4 h at 37°. The cells were pelleted by centrifugation at 2000 rpm for 15 min and resuspended in distilled water. This procedure was repeated four times to remove impurities which could affect the electroporation procedure. Ligated DNA samples were added to 50 µl of these cells and the mixture was placed into a 0.1 cm electroporation cuvette. The electroporator was used according to the manufacturer's instructions. The sample was pulsed once, 400 µl of SOC medium was added to remove the sample and this was added to a 15 ml Falcon tube containing a further 800 µl of SOC medium and shaken at 37° for 1 h. Each sample was spread onto three L-broth agar plates containing 100 µg/ml ampicillin and incubated at 37° overnight.

3.23. Small scale preparation of DNA

(a) Alkaline lysis

Following transformation of bacteria, colonies which grew on L-broth agar plates containing 100 µg/ml ampicillin were picked using sterile cocktail sticks into 10 ml 2YT broth containing 100 µg/ml ampicillin and 10 mM magnesium sulphate and shaken at 37° overnight. 1.5 ml of each culture was decanted into a 1.5 ml tube and centrifuged at 12,000 rpm for 3 min. The supernatant was discarded and the pellet was resuspended in 100 µl of GTE/lysozyme and incubated at RT for 10 min. 200 µl of SDS/NaOH was added, the tube mixed and placed on ice for 10 min. 150 µl of 3 M potassium acetate was added, the vial mixed and returned to ice for 10 min. The sample was centrifuged at 12,000 rpm for 10 min and the supernatant decanted into a fresh tube to which was added 400 µl of phenol/chloroform. The tube was mixed and centrifuged at 12,000 rpm for 5 min. The aqueous phase was removed into a fresh 1.5 ml tube to which was added 20 µl of 3 M sodium acetate pH 3 and 800 µl of ethanol. The tube was mixed and placed at -20° for 20 min before being centrifuged at 12,000 rpm for 10 min to pellet the precipitated DNA. The supernatant was removed and the pellet washed with 70% (v/v) ethanol before being lyophilised for 3 min. The pellet was resuspended in 50 µl 5 µg/ml RNase A (DNAse free).

(b) STET lysis

The bacterial pellet obtained from an overnight culture as in (a) was resuspended in 100 µl of STET/lysozyme in a 1.5 ml tube and placed in a heat block at 100° for 75 sec. The tube was then centrifuged at 12,000 rpm for 10 min, and the supernatant was transferred to a fresh tube containing 400 µl of 0.3 M sodium acetate pH 3 and mixed with 800 µl ethanol. The DNA was precipitated at -20° for 1 h, pelleted by centrifugation at 12,000 rpm for 10 min and then resuspended in 50 µl 5µg/ml RNaseA.

3.24. Producing glycerol stocks of cosmids or plasmids

Bacterial stocks were prepared from 10 ml overnight cultures grown at 37° in L-broth or 2YT broth. 1.5 ml was decanted into a plastic storage vial and centrifuged at 12,000 rpm for 3 min. The pellet was resuspended in 500 µl of sterile bactopeptone before adding 500 µl of sterile 80% (v/v) glycerol. The stocks were frozen on dry ice and stored at -20° or -70°.

3.25. Restriction endonuclease digestion

Digestion of DNA using restriction enzymes was carried out according to the manufacturer's instructions.

3.26. Partial digestion of cosmids in the presence of ethidium bromide

Cos24 was linearised at *Asp*718 sites in the presence of ethidium bromide to yield full length linear molecules and then treated with bacteriophage T4 DNA polymerase in the presence of the four deoxynucleoside triphosphates to produce blunt ends. After ligation this should result in the insertion of 4 bp into the *Asp*718 site, converting it from GGTACC to GGTACGTACC and thus introducing a shift in the translational reading frame from that point onwards. Optimal conditions for linearization were obtained by carrying out digestions of cos24 at a constant enzyme concentration in the presence of different concentrations of EtBr. The EtBr was removed by phenol:chloroform extraction and ethanol precipitation (see Section 3.23). Cleaved ends of partially digested cos24 were converted to blunt ends, electrophoresed through an agarose gel and extracted from the gel by electroelution (see Section 3.29a). The DNA was extracted with phenol:chloroform, ethanol precipitated and resuspended in 50 µl of H_2O. The linearised cosmid was checked by agarose gel electrophoresis, ligated and transfected into *E.coli* MAX efficiency DH5α cells. Resulting cosmids were screened for the loss of an *Asp*718 site and the gain of a novel *Sna*BI site. *Sna*BI cuts at the sequence TACGTA, which is generated when the 4 bp insertion occurs.

3.27. Production of blunt ends and ligation of cosmid or plasmid DNA

DNA fragments were treated with T4 DNA polymerase (3 units) in the presence of the four deoxynucleoside triphosphates (2mM) in 10 x React A buffer for 1 h at 37° to produce blunt ended DNA. The ligation mixture contained the DNA, 1 unit of T4 DNA ligase, 4 µl of 5 x ligase buffer, 2 µl of 10mM ATP and was made up to a final volume of 20 µl with water. The mixture was incubated at 16° overnight.

3.28. Agarose gel electrophoresis

100 ml of 0.8% (w/v) agarose was dissolved in 1 x TBE by boiling, then cooled at RT for 15 min before being poured into a taped gel plate (15 cm x 10 cm) containing a Teflon well-forming comb. When the gel was set, the tape was removed and the gel was placed into a horizontal gel electrophoresis apparatus containing 1 x TBE in the buffer tanks.

DNA samples were mixed with 0.25 volume of DF dyes before being loaded into the wells and electrophoresed at 25 V overnight or 75 V for 4-5 h.

For some experiments, a mini-horizontal gel apparatus was used (Anachem). A gel comprising 50 ml of 0.8% (w/v) agarose in 1 x TBE was cast in the apparatus, where it was electrophoresed for 2 h at 50 V.

In both cases the samples were stained with 0.5 μg/ml EtBr in 100 ml of 1 x TBE for 30 min at RT, and visualised by exposure to UV light. For analytical gels, short wave UV light was used, and for preparative purposes long wave UV light was used. The gels were photographed using a photo-imager system (Appligene).

In most cases, a *Hind*III digest of bacteriophage DNA was electrophoresed on the same agarose gel as a molecular weight marker, occasionally, a *wt* plasmid, cosmid or virus of known structure was electrophoresed alongside the samples as a control and doubled as an appropriate molecular weight marker.

3.29. Purification of restriction fragments from agarose gels

(a) Electroelution

Restriction fragments were separated electrophoretically on agarose gels and visualised under long wave UV light. Bands of interest were excised in the minimum volume of agarose using a sterile scalpel. Each slice was placed with 500 μl TBE into dialysis tubing which had previously been boiled in 1 x TBE. The tubing was sealed, making sure all air bubbles had been removed and the DNA was electroeluted in a horizontal gel kit at 100 V for 4 h. The polarity of the current was then reversed for 45 sec and the 1 x TBE containing the DNA was transferred into a sterile 1.5 ml tube. The solution was extracted with phenol:chloroform and ethanol precipitated (see Section 3.23), resuspended in H_2O and stored at -20°.

(b) Sephaglas

The fragment of interest was excised and transferred into a preweighed 1.5 ml tube in order to determine its weight. The DNA was eluted according to the manufacturer's instructions, and recovery was assessed by agarose gel electrophoresis.

(c) Spin-X tubes

The fragment of interest was excised, placed into a Spin-X tube and centrifuged at 12,000 rpm for 5 min at 4°. An equal volume of 0.3 M sodium acetate pH 3 was added to the sample and the tube was centrifuged again at 12,000 rpm for 5 min at 4°. One

twentieth of the volume remaining after centrifugation of 3 M sodium acetate and 2 volumes of ethanol were added to the tube. The DNA was precipitated at -20° for 1 h and pelleted by centrifugation at 12,000 rpm for 20 min. The DNA was washed with 70% (v/v) ethanol, dried, resuspended in H_2O and stored at -20°.

3.30. Transfection of BHK C13 cells

Regenerating virus

Cosmids were digested with *Pac* I to excise the HSV-1 DNA inserts, and equimolar amounts of the inserts representing the entire HSV-1 genome were mixed together and transfected into cells as follows.

(i) Calcium phosphate precipitation and DMSO shock

Samples of the cosmids in the set were prepared as described in Section 3.23 and mixed in approximately equal volumes. The mixture was precipitated with 87 µl of HeBS, 7 µl $CaCl_2$ and 2 µl of calf thymus DNA, and incubated at room temperature for 30 min, before being added to 80-90% confluent monolayers of BHK C13 cells on 35mm 6-well plates. The plates were incubated at 37° for 30 min, 3 ml of Dulbecco's medium was added and incubation was continued for a further 3.5 h at 37°. One ml of 20% (v/v) DMSO in HeBS buffer was added to the cells for 3 min 45 sec at RT then the DMSO solution was removed and replaced with 5 ml of methyl cellulose overlay. Plates were maintained at 37° for 4-5 days until plaques were observed.

(ii) Lipofection

Cosmid DNA was added to a tube containing 100 µl of Optimem medium. A mixture of 10 µl lipofectamine and 100 µl of Optimem medium was added and the mixture was allowed to stand at RT for 30 min to allow DNA-liposome complexes to form. 500 µl of Optimem medium was added and the final mixture was applied to 50-70% confluent monolayers of BHK C13 cells which had been prewashed with Optimem medium. The cells were incubated at 37° for 5 h before 3-5 ml of Dulbecco's medium containing 20% (v/v) NBCS was added. The cells were maintained in a 37° incubator overnight and then the medium was replaced with methyl cellulose overlay. The monolayers were incubated at 37° for 3-5 days until plaques were observed.

Constructing complementing cell lines

Plasmids were transfected into BHK C13 cells by either of the methods described above, except that the transfected cells were overlayed with Dulbecco's medium rather than methyl cellulose overlay. When the cells were fully confluent, they were removed from the plates by a trypsin/versene wash and each well was diluted into 18 ml of Dulbecco's

medium containing neomycin (G418) (400 μg/ml) and divided between the 6 wells of a 35 mm 6-well plate. Transformed cells were identified by their ability to grow in neomycin-containing medium.

3.31. Determination of differences in plaque area

Photographs of Giemsa-stained infected cell monolayers were taken using a camera-mounted microscope. Photographic prints were obtained for 30-100 different plaques for each virus and the plaques were cut out and weighed. Relative plaque area was determined by relating weight directly to plaque area.

3.32. Effect of interferon

Interferon-sensitive HFL cells were grown until 80% confluent in 35 mm 6-well plates. The medium was removed and replaced with 2ml of Dulbecco's medium containing 1000 U/ml interferon, or medium lacking interferon. The plates were incubated at 37° overnight, then the cells were washed twice with medium and infected with virus diluted in Dulbecco's medium at 5 pfu/cell for 1 h at 37°. The virus inoculum was removed and the cells were overlayed with methyl cellulose overlay and maintained at 37° for 24-48 h. The cells were then scraped into 2 ml of Dulbecco's medium, and the virus yield determined by titration on BHK C13 cells.

3.33. HSV-1 DNA preparation

Five roller bottles of BHK C13 cells were infected with virus in Dulbecco's medium at a moi of 0.001 pfu/cell and incubated at 37° until cpe was complete. Cells were shaken into the medium and pelleted by centrifugation at 2000 rpm for 10 min. The supernatant was retained on ice while the cells were resuspended in 10 ml RSB/0.5% NP40 and incubated on ice for 10 min. Nuclei were pelleted by centrifugation at 1000 rpm for 5 min. The supernatant was retained and the pellet re-extracted with RSB/0.5% NP40. The cytoplasmic supernatants and the supernatant from the infected cell culture were combined and centrifuged at 12,000 rpm for 2 h to pellet the virus, which was resuspended by sonication in 8 ml of NTE. To lyse the virus, EDTA (5%) and SDS (5%) were added and the mixture incubated for 5 min on ice followed by 5 min at 37°. Viral DNA was extracted twice with phenol:chloroform and once with chloroform by gently mixing equal volumes and incubating at RT for 5 min followed by 5 min on ice. Each extraction was centrifuged at 2000 rpm for 10 min, and the fluid upper aqueous layer was dialysed against 0.1 x SSC at RT overnight. The recovered DNA was stored at -70°.

3.34. *In vitro* transcription and translation

pGEM1 and pGEM2, which have T7 and SP6 promoters in different orientations separated by a multiple cloning site, were linearised at the *Sma*I site within the multiple cloning site region and incubated with calf intestinal phosphatase according to the manufacturer's instructions. A UL16-containing DNA fragment (29717 bp to 31213 bp in the HSV-1 sequence) was obtained from *Kpn*I q by digestion with *Bsu*36I and *Bss*SI in the appropriate buffers. The fragment was extracted from an agarose gel by the Sephaglas method (Section 3.29). The fragment was blunt-ended with the Klenow fragment of *E. coli* DNA polymerase I, ligated into the pGEM plasmids, and transformed into XL-1 cells. Ampicillin-resistant colonies were grown and plasmid DNA was prepared, digested, and electrophoresed to obtain clones containing the fragment in each orientation.

Using the Promega TNT coupled reticulocyte lysate system, a standard *in vitro* assay was set up according to the manufacturer's instructions. In most experiments, 0.5 µg of purified plasmid was used per 25 µl reaction containing 12.5 µl of TNT lysate, 2 µl of [^{35}S]-methionine or [^{35}S]-cystine and 0.5 µl of TNT reaction buffer, 0.5 µl of amino acid mixture lacking methionine or cystine, 0.5 µl of RNase inhibitor and 0.5 µl of the TNT T7 or SP6 RNA polymerase. Reactions were incubated at 30° for 90 min, and an aliquot was transferred to a tube containing boiling solution. The mixture was boiled for 5 min and subjected to SDS/PAGE.

3.35. Immune precipitation

Confluent monolayers of BHK C13 cells in 50 mm plates were infected at 5 pfu/cell in Dulbecco's medium. The medium was replaced by 3 ml of medium containing 50 µCi of [^{35}S]-methionine or [^{35}S]-cystine from 8-12 h pi or 4-24 h pi. The monolayers were washed with PBS A, air dried and stored at -70°. After thawing, the cells were lysed by the addition of 1 ml of Zweig's buffer or RIPA buffer. After incubating at RT for 1-2 min, the lysed cells were transferred into a 1.5 ml tube and incubated on ice for 1 h, then centrifuged at 12,000 rpm for 5 min. The supernatant was transferred to a fresh tube.

50 µl of serum was added to 200 µl of the extract produced above, and the mixture was placed on ice overnight. Protein A-Sepharose was prepared by mixing 1 g of protein A-sepharose with 4 ml of RIPA buffer or Zweig's buffer until resuspended, then centrifuging at 1000 rpm for 5 min. This process was repeated three times, then the protein A-Sepharose was resuspended in 1 ml of the appropriate buffer. 60 µl of protein A-Sepharose beads was added to these samples and incubation was continued on ice for

30 min with occasional mixing. The samples were centrifuged at 7000 rpm for 1 min, and the pellet was washed with 1 ml of Zweig's buffer or RIPA buffer and resuspended in the appropriate buffer. The pellet was washed thus four times and the beads were finally resuspended in 60 µl of boiling mix and boiled for 5 min. 40 µl of each sample was electrophoresed on a 12% polyacrylamide gel. The gel was fixed for 1h in fix solution, destained three times for 30 min in destain solution, soaked for 1h in En^{3}Hance, washed in H_2O for 15 min and dried on a gel drier at 80° for 1.5 h. The gel was then exposed to photographic film.

3.36. Western blotting

Protein samples were electrophoresed on a single concentration polyacrylamide gel and electroblotted in Western blotting transfer buffer to a Hybond ECL membrane. The membrane was washed twice for 30 min in PBS A containing 0.05% (w/v) BSA and 5% (w/v) Marvel (low fat milk substitute). This was followed by two 15 min washes in PBS A containing 0.05% (w/v) BSA. The antibody produced against the protein under investigation was diluted as appropriate in PBS A/Tween 20/1% (w/v) BSA, added to the membrane and incubated at room temperature for 2-3 h. The membrane was subjected to two more 15 min washes in PBS A containing 0.05% (w/v) BSA prior to the addition of 1:1000 dilution of protein A conjugated to horseradish peroxidase and incubated at RT for 1-2 h. A further two 15 min washes in PBS A containing 0.05% (w/v) BSA were followed by a 1 min incubation in a 1:1 mixture of reagent 1 and 2 from the ECL western blotting detection kit, which causes a light emitting reaction. The treated membrane was then placed in a plastic bag, taking care to remove air bubbles, and exposed to photographic film for an appropriate time (1 sec-1 min).

3.37. Analysis of viral growth *in vivo*

Intracranial (IC) and footpad (FP) injections (Project Licence No. PPL/60/01357) were carried out by Dr. A. MacLean (under Animal Licence No. PIL/60/5294).

(a) Neurovirulence

Three week old BALB/c mice were anaesthetised using halothane and given intracranial injections into the central region of the left cerebral hemisphere. Individual virus stocks were injected at doses of 10^2 to 10^5 pfu/mouse in 25 µl in PBS/calf (three or four mice were inoculated with each virus dilution). Stocks were titrated on the day of the inoculation to ensure that the correct dose had been administered. The mice were

monitored twice daily for 14 days post inoculation for signs of illness or death. The LD_{50} for each virus was calculated according to the formula of Reed and Muench (1938).

In order to determine the genotype of replicating viruses, the brains of three-week old BALB/c mice that had been infected with 10^7 pfu/mouse were removed at 5 or 7 days post inoculation and homogenized in 1 ml PBS/calf. The progeny virus was titrated on BHK C13 cell monolayers. The stability of viral genotypes were examined by probing infected cell DNA after Southern blotting (see Section 3.14).

(b) Latency

Four week old BALB/c mice were inoculated in the right rear footpad with virus stocks at doses of 10^4 to 10^7 pfu/mouse in 25 µl of PBS/calf. Following inoculation, virus stocks were titrated to ensure that the correct dose had been administered. After 6 weeks, all surviving mice were killed and dissected, and the nine ipsilateral dorsal root ganglia supplying the lower limb of each mouse were separately cultured in ETF10 in microtitre plates. They were screened every second day for the presence of infectious virus by transferring the culture supernatant to BHK C13 cell monolayers. The cells were incubated for 2 days at 37° and examined for the presence of plaques or cpe. Plaques were picked at various stages of reactivation and viral phenotypes examined by probing infected cell DNA following Southern blotting (see Section 3.14).

3.38. DNA sequencing

The ends of cos46 were sequenced to confirm that the it overlapped with the cosmids adjacent to it in the set. Also, mutated cosmids were sequenced to confirm the precise nature of the mutations introduced.

(a) DNA preparation and ligation

Bacteriophage M13 mp19 RFII DNA was digested sequentially with *Sma*I then *Eco*RI in the appropriate buffers. Cosmid DNA (2 µg) was digested with *Sma*I (*Sma*I cuts HSV-1 DNA frequently but does not cut the cosmid vector). The digested DNAs were electrophoresed on an agarose gel and the appropriate fragments were recovered from the agarose using the Sephaglass method (Section 3.29). The DNA fragments were then digested with *Eco*RI (which cuts in at each end of the cosmid vector), extracted with phenol:chloroform, ethanol precipitated and resuspended in 50 µl of H_2O (see Section 3.23). Ligations were set up containing 1 µg of M13 DNA, 5 µg of cosmid DNA, 1 x ligation buffer, 10 mM ATP and 1 unit of T4 DNA ligase. Two control ligations were

also set up, both without the cosmid DNA and one also without ligase. Ligations were incubated at 16°C overnight and then frozen at -20°.

(b) Transformation of *E. coli*

200 µl of an overnight culture of XL-1 cells was added to 10 ml of 2YT broth and shaken at 37° for 2 h. The cells were pelleted by centrifugation at 2000 rpm for 5 min, resuspended in 1 ml of TSB/DMSO and incubated on ice for 10 min. 100 µl of cells and 5 µl of a 1:5 dilution of the ligations were added to precooled 15 ml Falcon tubes and incubated on ice for 15 min. The mixture was placed at 42° for 2 min before adding 3 ml of melted top agar solution (at 42°) containing 96 µl of 4% Xgal, 160 µl of 30 mg/ml IPTG and 1.6 ml of the original overnight bacterial culture. The mixture was poured onto L-broth agar plates, and the plates incubated inverted at 37° overnight.

(c) Preparation of DNA templates

1 ml of an overnight culture of XL1 cells was added to 100 ml of 2YT broth and 1.2 ml was aliquoted into each well of a 24 well plate. Clear plaques were transferred to the wells using sterile cocktail sticks and the plates were incubated in a shaking benchtop incubator at 37° for 6 h. 1 ml of culture was transferred to a 1.5 ml tube and centrifuged at 12,000 rpm for 5 min to pellet the bacteria. The supernatant was transferred to a fresh tube, mixed with 120 µl PEG/NaCl and incubated at 4° overnight. The bacteriophage was pelleted by centrifugation at 12,000 rpm for 5 min and resuspended in 100 µl TE and 50 µl of phenol/TE by vortexing. After 1 h at RT the tube was vortexed again and centrifuged at 12,000 rpm for 2 min. The supernatant was transferred to a fresh tube and mixed with 20 µl of sodium acetate and 250 µl of ethanol. After incubation at -20° overnight, the DNA was pelleted by centrifugation at 12,000 rpm for 10 min at RT, washed with 70% (v/v) ethanol and lyophilised. The pellets were resuspended in 30 µl of TE and stored at -20°.

(d) DNA sequencing reactions

2 µl of each template was added to a 96 well microtitre plate, followed by 10 µl of annealing mix. The plate was vortexed briefly and incubated at 37° for 30 min. 2 µl of each annealed template was dispensed into 4 wells of a 96 well microtitre plate. 2 µl of [^{35}S]-G, -A, -T or -C mixture plus Klenow was aliquoted onto the side of one well each. The plate was covered, centrifuged briefly in a tabletop centrifuge (Sorvall RT6000B) and placed at 37° for 10 min. 2 µl of chase solution was added to the sides of each well, the plate was centrifuged briefly and incubated at 37° for 10 min. The plates were stored at -20°.

(e) Gel electrophoresis

Clean siliconised sequencing gel plates were assembled into a sandwich using sealing tape, with two large foldback clips on each side positioned over the spacers. 60 ml of top gel mix and 12 ml of bottom gel mix were aliquoted into separate beakers, to which 120 µl and 24 µl of APS, respectively, was added. The same volumes of TEMED were added and the solutions mixed. 12 ml of polymerising top gel mix was taken up into a 25 ml pipette, followed by all of the polymerising bottom gel mix, allowing a few bubbles to disturb the interface. This mixture was dispensed into the plate sandwich followed by the remaining top gel mix. The sandwich was then placed almost horizontally and two narrow well sharkstooth combs were placed adjacently into the top 5 mm of the gel, with the teeth uppermost. The gel was allowed to set for 15-30 min, the tape was removed from the bottom of the plates and the sandwich was placed into a sequencing apparatus in which the electrophoresis tanks were filled with 0.5 x TBE. The combs were removed and the wells washed with 0.5 x TBE using a syringe. The combs were replaced with the teeth touching the top of the gel. 2 µl of formamide-dyes was added to each well of the microtitre plate containing the sequencing reactions. The trays were vortexed briefly, then placed into a boiling water bath for 1 min and chilled on ice. 2 µl of each sample was loaded for each template, (in the order G, A, T and C) and the gel was electrophoresed at 60 W until the bromophenol blue dye reached the bottom of the gel (approximately 2.5 h). The plates were separated and the plate containing the gel was fixed with 3-4 10 min washes of 10% acetic acid in a fume hood. The fixed gel was transferred to 3MM paper, covered with clingfilm, dried for 45 min at 80° on a gel drier and autoradiographed.

Chapter 4

Results

4.1. COSMIDS CONTAINING MUTATIONS IN UL14, UL15 AND UL17

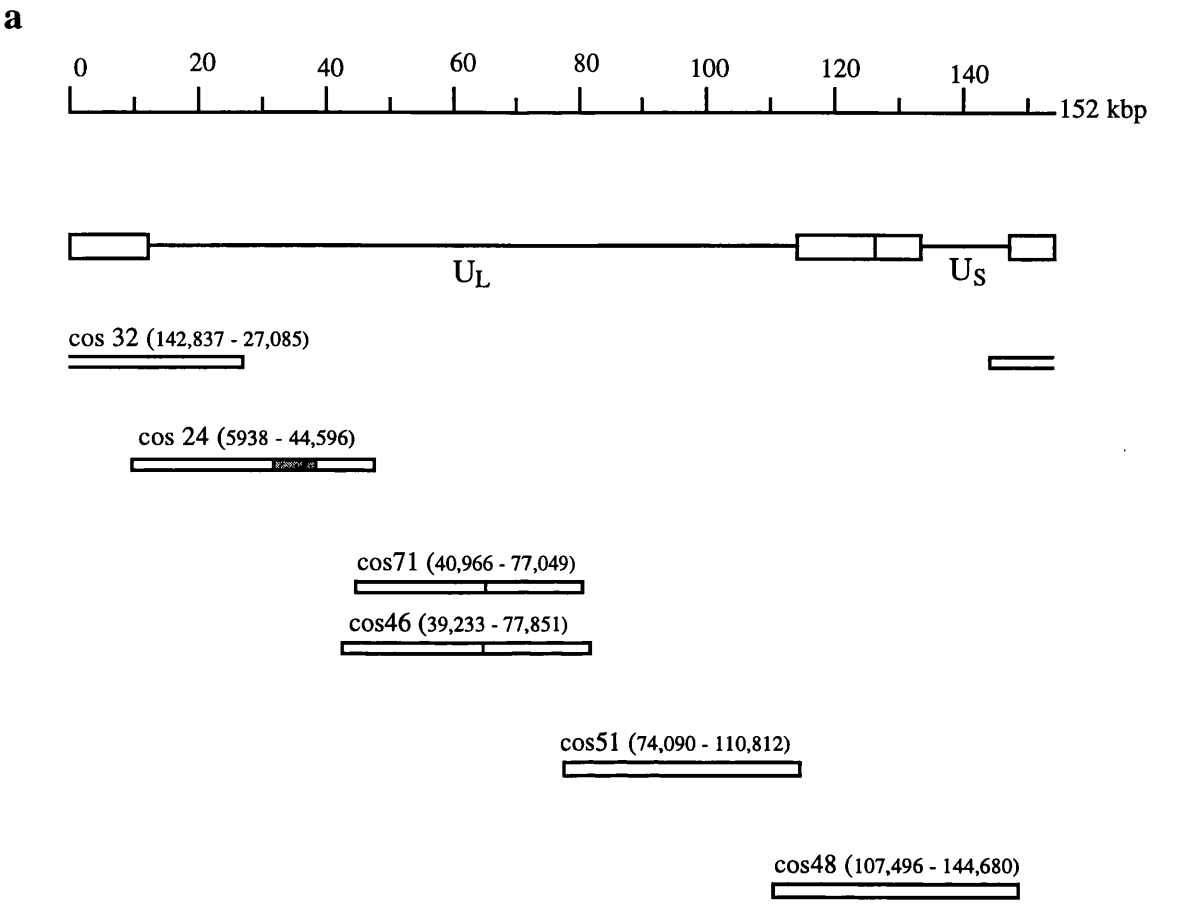
4.1.1. Status of ori_L in the cosmid set

Cunningham and Davison (1993) developed a cosmid-based system of mutagenesis for producing mutations in HSV-1 genes. Of the three cosmid sets generated, one, comprising five cosmid clones (cos32, cos24, cos71, cos51, cos48), which together contain the coding sequence of the entire HSV-1 genome (Fig. 13), was initially used in this study with the aim of producing frameshift mutants in HSV-1 genes UL14 to UL17. Cos24 contains genes UL14-UL17 in a region which does not overlap with adjacent cosmids, and thus mutants can in principle be generated in these genes without generating *wt* virus. UL14 is a small gene which is orientated in the opposite direction to UL15. UL15 is the only spliced gene in U_L and contains UL16 and UL17 entirely in the intron on the opposing strand (see Fig. 13b).

Cunningham and Davison (1993) found that ori_L is readily lost from cosmids. This is consistent with earlier work showing that ori_L is unstable in plasmids (Weller *et al.*, 1985; Hardwicke and Schaffer, 1995). Cunningham and Davison (1993) found that cosmids containing the ori_L region had either lost ori_L entirely and were thus ori_L^- or were composed of a mixture of ori_L^+ and ori_L^- cosmids, with the former in the minority. They also noted that ori_L^+ virus was invariably produced from cosmids that are predominantly ori_L^-, presumably because of the ability of the minority of ori_L^+ molecules to replicate and predominate in the viral progeny. Cunningham and Davison (1993) noted that cos71 is completely ori_L^-, and therefore it was necessary at the initiation of this work to replace cos71 by a cosmid containing an ori_L^+ population. A candidate cosmid (cos46) from the library was investigated.

The status of ori_L in cos46 was checked by probing *Kpn*I digests with a plasmid containing *Kpn*I v, which contains the ori_L spanning region (although this fragment itself lacks the complete ori_L). Cos14, from another cosmid set, which contains ori_L^+ molecules, and an ori_L^- derivative of cos14 were used as controls. Fig. 14 shows that four different cos46 preparations were ori_L^+.

Restriction endonuclease (RE) analysis showed that cos46 overlaps the flanking cosmid, cos51. The restriction fragment pattern of cos46 following digestion with *Eco*RI confirmed that it overlapped cos51 since both contain the *Eco*RI L fragment. The ends of the cos46 insert were sequenced to define precisely the extent of overlap with both adjacent cosmids. Cos46 was digested with *Sma*I plus *Eco*RI, the ends excised from an agarose gel and inserted into *Sma*I plus *Eco*RI digested M13mp19. The ends of the



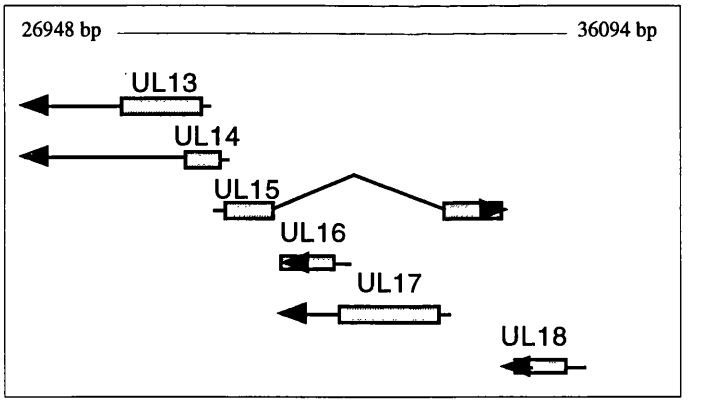


Fig. 13. Location of cosmid inserts with respect to the HSV-1 genome

a Locations of cosmid inserts are indicated by open bars and their precise locations in the HSV-1 sequence are given in bp. The position of the *ori*$_L$ region is denoted by a vertical line in cos71 and cos46 and the area containing UL14-UL17 is shaded in cos24.

b Detailed representation of the position of UL14-UL17. The shaded boxes represent protein coding sequences, horizontal lines indicate mRNA and the arrows indicate direction. The nucleotide positions in the HSV-1 sequence of the end of the UL13 and the beginning of the UL18 coding regions are given in bp.

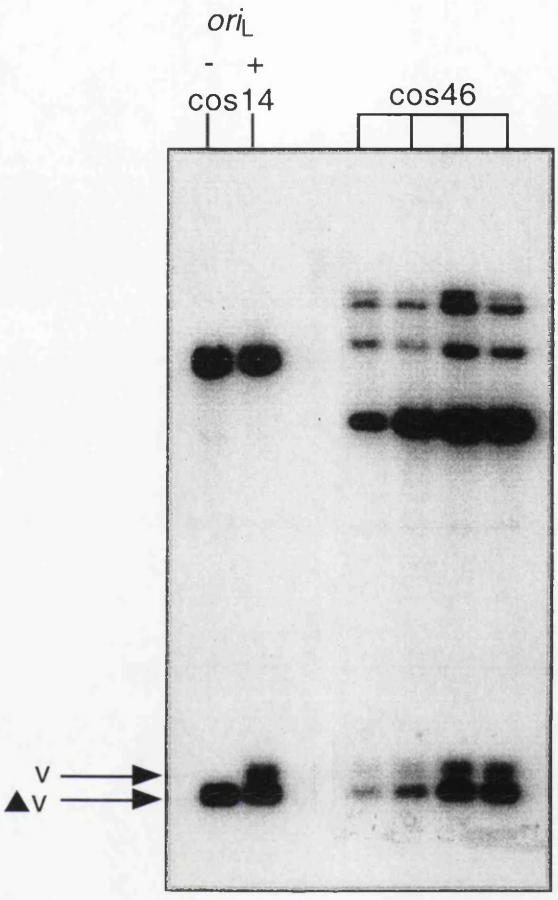


Fig. 14. Status of *ori*$_L$ in cos46

An autoradiograph of a Southern blot in which *Kpn*I fragments from four different preparations of cos46 and two varients of cos14 (one consisting entirely of *ori*$_L$- molecules (-) and the other containing a majority of *ori*$_L^-$ and a minority of *ori*$_L^+$ molecules (+)) were transferred from a 0.8% (w/v) agarose gel and probed with a plasmid containing *Kpn*I v, which contains the *ori*$_L$ region. Fragments representing the full length (v; 1933 bp) and deleted (▲v; approximately 1800 bp) forms of *Kpn*I v are indicated. The more intense bands near the top of the autoradiograph are due to hybridisation of vector sequences, and the less intense bands near the top of the autoradiograph are due to partial digestion of the cos46 samples.

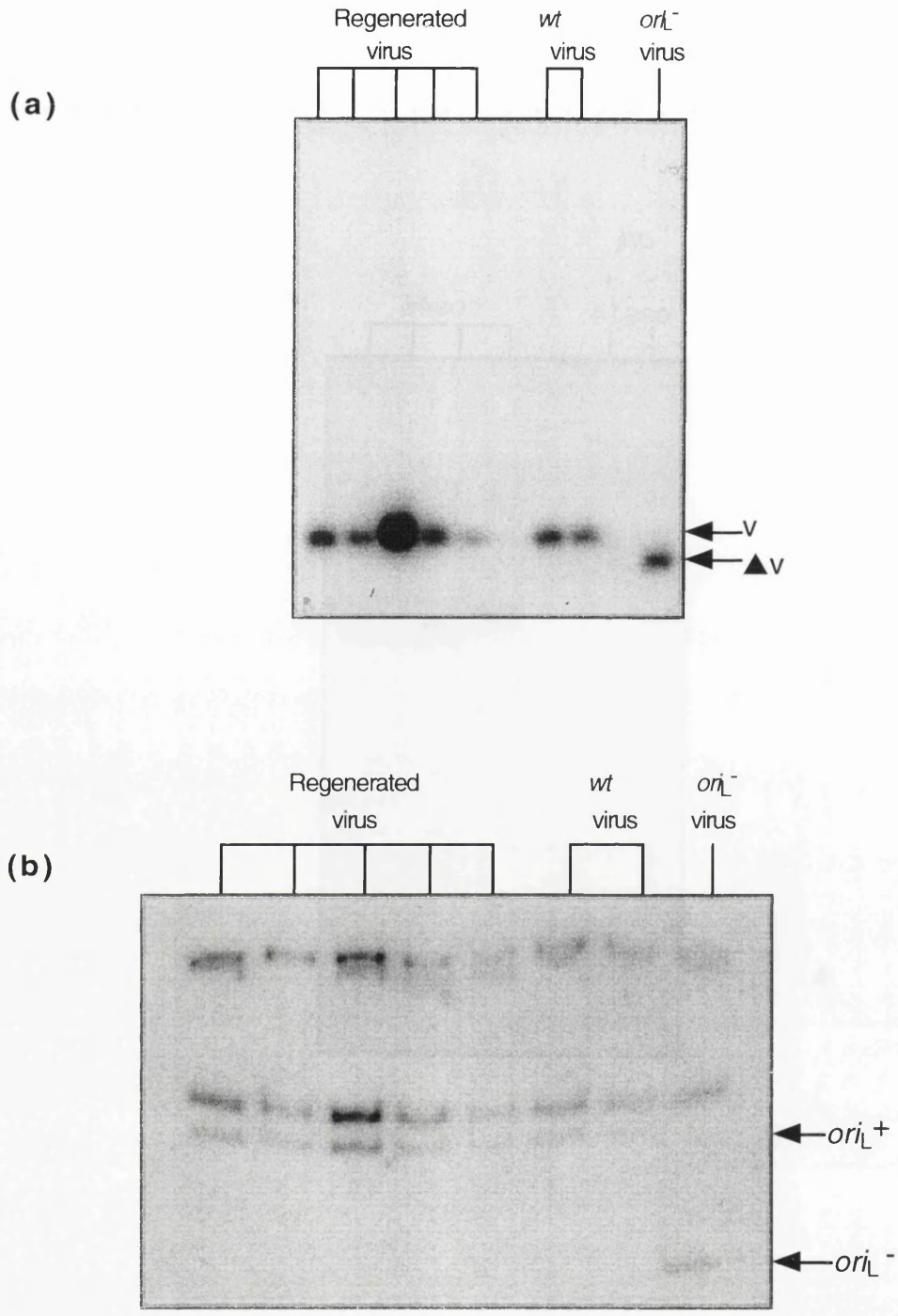


Fig. 15. Status of *ori*$_L$ in *wt* and reconstructed viruses

An autoradiograph of a Southern blot in which infected cell DNA was digested with (a) *Kpn*I or (b) *Bam*HI plus *Bst*EI, and electrophoresed through (a) a 0.8% agarose or (b) a 5% polyacrylamide gel, transferred to a nitrocellulose membrane and probed with a plasmid containing *Kpn*I v. The positions of *Kpn*I v (full length) and ▲v (deleted) are shown in (a) and the locations of *ori*$_L^+$ and *ori*$_L^-$ fragments are shown in (b). The *ori*$_L^-$ virus was prepared by cosmid recombination and provided by C. Cunningham.

cos46 insert were found to be located at 39233 and 77851 bp in the HSV-1 DNA sequence and therefore, cos46 overlaps cos24 by 5363 bp and cos51 by 3761 bp (see Fig. 13).

4.1.2. Regeneration of *wt* virus

The five cosmids (cos32, cos24, cos46, cos51, cos48) were digested with *Pac*I to excise the intact inserts, since *Pac*I does not cleave HSV-1 DNA. Approximately equimolar amounts of digested cosmids were mixed together (they were quantified by comparison with DNAs of known concentration (obtained commercially) by agarose gel electrophoresis) and transfected into BHK C13 cells and plaques were picked at 2-4 days post transfection. Small viral stocks were made from each of the picked plaques, and infected cell DNA was prepared. *Kpn*I or *Bam*HI plus *Bst*EI digests were probed with a plasmid containing *Kpn*I v (Fig. 15). All of the five picked plaques were found to be ori_L^+.

4.1.3. Construction of mutant cosmids

*Asp*718, which cuts (at the sequence G'GTACC) seven times in the cos24 insert but not in the vector, was used to produce frameshift mutations. Fig. 16 shows *Kpn*I profiles of the five parental cosmids. *Kpn*I recognises the same site as *Asp*718 but cleaves differently (at GGTAC'C).

Optimal conditions for linearising cos24 with *Asp*718 were established with respect to enzyme dilution and EtBr concentration. Linearised cos24 DNA was extracted from agarose gels by electroelution, treated with T4 DNA polymerase in the presence of the four dNTPs to produce flush ends and ligated. This results in a 4 bp insertion (GGTAC**GTAC**C), which alters the reading frame of the gene from that point onwards, and creates a new *Sna*BI site (GGTACGTACC) in place of the lost *Asp*718 (*Kpn*I) site. Fig. 17 shows a map of the fragments obtained following digestion of cos24 with *Kpn*I or *Sna*BI.

After transfection, cosmids were obtained with insertions in UL6, UL7, UL14, UL15 or UL17. Each mutation was detected by screening for loss of the appropriate *Kpn*I (*Asp*718) site and gain of a new *Sna*BI site (Fig. 18). Thus, for example, the mutation in UL14 results in fusion of *Kpn*I q (3819 bp) and f (10837 bp), giving rise to a novel 14.6 kbp fragment. The creation of a novel *Sna*BI site generates fragments of 6062 bp and 228 bp (electrophoresed from the bottom of the agarose gel) from the parental 6.29 kbp fragment. The UL6, UL7, UL14 and UL15 mutations were confirmed by *Kpn*I and *Sna*BI RE analysis. The UL17 mutation was confirmed by *Kpn*I digestion, but the

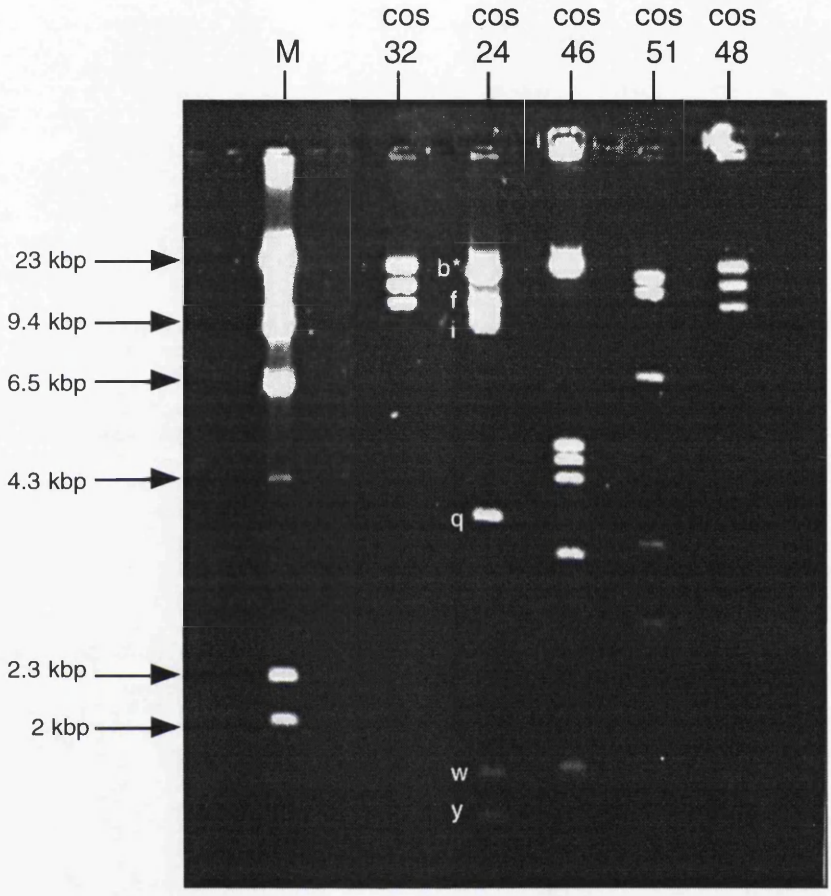


Fig. 16. *Kpn*I restriction profiles of parental cosmids

An EtBr-stained 0.8% (w/v) agarose gel of *Kpn*I digests of the five cosmids. HSV-1 *Kpn*I fragments generated from cos24 are shown; b* contains part of *Kpn*I b and b' plus the cosmid vector (see Fig. 17). M is a *Hind*III digest of bacteriophage lambda DNA used as size markers.

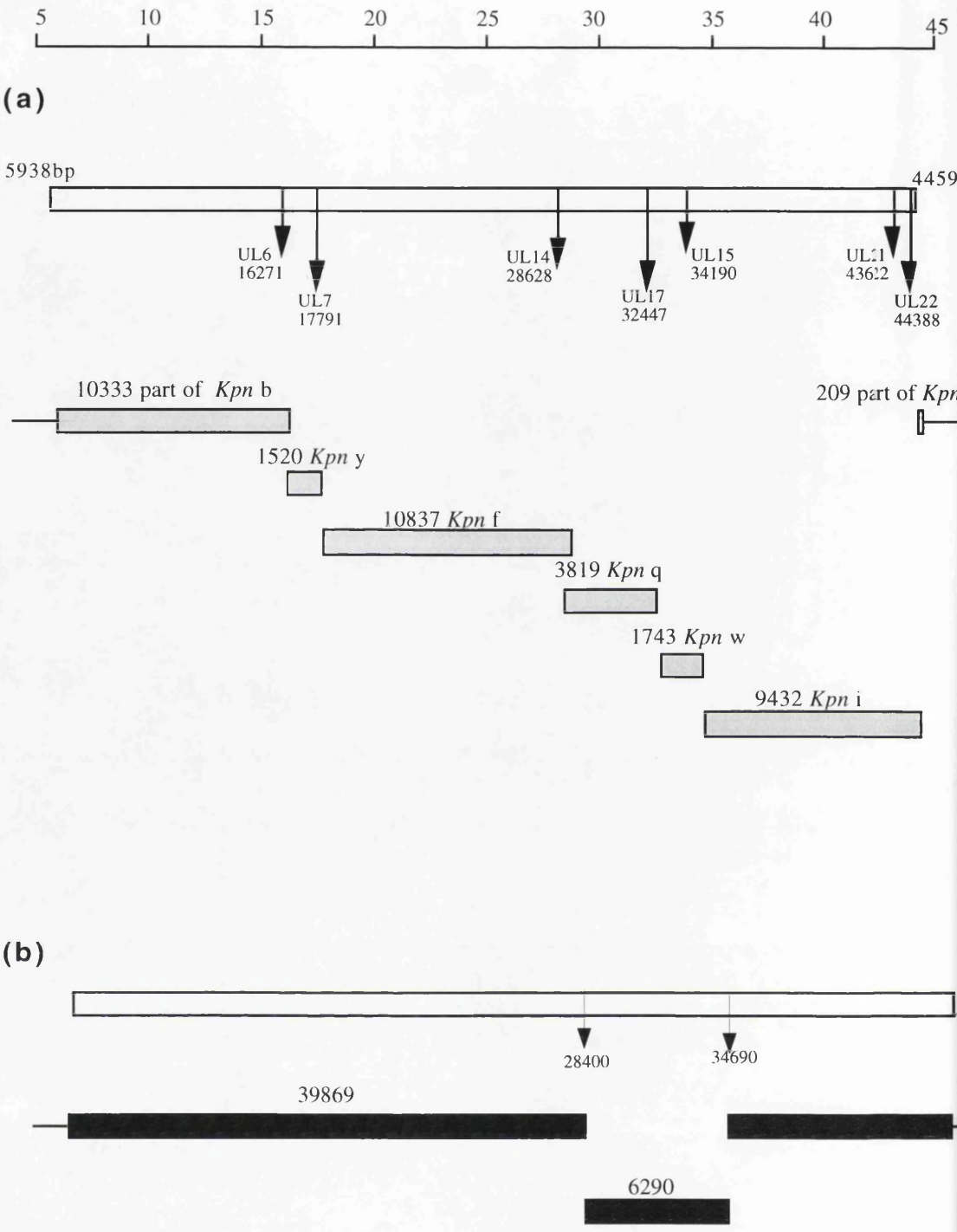
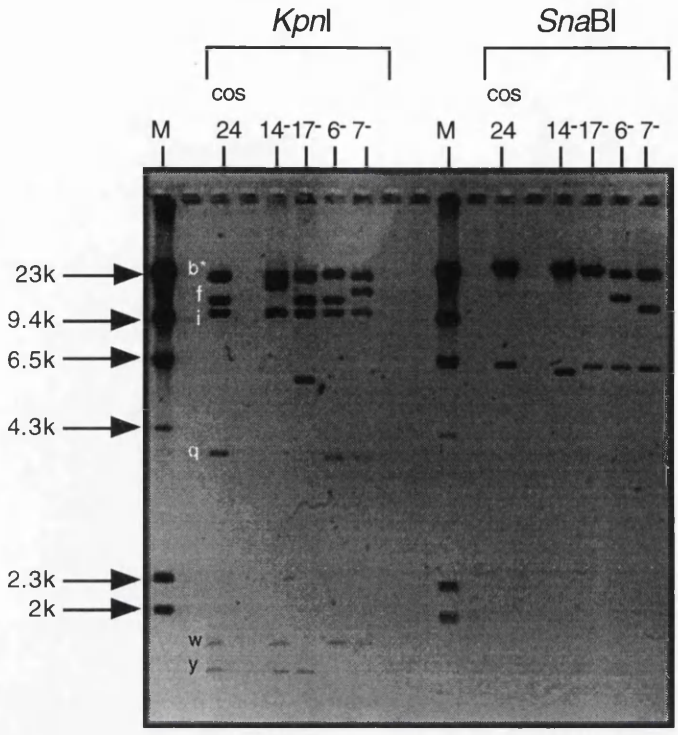


Fig. 17. *Kpn* I (*Asp* 718) and *Sna*BI sites in cos24

(a) Fragments produced by digestion of cos24 with *Kpn*I or *Asp*718 are shown as shaded rectangle The name and size in bp of each fragment is given. The positions of the seven restriction sites in th HSV-1 sequence are given in bp and denoted by arrows. The horizontal lines at the end of the frag represent vector sequences, which lack *Kpn*I sites.

(b) Fragments produced following digestion of cos24 with *Sna*BI are shown as filled rectangles. The sizes of the two fragments are given in bp. The position of the restriction sites in the HSV-1 sequenc given in bp and denoted by arrows. The horizontal lines at the end of fragments indicate vector sequences, which lack *Sna*BI sites.



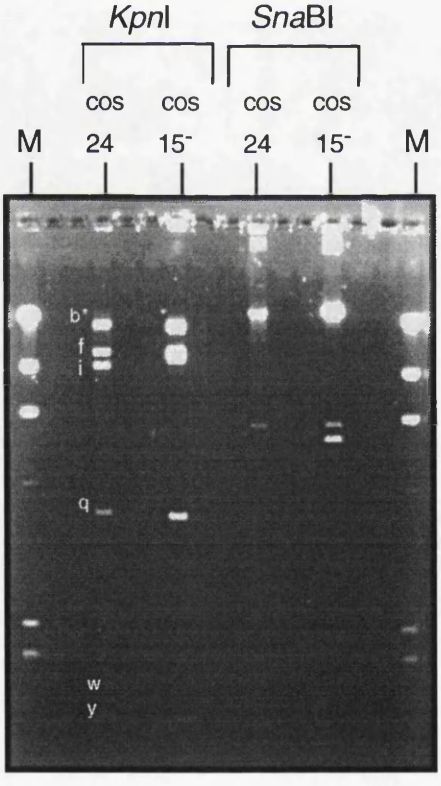


Fig. 18. Identification of mutant cosmids by RE analysis

EtBr-stained gels of cosmids with 4 bp insertions at sites in UL14, UL15, UL17, UL6 and UL7 (denoted 14^-, 15^-, 17^-, 6^-, 7^-) were screened for the loss of a *Kpn*I site and gain of a *Sna*BI site. Cos24 was used as a control and the HSV-1 *Kpn*I fragments are indicated (b* = part of *Kpn*I b plus b' and the cosmid vector). Digestion of 15^- with *Sna*BI produced a partial *Sna*BI digest. M is a *Hind*III digest of bacteriophage lambda DNA used as size markers.

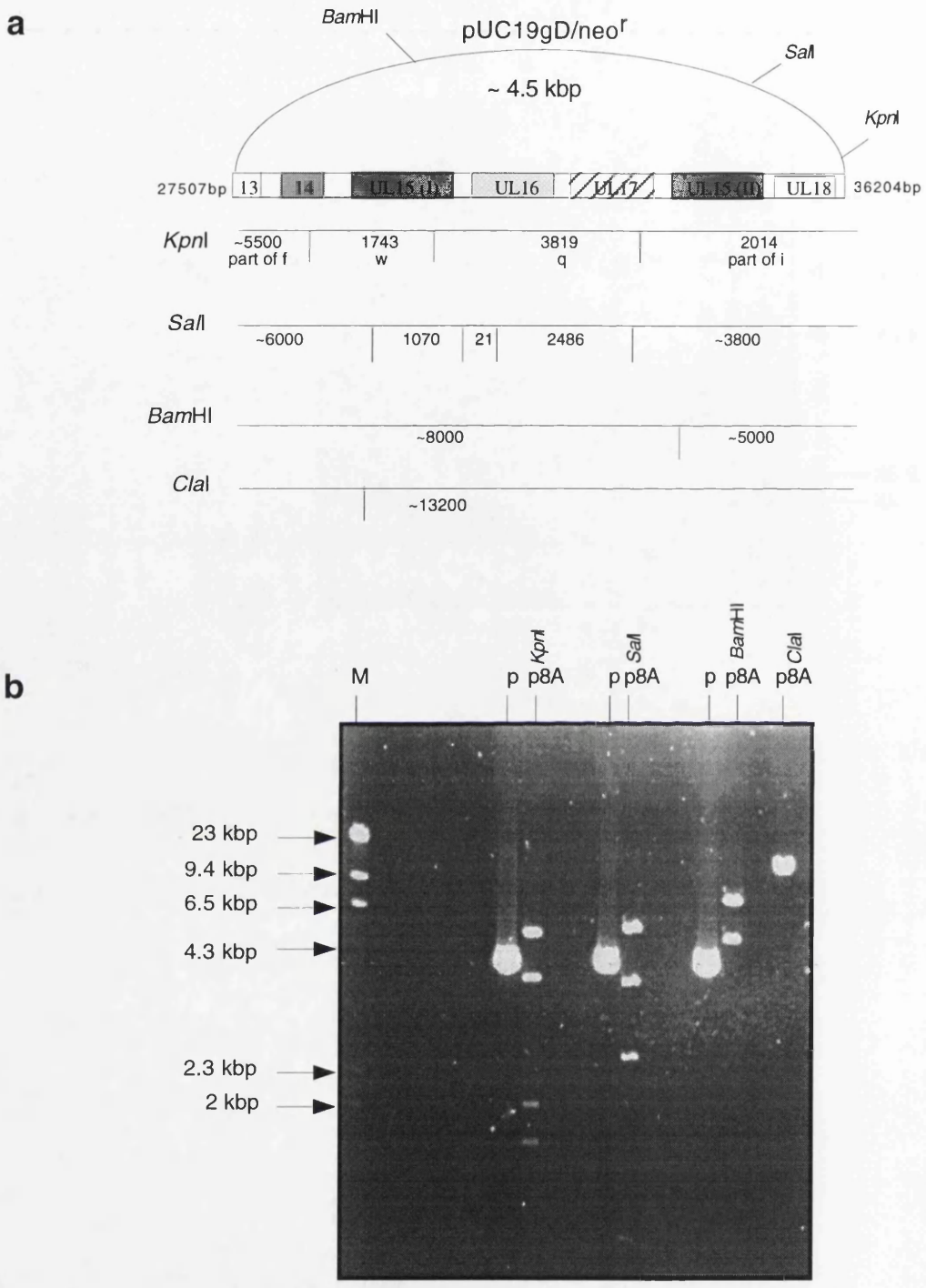


Fig. 19. The structure of plasmid p8A, containing UL14-UL18

a RE map of p8A for *Kpn*I, *Sal*I, *Bam*HI and *Cla*I. Fragment sizes are given in bp.

b An EtBr-stained gel of pUC19gD/*neo*r linearised with *Sph*I (p) or p8A digested with *Kpn*I, *Sal*I, *Bam*HI or *Cla*I. M is a *Hind*III digest of bacteriophage lambda as size markers.

anticipated 4 bp insertion may not have take place since a novel *Sna*BI site was not detected.

An attempt was made to generate additional mutant cosmids using *Kpn*I and *Alw*44I. *Kpn*I recognises the same site as *Asp*718 but cleaves to leave a 3' 4 bp overhang, so that mutated cosmids would contain a 4 bp deletion. *Alw*44I cleaves at G'TGCAC, present in UL14, UL15, UL16 and UL17, and thus mutated cosmids would contain a 4 bp insertion. However, mutant cosmids were not obtained using these enzymes. Since in some experiments at least 200 colonies were screened in order to obtain a single mutant cosmid, and cosmids with insertions in UL14, UL15 and UL17 had already been produced using *Asp*718, work with these two enzymes was not pursued. In addition, two UL16$^-$ mutant viruses had already been produced in the laboratory by C. Cunningham using a different cosmid set.

4.1.4. Candidate complementing cell lines

At the beginning of this work little was known about the nature of UL14-UL17. Poon and Roizman (1991) had shown that a UL16$^-$ deletion mutant was viable in cell culture, and also suggested that UL17 is an essential gene because they were unable to produce a UL17$^-$ mutant. Given the possibility that UL14, UL15 and UL17 might be essential, cell lines potentially expressing these genes were constructed.

a. Cos24 cell line

A candidate complementing cell line, produced by transfecting cos24 into BHK C13 cells was provided by C. Cunningham. The cosmid vector contains the neomycin resistance gene and the resulting cell line was neomycin (G418) resistant. This cell line has the potential to express UL1 to UL21, and has been shown to complement UL9$^-$ and UL12$^-$ mutants but not UL6$^-$ or UL8$^-$ mutants. (C. Cunningham and A.J. Davison, personal communication).

b. 8A cell line

As an initial step, an 8.6 kbp *Sph*I fragment from cos24 containing the entire coding sequences of UL14 to UL18 was inserted into the *Bgl*II site in pUC19gD/*neo*^{r} to give plasmid p8A (Fig. 19A). pUC19gD/*neo*^{r} consists of pUC19, the neomycin resistance gene under the control of the SV40 promoter and the HSV-1 US6 (gD) promoter (which may be used to drive the inserted genes if their own promoter is absent) upstream from two unique restriction sites (*Bgl*II and *Kpn*I), and was provided by Dr A. Patel. The structure of p8A was assessed by RE analysis with *Kpn*I, *Sal*I, *Bam*HI or *Cla*I. This analysis confirmed that p8A contains the expected insert, since the RE profiles shown in

Fig. 19B are as predicted in Fig. 19A. p8A was transfected into BHK C13 cells by lipofection and 98 neomycin-resistant cell lines were isolated from single colonies.

c. p14, p15 and p17 cell lines

Cell lines p14, p15 and p17 were produced using plasmids containing UL14, UL15 or UL17 individually.

*Bgl*II fragments containing UL14 or UL17 were inserted into pUC19gD/*neo*^{r} under their own promoters. The UL15 coding region (lacking the intron) was isolated from a modified form of pMJ511 and inserted into pUC19gD/*neo*^{r} under control of the US6 (gD) promoter. pMJ511 is a vaccinia virus transfer vector containing the fused exons of UL15 under the control of a vaccinia promoter, and was constructed from a genomic clone by Dr A.J. Davison. A novel *Bgl*II site was inserted at the 5' end of the coding region by Dr V.G. Preston to give the modified form of pMJ511. The structures of plasmids containing UL14 (p14), UL15 (p15) or UL17 (p17) were confirmed by RE analysis (Figs. 20 and 21).

BHK C13 cells were transfected with p14 (orientation 1), p15 (orientation 2) or p17 (orientation 1) by calcium phosphate precipitation and DMSO treatment. Six neomycin-resistant cell lines were isolated using p14, 15 with p15 and two with p17.

4.1.5. Generation of viruses

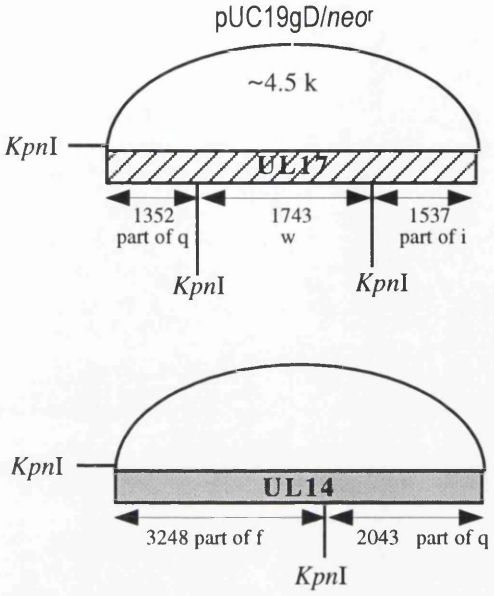
a. Cos24 cell line

The cos24 cell line and BHK C13 cells were transfected with the UL14, UL15 and UL17 mutant cosmid sets in a number of experiments. Viral plaques were obtained only from the UL14 set. Plaques were picked and small viral stocks were produced. *Kpn*I digests of infected cell DNA were probed with plasmids containing *Kpn*I q or f, and the results showed that all progeny were *wt* in that they produced *Kpn*I q and f and not the fused fragment (Fig. 22). Reversion of the lesion introduced into UL14 indicates that the mutant is severely disabled or not viable in BHK C13 cells and that the cos24 cell line does not complement the mutation.

b. 8A cell line

Fifteen cell lines were transfected with the three mutant cosmid sets. Four cell lines produced virus after transfection with the UL14, UL15 and UL17 mutant cosmid sets. Plaques were picked and small viral stocks produced on the 8A cell line originally tested. *Kpn*I-digested infected cell DNA was probed with plasmids containing *Kpn*I q or w. Fig. 23 shows that only *wt* progeny were produced in that they contained *Kpn*I q and w

a



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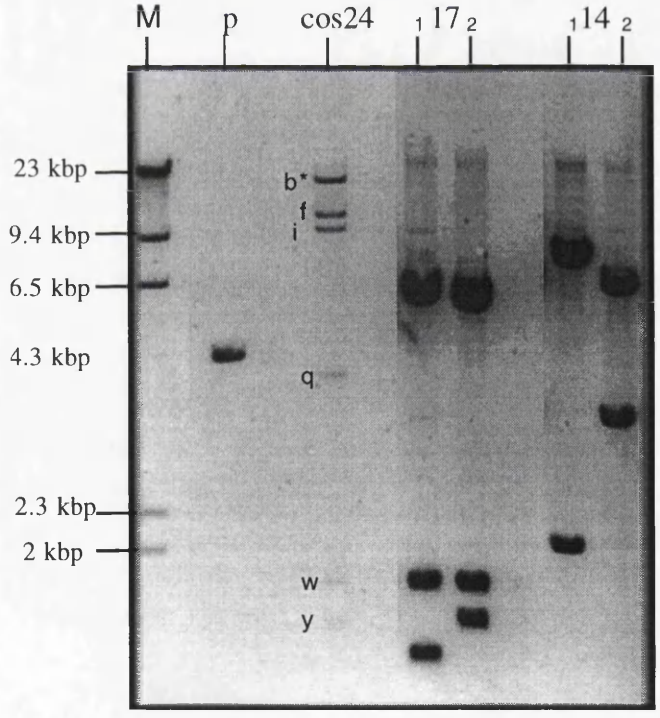


Fig. 20. The structures of p14 and p17

a RE map of p14 and p17 for *Kpn*I. Fragment sizes are given in bp.

b Representative pannels of a single EtBr-stained gel of p14 and p17 digested with *Kpn*I are spliced together. Plasmids contain the UL14 or UL17 fragment in both orientations (1 or 2). Cos24 digested with *Kpn*I, linearised pUC19gD/*neo*r vector (p ~4.5 kbp) and a *Hind*III digest of bacteriophage lambda DNA (M) were used as size markers.

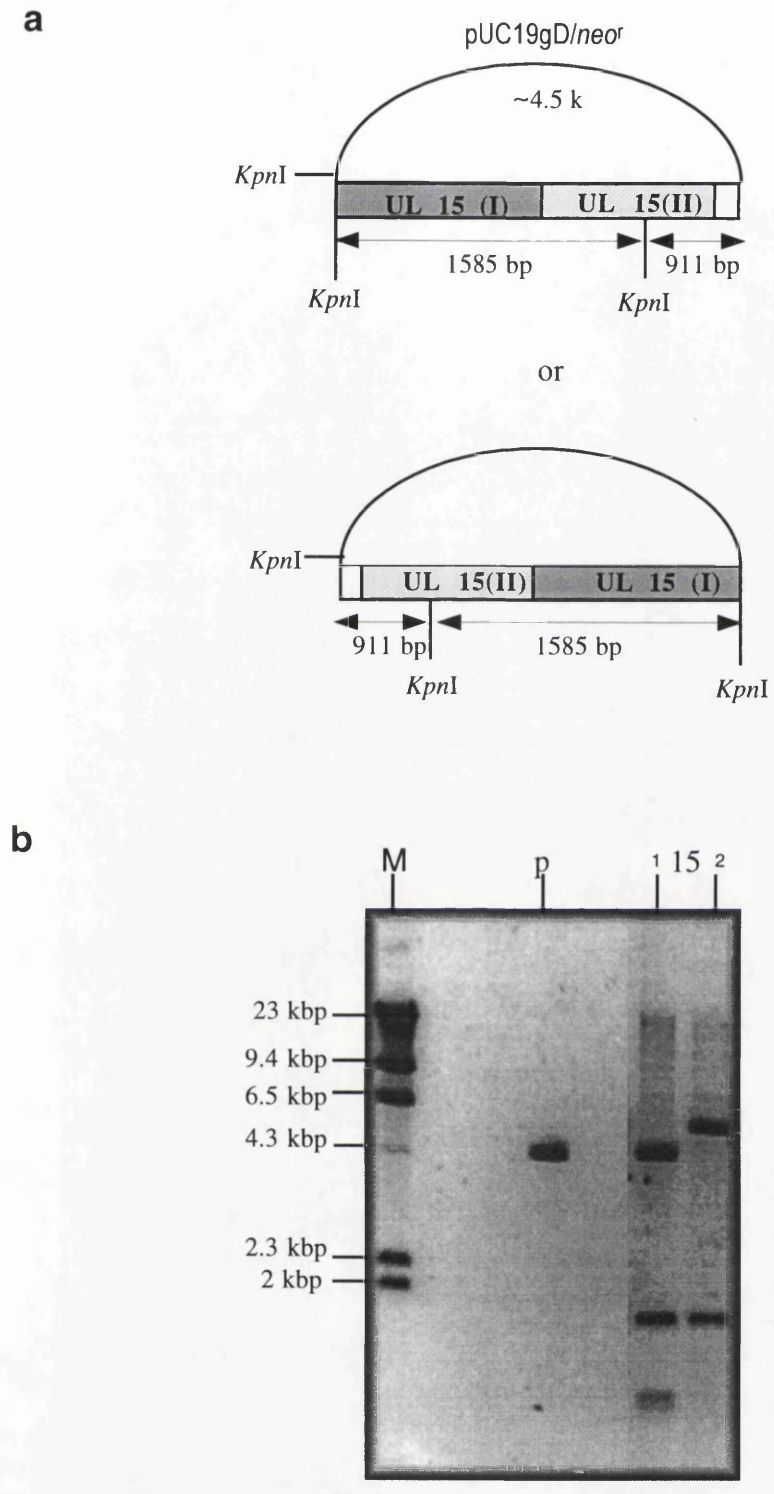


Fig. 21. The structure of p15

a RE map of p15 for *Kpn*I. Fragment sizes are given in bp. I and II indicate the two exons of UL15.

b An EtBr-stained gel of p15 digested with *Kpn*I. Plasmids containing the UL15 fragment in both orientations (1 or 2) are shown. Linearised pUC19gD/*neo*r vector (p ~4.5 kbp) and a *Hind*III digest of bacteriophage lambda DNA (M) were used as size markers.

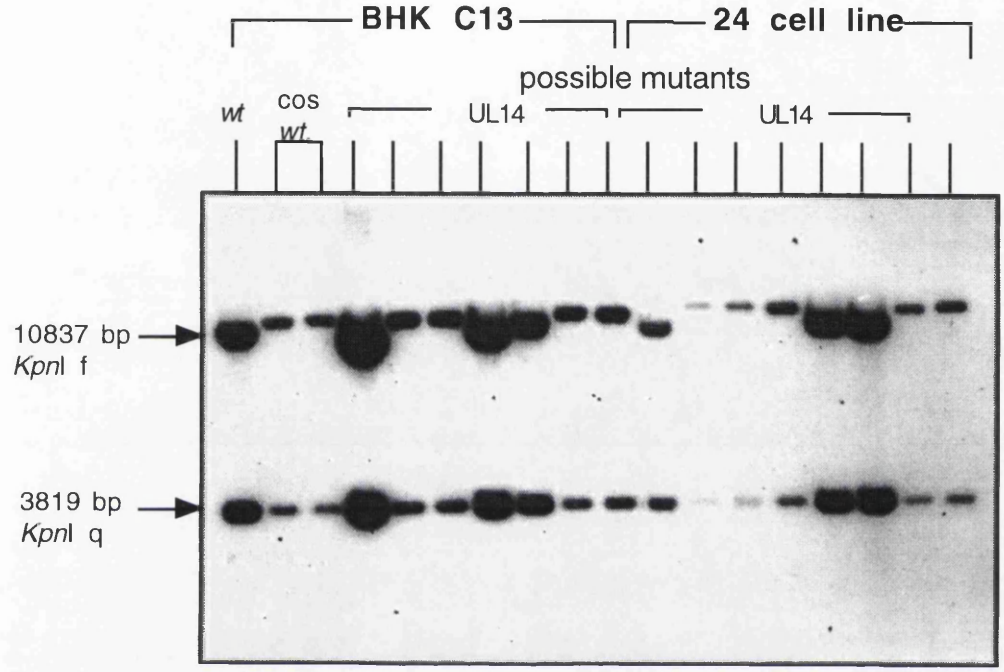


Fig. 22. Genotype of virus generated from the UL14⁻ cosmid set

An autoradiograph of a Southern blot in which infected cell DNA produced from *wt*, cos*wt* (*wt* HSV-1 produced from the parental cosmid set), and possible UL14 mutants picked from BHK C13 cells or the cos24 cell line was digested with *Kpn*I, transferred from a 0.8% (w/v) agarose gel and probed with plasmids containing *Kpn*I q and f. The positions and sizes in bp of these fragments is shown. Apparently faster migrating forms of *Kpn*I f in some lanes are due to a loading artifact.

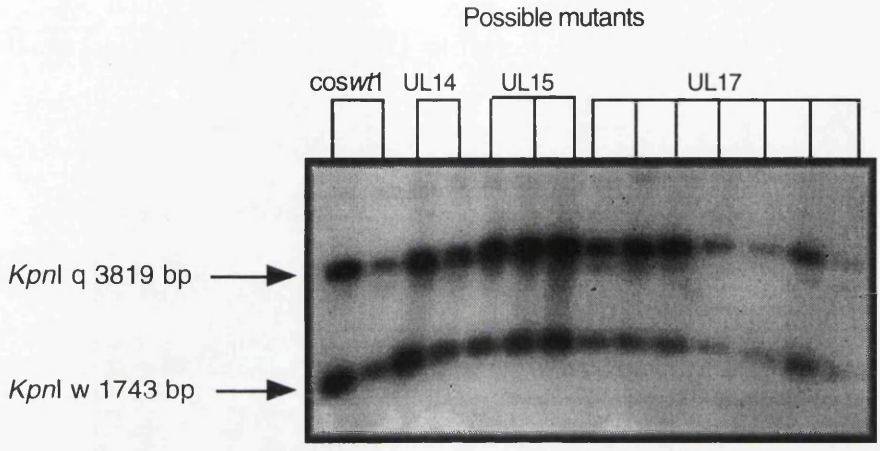


Fig. 23. Genotype of virus generated from one p8A cell line

An autoradiograph of a Southern blot in which infected cell DNA was digested with *Kpn*I and transferred from a 0.8% (w/v) agarose gel to a membrane which was probed with plasmids containing *Kpn*I q and w. The position and size of these fragments are shown.

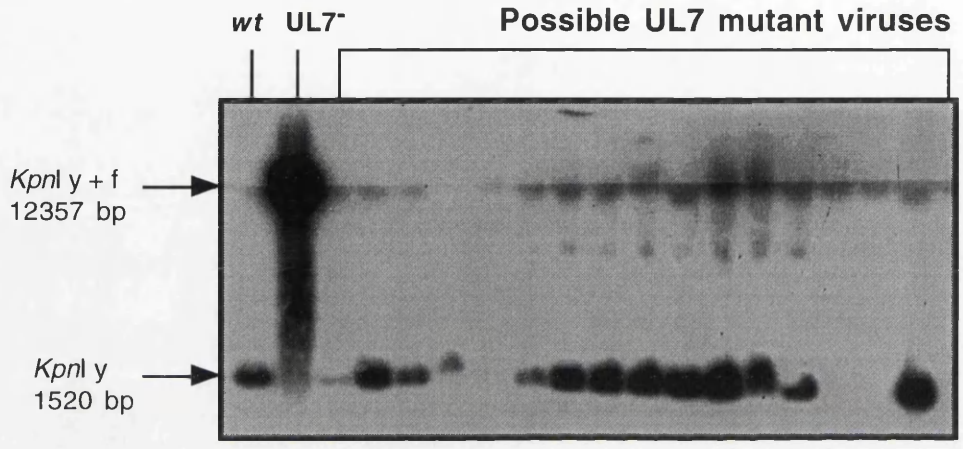


Fig. 24. Genotype of virus generated from the UL7$^-$ cosmid set

An autoradiograph of a Southern blot in which infected cell DNA was digested with *Kpn*I, transferred from a 0.8% (w/v) agarose gel and probed with a plasmid containing *Kpn*I y. The UL7$^-$ lane contains a mutated cosmid and illustrates the mutant banding pattern due to fusion of two fragments. The size and position of detected fragments are shown. The faint bands at the same level as the UL7$^-$ band may be due to non-specific hybridisation.

and not the fused fragment. Reversion to *wt* showed that disruption of UL14, UL15 or UL17 is severely detrimental or lethal to viral growth and that the cell lines tested were not able to complement the defects.

c. p14, p15 and p17 cell lines

All of the cell lines were screened using the appropriate mutant cosmid sets and at least 10 plaques were picked from each for preparation of small viral stocks. Infected cell DNA was digested with *Kpn*I and probed with a plasmid containing the appropriate *Kpn*I fragment. Only *wt* plaques were produced (data not shown), indicating that the cell lines were not able to complement the defects and confirming the severely detrimental effects of mutations in UL14, UL15 or UL17.

4.1.6. Generation of virus from the mutant UL7 cosmid set

In addition to the cosmids with mutations in UL14, UL15 and UL17, a mutant cosmid with a 4 bp insertion in UL7 was made (see Section 4.1.3). The status of UL7 in cell culture is unknown. The mutation is located in a region of the cos24 which overlaps cos32 and thus *wt* and mutant virus can be produced following transfection of the mutant cosmid set. A cell line that complements UL6$^-$ and UL8$^-$ mutants and which therefore may express UL7 was provided by Dr A. Patel. Transfection of this cell line and BHK C13 cells with the UL7$^-$ cosmid set resulted in approximately equal numbers of plaques. Probing of infected cell DNA with a plasmid containing *Kpn*I y showed that all of the plaques picked from the candidate complementing cell line were *wt* in that they contained *Kpn*I y and not the larger fused fragment (Fig. 24). Plaques picked from BHK C13 cells were also found to be *wt* (data not shown). This indicates that the cell line does not complement the mutation and that the UL7$^-$ mutant is severely disabled or not viable.

4.1.7. Mass spectrometric analysis and identification of proteins

Proteins whose predicted sequences are present in databases may be identified by newly developed techniques involving mass spectrometry of electroblotted proteins digested with proteases (e.g. trypsin), and computer-aided comparison of the observed peptide masses with the masses predicted from theoretical cleavage of protein sequences in a database.

The protein products of the EHV-1 counterparts of UL16 and UL17 have been identified in small amounts in the EHV-1 tegument using this technique (A.J. Davison, personal communication). Thus, an attempt was made to identify the UL16 and UL17 proteins in HSV-1 virions. Attempts to identify the UL16 protein are described in Section 4.2.3.

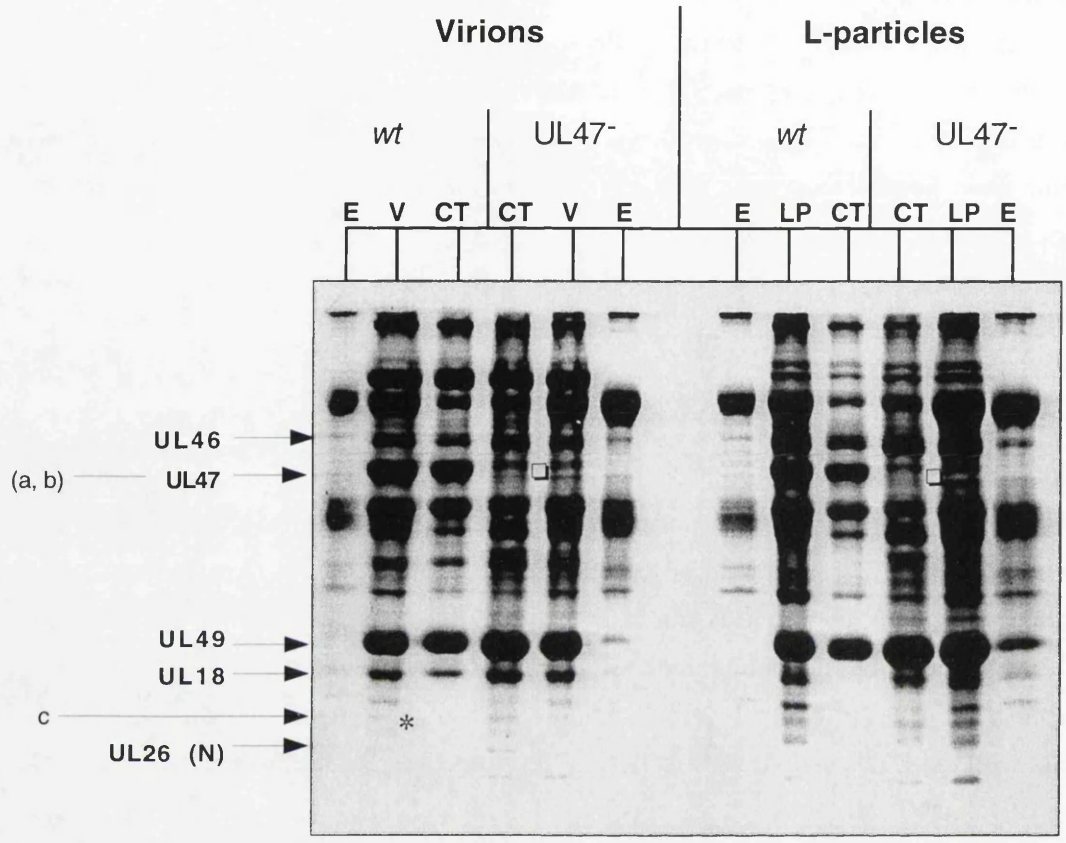


Fig. 25. Identification of proteins using mass spectrometric analysis

A coomassie blue stained gel of virions and L-particles from *wt* virus and a UL47$^-$ mutant. Bands a (upper) and b (lower) (❑) were identified in the UL47$^-$ virus samples and a number of lower molecular weight bands were identified, including band c (*). These three proteins were analysed by laser desorption mass spectrometry (see Figs. 26 and 27). Known HSV-1 virion proteins are shown in bold type on the left. V=virions, LP=L-particles, E=envelope fraction, CT=capsid/tegument fraction.

The UL17 protein (predicted mass 74584.1 Da) was expected to comigrate with the UL47 protein (predicted mass 73818.7 Da) on polyacrylamide gels. UL47 is a major component of virions, so a UL47$^-$ virus was utilised so that minor proteins that might comigrate with the UL47 protein could be analysed. The UL47$^-$ virus used contained a 4 bp insertion at the *Kpn*I site at position 103010 bp in the the HSV-1 sequence and was produced by C. Cunningham. In Fig. 25, two bands (a and b) are evident in the region of the Coomassie blue-stained gel which usually would be obscured by the UL47 protein. These bands were isolated from a stained membrane, digested with trypsin and subjected to laser desorption mass spectrometric analysis. It was not possible to distinguish between the two bands during excision and so they were analysed together. Fig. 26a shows a typical mass spectrum obtained from analysis of bands a + b. The peaks are small and the background is high, as is typical of minor proteins. The values of all credible peaks were entered into the Massmap search program, which compares them with the profiles of proteins whose sequences are present in a database (Genbank). Fig. 26b shows the proteins identified from the results obtained in Fig. 26a. From the thousands of proteins searched, UL46 and UL17 were identified as the best two matches. Fig 26c shows the masses which are unique to each protein or which are shared by both. UL17 and UL46 share four peptide masses, but each has enough unique masses to indicate its presence in this region. The UL46 protein (predicted mass 78246.3 Da) normally migrates with a greater apparant mass by SDS-PAGE (see Fig. 25), and the minor form detected in bands a + b may be an alternatively processed form.

In Fig 25, a number of faint bands are also visible in the 20-40 kDa region of the stained gel. Analysis of band c (as described above) indicated the presence of the UL14 protein in conjunction with other proteins. Fig. 27a shows the mass spectrum obtained. Analysis of these peptide peaks indicated the presence of UL14 and UL49 (Fig. 27b). Fig. 27c shows the peptide peaks unique to each protein.

Since the proteins analysed were present in small amounts, it was difficult to produce enough material to analyse and so identification has been made at the limits of detection. However, various rounds of Massmap analysis from a number of different experiments indicate that the UL17 and the UL14 proteins are present in small amounts in HSV-1 virions.

4.1.8. Assessment of an antibody potentially identifying the UL17 protein

A β-galactosidase fusion protein rabbit antiserum potentially directed against the UL17 protein (residues 458 to 531) was produced by C. Cunningham but had not been evaluated, and so was used as a way of identifying the UL17 protein in Western blots.

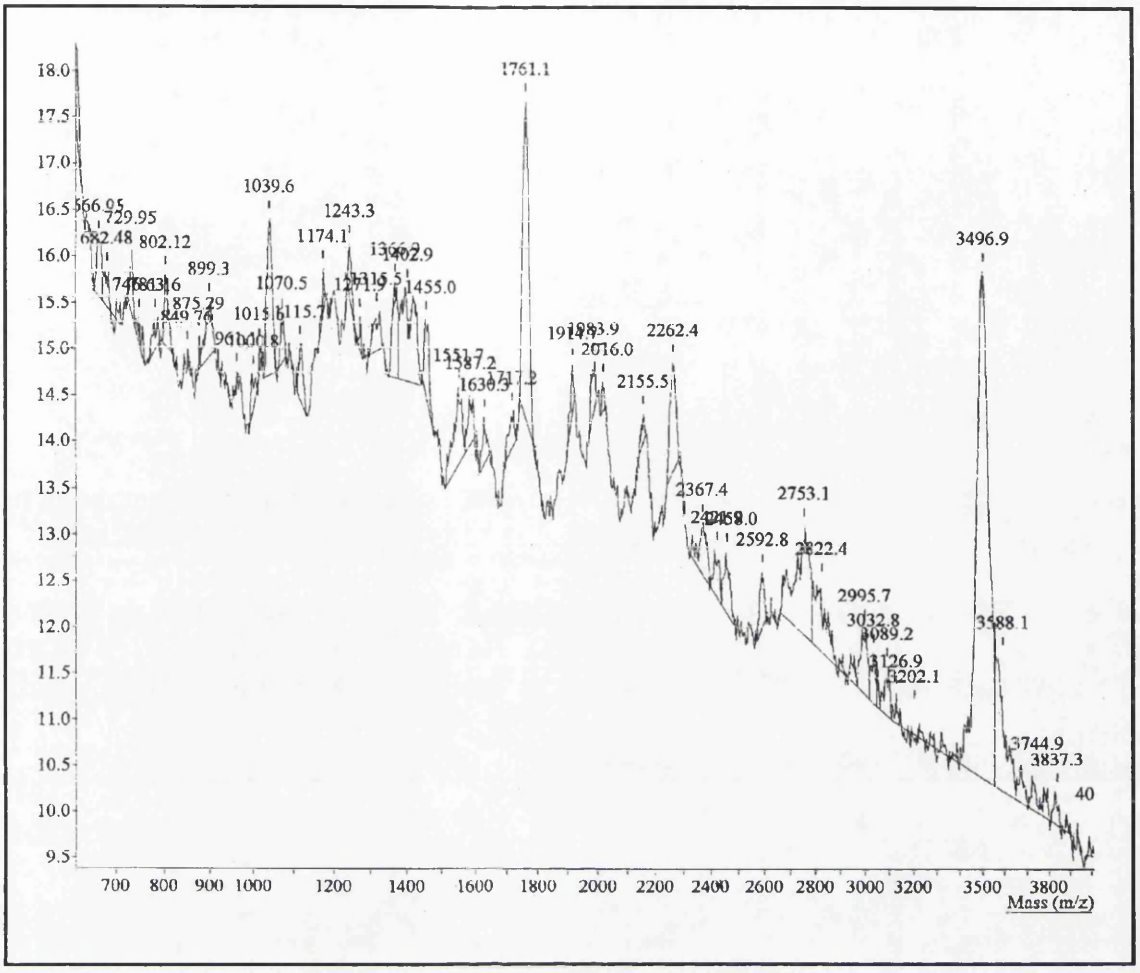
Fig. 26. Mass spectrometric analysis of proteins a and b which comigrate with UL47

a A mass spectrum generated from analysis of bands a and b (see Fig. 25). All of the masses represent the protonated forms. The 3496.9 peak is the insulin B chain marker used to calibrate the spectrum.

b The Massmap program was used to identify the proteins by comparison of credible peaks from **a** with those of proteins stored in a database (Genbank). The search mass uncertainty was ±3.3 Da and the search mass range 70-80000 Da. The results identified HSV-1 proteins UL17 and UL46 as the two best matches.

c The masses unique to each protein are shown: UL17 (4), UL46 (7) and those which the two proteins have in common (4).

a



b

Valid Search Masses: 2995.7 2753.1 2592.8 2459.0 2367.4 2262.4 2155.5 2016.0 1983.9 1914.7 1761.1 1587.2 1551.2 1455.0 1402.9 1366.9 1243.3 1174.1 1070.5 1039.6 899.3 802.1 729.95 1630.3
Search mass uncertainty= ± 3.3
70000 < Protein mass < 80000
Enzyme: TRYP_PAR
Scoring scheme: MOWSE

Score	# Hits	Mol. Wt.	Accession	Description
543	11	78246.3	gi_221767	UL46
543	11	78246.3	gi_59546	UL46 gene product (AA 1 - 718)
457	10	77359.4	gi_330058	alpha trans-inducing factor 77kb UL46
316	8	74584.1	gi_221738	UL17
316	8	74584.1	gi_59517	UL17 gene product (AA 1 - 703)
311	8	72239.9	gi_323567	polyprotein
285	8	73199.1	gi_209899	pTP protein

c

UL17 peaks	UL46 peaks	Peaks in common
1039.6	1070.5 x 2	729.95
1243.3	1455.0	1761.1
1402.9	1551.7	1914.7
2995.7	1587.2	1983.9
	2155.5	
	2753.1	

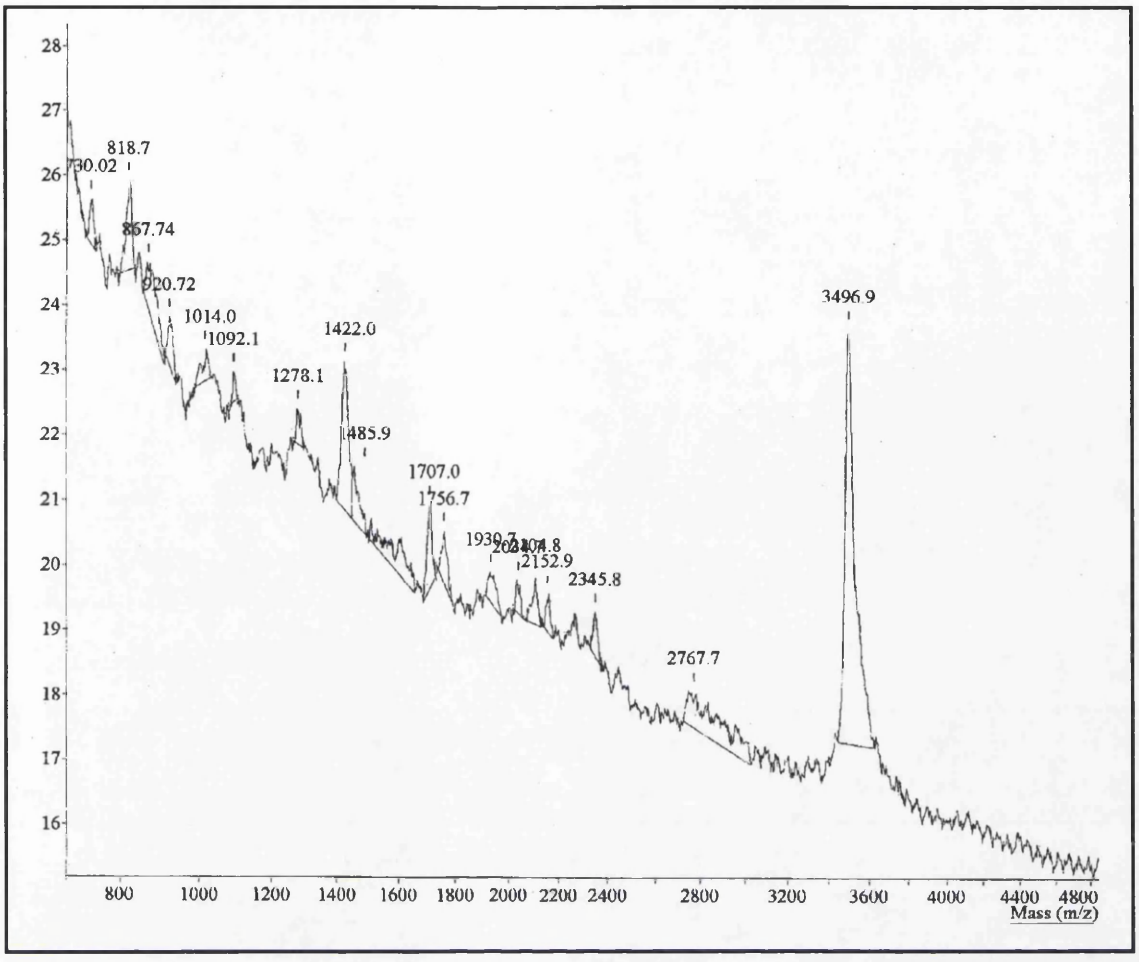
Fig. 27. Mass spectrometric analysis of protein c

a A mass spectrum generated from analysis of band c (see Fig. 25). All of the masses represent the protonated forms. The 3496.9 peak is the insulin B chain marker used to calibrate the spectrum.

b The Massmap program was used to identify the proteins by comparison of credible peaks from **a** with those of proteins stored in a database (Genbank). The search mass uncertainty was ±3 Da and the search mass range 20-40000 Da. The results identified HSV-1 proteins UL14 and UL49.

c The peaks unique to each protein are shown: UL14 (4) and UL49 (4).

a



b

```
Valid Search Masses: 2767.7 2345.8 2152.9 2104.0 2034.7 1930.7 1756.7 1707.0 1422.0 1278.1
1092.1 1014.0 920.7 818.7 730.0
Search mass uncertainty= + 3.0
20000 < Protein mass < 40000
Enzyme: TRYP PAR
Scoring scheme: MOWSE
```

Score	# Hits	Mol. Wt.	Accession	Description
237	4	23457.6	gi_221736	UL14
237	4	23457.6	gi_59846	23K protein (aa 1-215) UL14
237	4	23457.6	gi_59515	UL14 gene product (AA 1 - 215)
230	4	23254.1	gi_297553	ORF of RNA1
216	4	23330.2	gi_303990	protease
216	4	23336.2	gi_209861	23kD protease
214	4	21239.9	gi_221130	thymidine kinase
212	4	21427.0	gi_221127	thymidine kinase
198	5	32721.3	gi_385350	DL1
195	4	23787.0	gi_424388	envelope glycoprotein
188	4	20082.3	gi_381646	30K protein
185	3	20620.7	bbm_332425	putative nonstructural protein NS3 [huma
184	4	21715.8	gi_335255	coat protein
184	4	21715.8	gi_62288	coat protein
183	4	22971.9	gi_535502	surface envelope glycoprotein
181	4	28622.6	gi_559140	IAP1-like protein; atg conforms to Kozak
168	5	36886.0	bbm_191594	exoMTV vSAG=viral superantigen type II m
165	5	39227.7	gi_583947	HCMVUL23 (US22 FAMILY) (AA 1-342)
156	3	25868.1	gi_403852	NS1 protein
156	3	25883.0	gi_324835	nonstructural protein ns1
156	3	25869.9	gi_60804	NS1 protein
156	3	25868.1	gi_60717	NS1 protein (AA 1-230)
153	3	27854.5	gi_324819	membrane protein M1
152	4	23936.0	gi_424722	envelope glycoprotein
152	4	23914.0	gi_424679	envelope glycoprotein
152	4	32255.5	gi_221770	UL49
152	4	32255.5	gi_59549	UL49 gene product (AA 1 - 301)
150	5	29431.4	gi_468719	29K protein
149	3	27788.3	gi_438080	Matrix Protein

c

UL14 peaks	UL49 peaks
1014.0 x 2	1092.1
2152.9	1422.0
2345.8	1707.0
	2034.7

Pre-immune and immune sera reacted similarly with several virion proteins from *wt* or the UL47$^-$ mutant, despite carrying out reactions under a range of conditions (data not shown). The antiserum also failed to identify a convincing candidate protein in infected cells. These results imply that the antisera does not contain antibodies against UL17.

4.2. CHARACTERISATION OF UL16⁻ MUTANTS

4.2.1. UL16⁻ MUTANTS

The cosmid system does not lend itself readily to generating revertants since there is no general basis on which to identify them. An alternative approach is to generate a number of independent mutants with lesions in the target gene in order to resolve potential problems associated with spontaneous secondary mutations. Therefore, during this work six UL16⁻ mutants were analysed. Having produced the mutants, it was found that revertants could be isolated on the basis of plaque size (see Section 4.2.2.1).

4.2.1.1. Production of mutants

Two UL16⁻ mutants (1a and 1b) which have a 4 bp insertion at the *Bgl*II site (A'GATCT) at position 31096 bp were produced from two independently derived mutant cosmids by C. Cunningham using a different cosmid set from that described in Section 4.1 (set C in Cunningham and Davison, 1993). C. Cunningham also provided cosmids with a 16 bp insertion containing stop codons in all reading frames (GATCTAATCTAGATTA) proportedly inserted at each of the three *Sau*3AI ('GATC) sites in UL16, and a further four UL16⁻ viruses were produced from these. During the course of this work, therefore, six mutants with insertions in UL16 were produced: 1a and 1b first (generated by C. Cunningham), followed by mutants 1c, 1d, 2a and 3a (generated in the course of this work). Also, two independent *wt* viruses were derived from the parental cosmid set (cos*wt*1 and cos*wt*2). The locations of the mutations are summarised in Table 13 and Fig. 28.

4.2.1.2. Mutant genotypes

Infected cell DNA from mutants 1a and 1b was digested with *Kpn*I plus *Bgl*II and the infected cell DNA of mutants 1c, 1d, 2a and 3a with *Xba*I plus *Eco*RI. Southern blots were probed with a plasmid containing *Kpn*I q. Fig. 29 shows the results for 1a and 1b. *Wt* virus exhibits bands of 2041 bp and 1351 bp; an additional 426 bp fragment would have electrophoresed from the bottom of the gel. The mutants exhibit the 2041 bp fragment plus a novel 1777 bp fragment resulting from fusion of the 1351 and 426 bp fragments. The results in Fig. 30 confirm the genotypes of the other UL16⁻ viruses. *Wt* virus exhibits one large band of 15252 bp. The insertion of the oligonucleotide creates a novel *Xba*I site which causes the cleavage of the 15252 bp fragment into two smaller fragments.

Table 13. UL16⁻ mutants

Viruses	Insertion at position	Type of insertion	Predicted length of UL16 protein fragment (residues)[a]
cos*wt*1	none	none - *wt*	373 (full length)
cos*wt*2	none	none - *wt*	373 (full length)
1a	31096 bp	4 bp	84 (18)
1b	31096 bp	4 bp	84 (18)
1c	31096 bp	16 bp	66 (0)
1d	31096 bp	16 bp	66 (0)
2a	30671 bp	16 bp	208 (0)
3a	30181 bp	16 bp	371 (0)

[a] The number in parenthesis indicates the additional residues produced after the insertion.

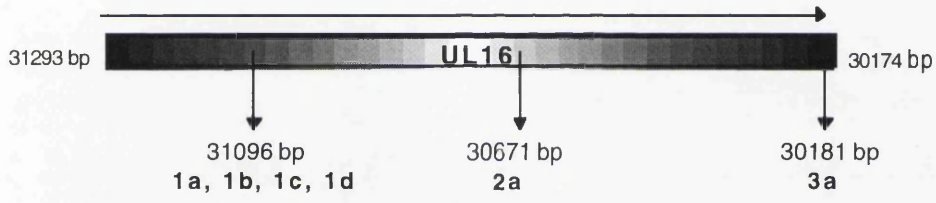


Fig. 28. Positions of insertion mutations in UL16

The ends of the UL16 coding sequence are given with respect to the HSV-1 DNA sequence and the direction of transcription is shown by a horizontal arrow (Table 13 contains further details).

Mutant cosmids 1c, 1d, 2a and 3a were also sequenced to confirm the sites into which the oligonucleotide had been inserted, by ligating an *Xba*I/*Hae*III digest of the cosmid with *Xba*I/*Hae*III digested M13mp19 (see Section 3.38). As an example, Fig. 31 shows the sequence of the mutation in 2a, and confirms that the insertion occurred at 30671 bp in the HSV-1 sequence.

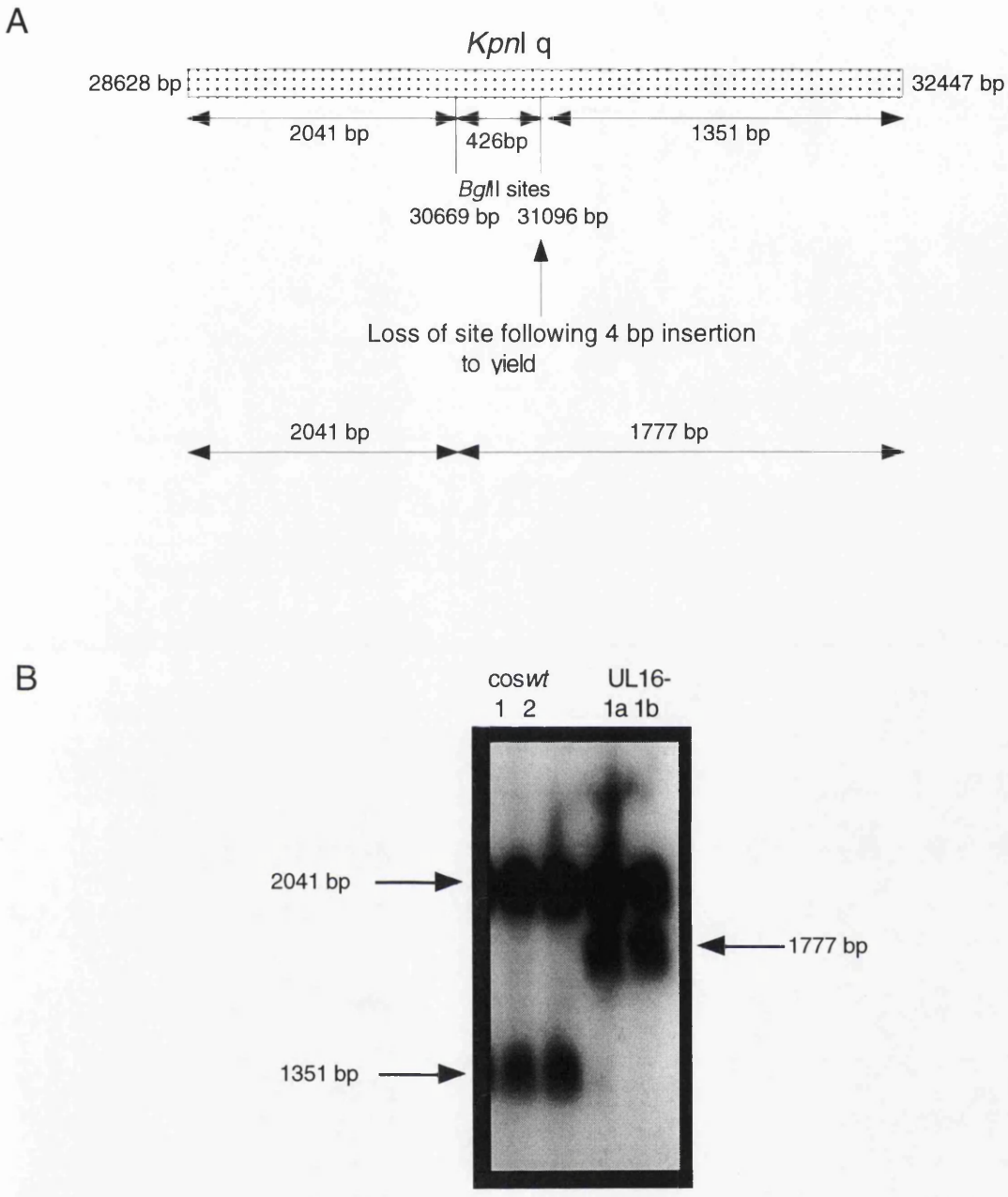


Fig. 29. Genotypes of UL16$^-$ mutants 1a and 1b

A A map of the 3819 bp *Kpn*I q fragment is shown as a shaded rectangle. The two *Bgl*II sites are denoted by vertical lines and the sizes of fragments generated following *Bgl*II digestion are given. After insertion of 4 bp at 31096 bp, the *Bgl*II site is lost, resulting in the generation of a novel fragment. Not drawn to scale.

B An autoradiograph of a Southern blot in which infected cell DNA was digested with *Kpn*I plus *Bgl*II and probed with a plasmid containing *Kpn*I q. The sizes of the fragments detected are shown.

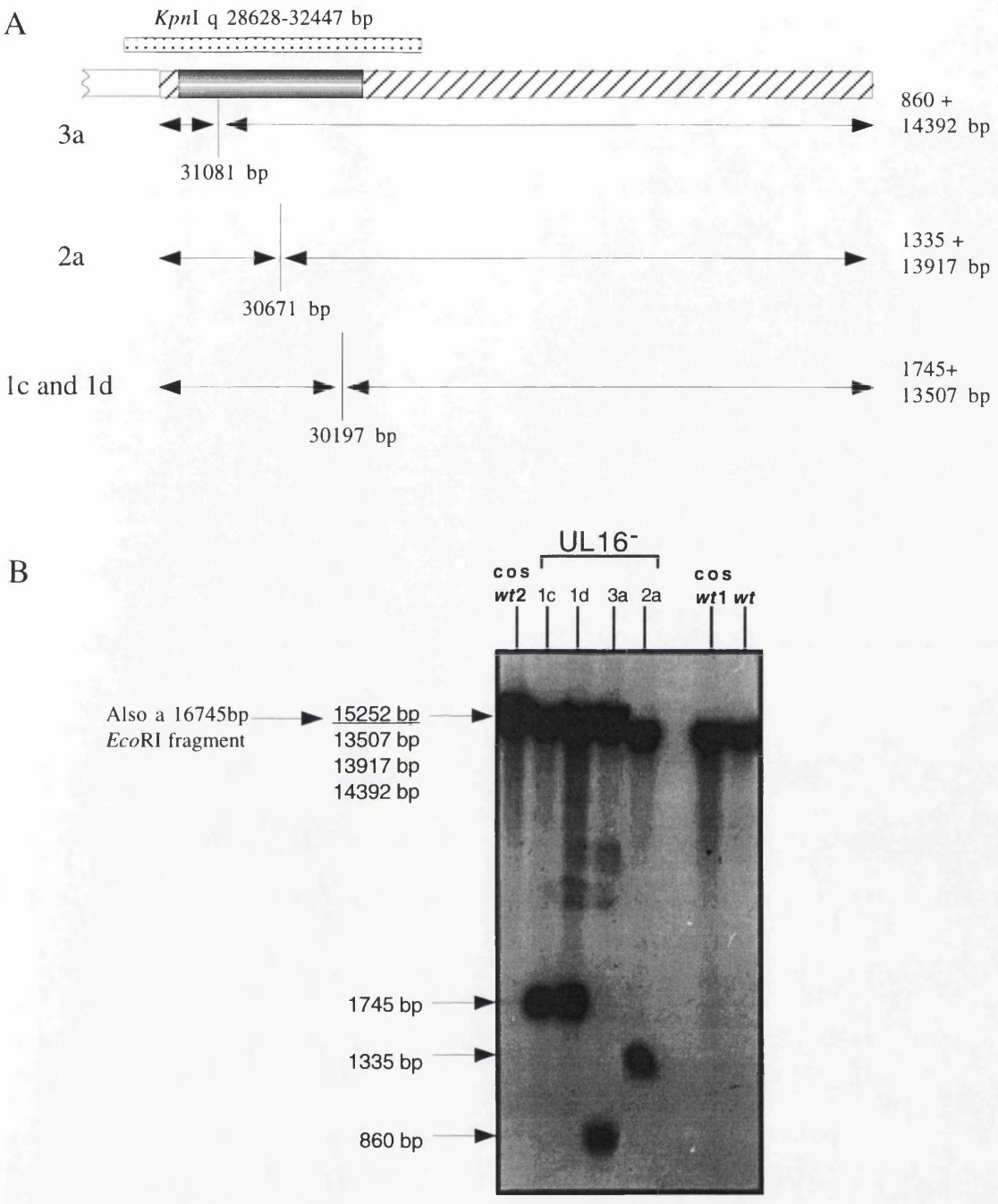


Fig. 30. Genotypes of UL16⁻ mutants 1c, 1d, 2a and 3a

A A map of the 15252 bp *Eco*RI/*Xba*I fragment (hatched rectangle) which contains UL16 (shaded rectangle). The *Sau*3AI sites into which the oligonucleotide was inserted are denoted by vertical lines. Insertion results in the gain of a novel *Xba*I site. The sizes of the two predicted novel fragments following digestion with *Eco*RI plus *Xba*I are given on the right. The region covered by the *Kpn*I q probe is shown by a shaded rectangle. As well as binding to these fragments, the probe also binds to an HSV-1 16745 bp *Eco*RI fragment, the right end of which is denoted by an open box.

B An autoradiograph of a Southern blot in which infected cell DNA was digested with *Xba*I plus *Eco*RI and probed with a plasmid containing *Kpn*I q. The sizes of the resulting bands are given in bp.

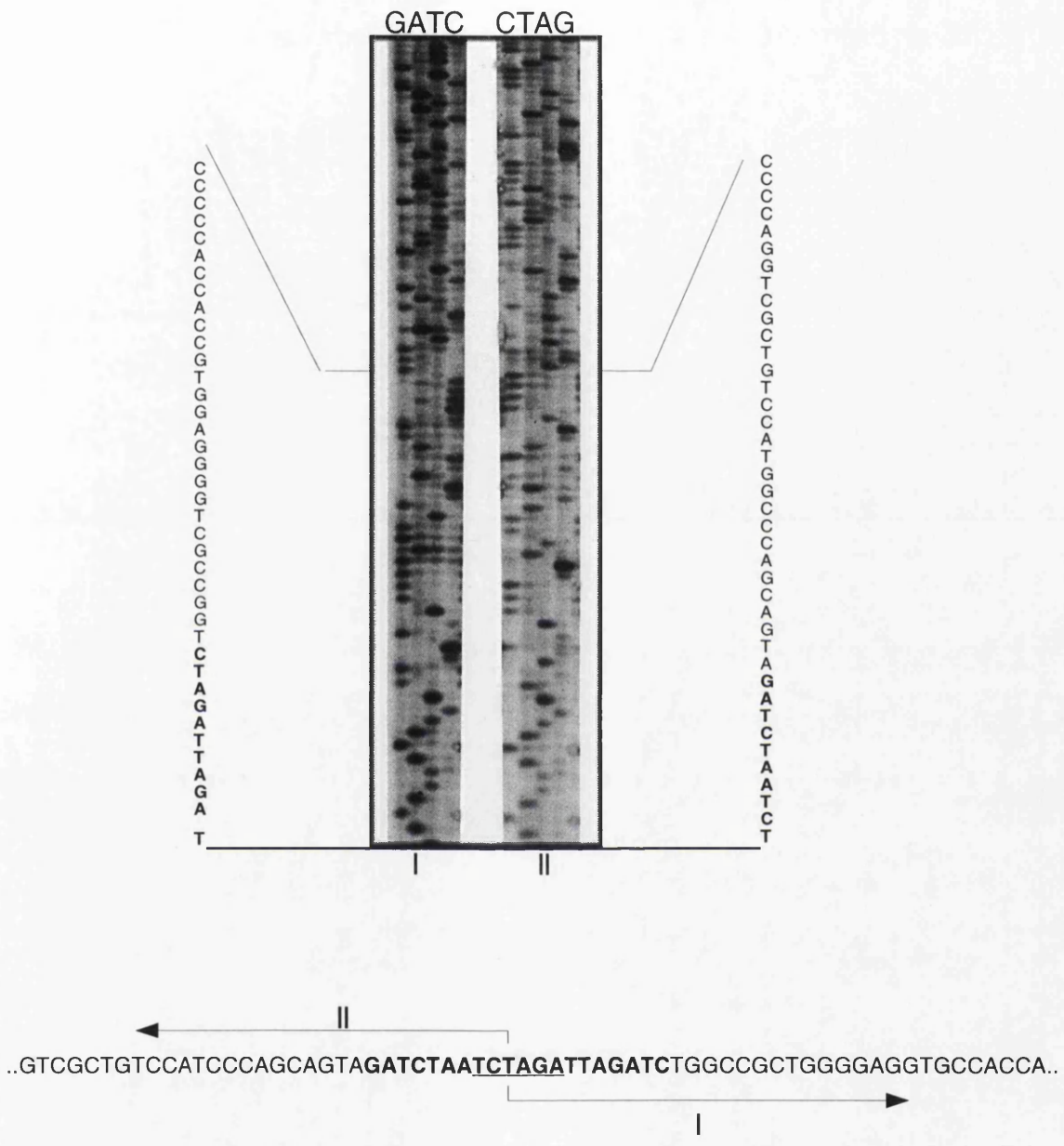


Fig. 31. Confirmation of the lesion in mutant 2a by sequencing

An autoradiograph of a sequencing gel in which the position of the insertion of an oligonucleotide into the *Sau* 3AI site at position 30671 bp within gene UL16 is confirmed. The sequence from the autoradiograph corresponds to the expected sequence shown below the figure. The novel *Xba*I site generated is underlined.

4.2.2. *IN VITRO* ANALYSIS OF UL16⁻ MUTANTS

Mutants 1a and 1b were constructed prior to the commencement of this work and were used in all of the experiments. Additional mutants were used in selected experiments. HSV-1 *wt* and cos*wt*1 or cos*wt*2 were used as *wt* controls.

4.2.2.1. Plaque area

In initial virus titrations on BHK C13 cells it became obvious that the mutants 1a and 1b had a smaller plaque size than the *wt* viruses. In an experiment designed to allow statistical comparisons of this aspect of the mutant and *wt* viruses, plaque area was measured by photographing Giemsa-stained infected cell monolayers, cutting out the plaques from prints and weighing them. Mutant virus plaque size was 14-19% (1a) and 24-35% (1b) that of *wt* plaques on BHK C13 cells, a hamster fibroblastoid cell line (Table 14 a and Fig. 32A). Cos*wt*1 plaques were the same size as *wt* (80-102%). The viruses were also titrated on MeWo cells, a human fibroblastoid cell line, and mutants produced plaques 31-64% (1a and 1b) the size of *wt* (Table 14b).

Table 14. Relative plaque areas

(a) BHK C13

	Expt 1[a] 2 days pi		Expt 2[b] 2 days pi		Expt 3[c] 4 days pi	
Virus	No of plaques examined	% of *wt* plaque area[d]	No of plaques examined	% of *wt* plaque area	No of plaques examined	% of *wt* plaque area
cos*wt*1	60	80 ±15	54	102 ±20	31	87 ±12
1a	111	15 ±6	87	14 ±5	52	19 ±6
1b	107	25 ±7	82	24 ±8	46	35 ±10

[a] = 69 *wt* plaques examined (100%±9) [b] = 39 *wt* plaques examined (100%±15) [c] = 35 *wt* plaques examined (100%±12) [d] = ±SD

(b) MeWo

	Expt 1[a] 2 days pi		Expt 2[b] 2 days pi		Expt 3[c] 4 days pi	
Virus	No of plaques examined	% of *wt* plaque area[d]	No of plaques examined	% of *wt* plaque area	No of plaques examined	% of *wt* plaque area
cos*wt*1	48	99 ±15	54	60 ±10	40	124 ±20
1a	45	64 ±10	72	31 ±9	51	46 ±10
1b	52	61 ±8	49	31 ±7	46	64 ±12

[a] = 45 *wt* plaques examined (100%±18) [b] = 48 *wt* plaques examined (100%±10) [c] = 41 *wt* plaques examined (100%±15) [d] = ±SD

The effect of the UL16 mutation seems to be more marked on BHK C13 cells. The difference in the plaque size between the two mutants was not significantly different.

The previous three experiments were independent, but they were carried out within a few days of each other. Therefore, the experiment was repeated at a later date and extended to include additional mutants and cos*wt*2. The results (Table 15 and Fig. 32B) show that mutants 1a-d (which are mutated at the same site) have similarly sized plaques on BHK C13 cells (20-27% the size of *wt*). The two cos*wt* viruses and mutant 3a (which is missing only the last 2 residues of the UL16 protein) had plaques the same size as *wt*. Mutant 2a gave plaques that were only 4% the size of *wt*.

Table 15. Relative plaque areas on BHK C13 cells

	Expt 4[a] 2 Days pi	
Virus	No of plaques examined	% of *wt* plaque area ±SD
cos*wt*1	30	109 ±18
cos*wt*2	44	92 ±14
1a	32	26 ±8
1b	39	25 ±5
1c	68	20 ±6
1d	53	27 ±7
2a	132	4 ±2
3a	37	97 ±12

[a] = 40 *wt* plaques examined (100%±14)

These results indicate that the last two residues of UL16 are dispensible for formation of plaques of *wt* size, and suggest that the larger portion of the UL16 protein produced by 2a may interfere with the normal function of the protein to a greater extent than the portion of the protein produced by mutants 1a-d. The possibility that 2a contains another mutation which is contributing to plaque size is considered unlikely (see below).

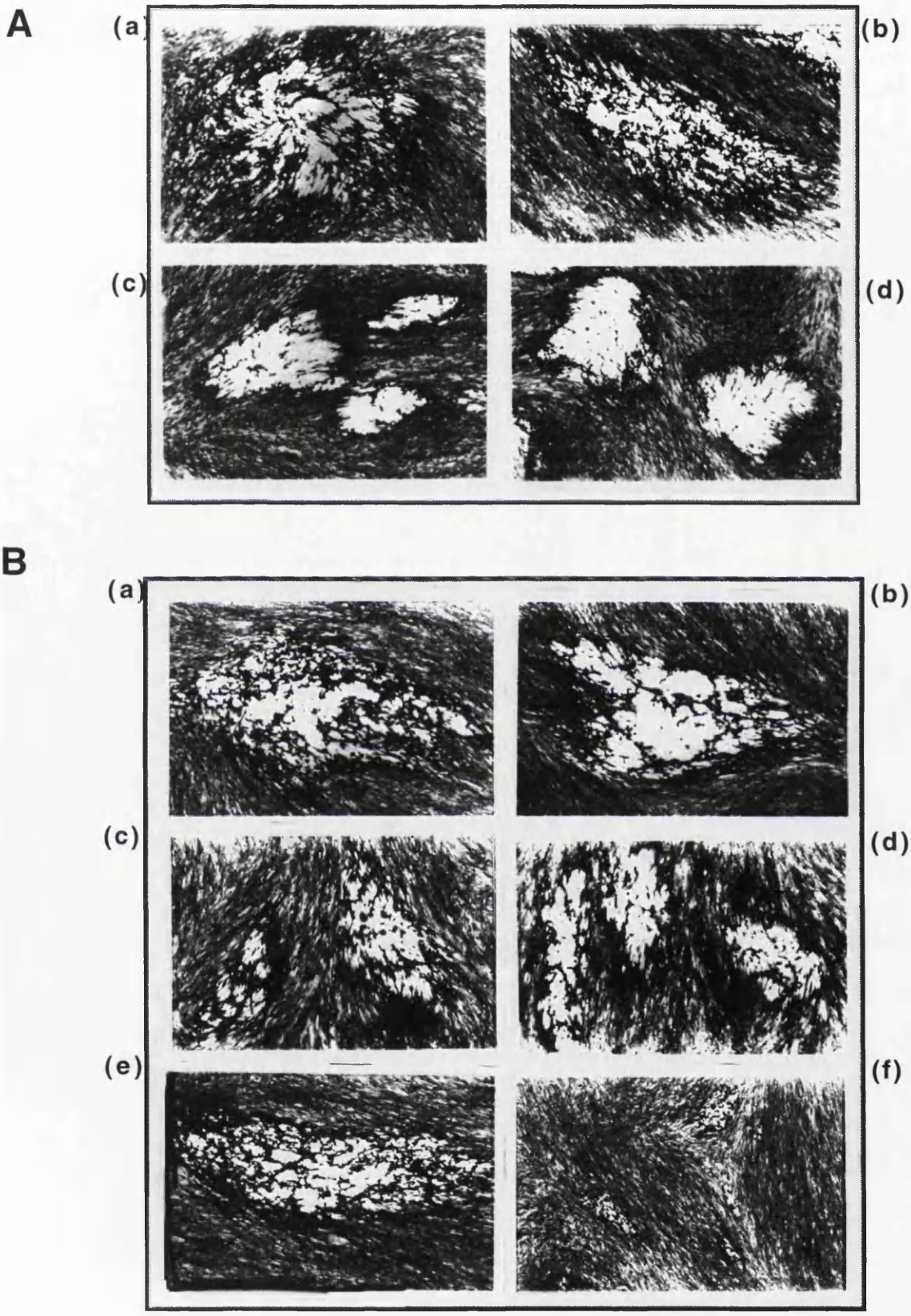


Fig. 32. Differences in plaque size

Photographs were taken of Giemsa-stained plaques at 2 d pi in two separate experiments, A and B. Typical plaques are shown. *(a) wt*, (b) cos*wt*1, (c) 1a, (d) 1b, (e) 3a, (f) 2a. See Tables 14 and 15 for details.

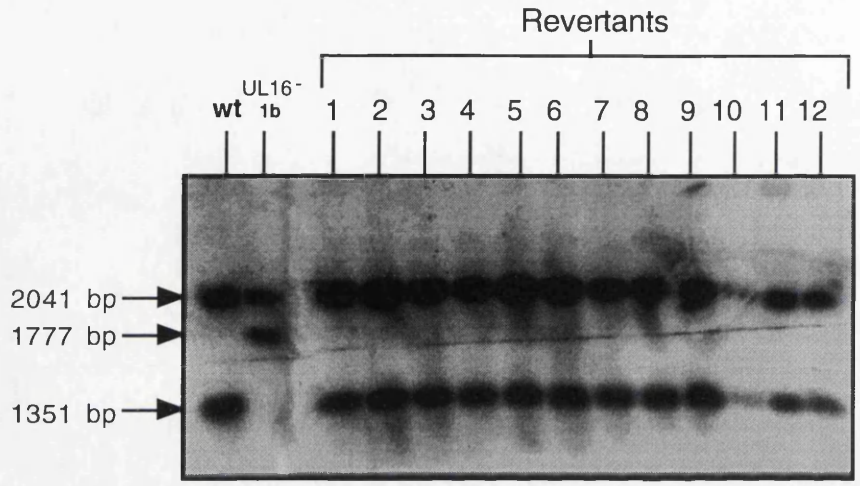


Fig. 33. Status of recovered virus after co-transfection of UL16⁻ 1b DNA and a plasmid containing *wt* UL16

An autoradiograph of a Southern blot in which infected cell DNA prepared from large plaques (1-12) were digested with *Kpn*I, transferred to a membrane and probed with a plasmid containing *Kpn*I q. The UL16⁻ 1b lane shows the mutant banding pattern from fusion of two fragments (see Fig. 29 for details). The sizes of the detected fragments are given in bp.

Revertants or rescuants (1b rev and 2a rev) were produced from mutants 1b and 2a to confirm the relationship between loss of the intact UL16 protein and small plaque size, and for use in *in vivo* analysis (see Section 4.2.4.1.).

Mutant 1b DNA was cotransfected into BHK C13 cells with plasmid p1, which contains UL16 in its entirety, linearised with *Sph*I (see Section 4.2.4.1). Revertant or rescuant (large) plaques were present at a higher proportion than would be expected from a plasmid recombination experiment, suggesting that a proportion had arisen by intramolecular recombination within the 4 bp direct repeat at the site of the lesion. Large plaques were picked, and *Kpn*I/*Bgl*II digested infected cell DNA was produced and probed with a plasmid containing *Kpn*I q. All recovered viruses were *wt* (Fig. 33). Revertants or rescuants were also obtained from mutant 2a (data not shown).

Examination of the plaque size of revertants and mutants was carried out as described previously. Revertants from mutants 1b and 2a produced plaques the same size as *wt*, while the original mutants produced small plaques as before (Table 16). These results indicate that the mutations in UL16 are directly linked to plaque size.

Table 16. Relative plaque areas on BHK cells.

	Expt 5[a] 2 days pi	
Virus	No of plaques examined	% of *wt* plaque area ±SD
cos*wt*1	40	107 ±10
1b	79	34 ±9
2a	95	7 ±3
1b rev	36	97 ±12
2a rev	42	101 ±18

[a] = 35 *wt* plaques examined (100%±9)

4.2.2.2. Growth characteristics

The growth characteristics of the mutants under conditions of high or low moi were investigated, since the smaller plaque size of the mutants could be due to a slower growth rate or to a lower yield of infectious virus. BHK C13 cells were infected with mutant 1a, mutant 1b, cos*wt*1 or *wt* virus at high (10 pfu/cell) or low (0.01 pfu/cell) moi and yields were calculated during the period 0-36 h pi for high moi and 0-96 h pi for low moi. The

cells were scraped into 2 ml of medium, sonicated and titrated on BHK C13 cells. The results (Fig. 34a and b) show that mutants 1a and 1b grew to about 20-30% of the final titre of the two *wt* viruses in both high and low multiplicity infections. It is of interest that the mutants show a delay in the initiation of growth, this corresponds to the results obtained from EM analysis (Section4.2.2.5).

In a second experiment, BHK C13 cells were infected with each of the mutants and *wt* viruses at low moi. Fig. 35 shows that mutants 1a-d grew to about 20-30% of the final titre of the two *wt* viruses. The growth curve of mutant 3a was similar to that of *wt*, whereas mutant 2a was about 15,000-fold reduced in yield. The growth characteristics correlate with the plaque area results: mutants 1a-d were impaired, mutant 3a was indistinguishable from *wt* virus and mutant 2a was severely impaired.

4.2.2.3. Growth on different cell lines

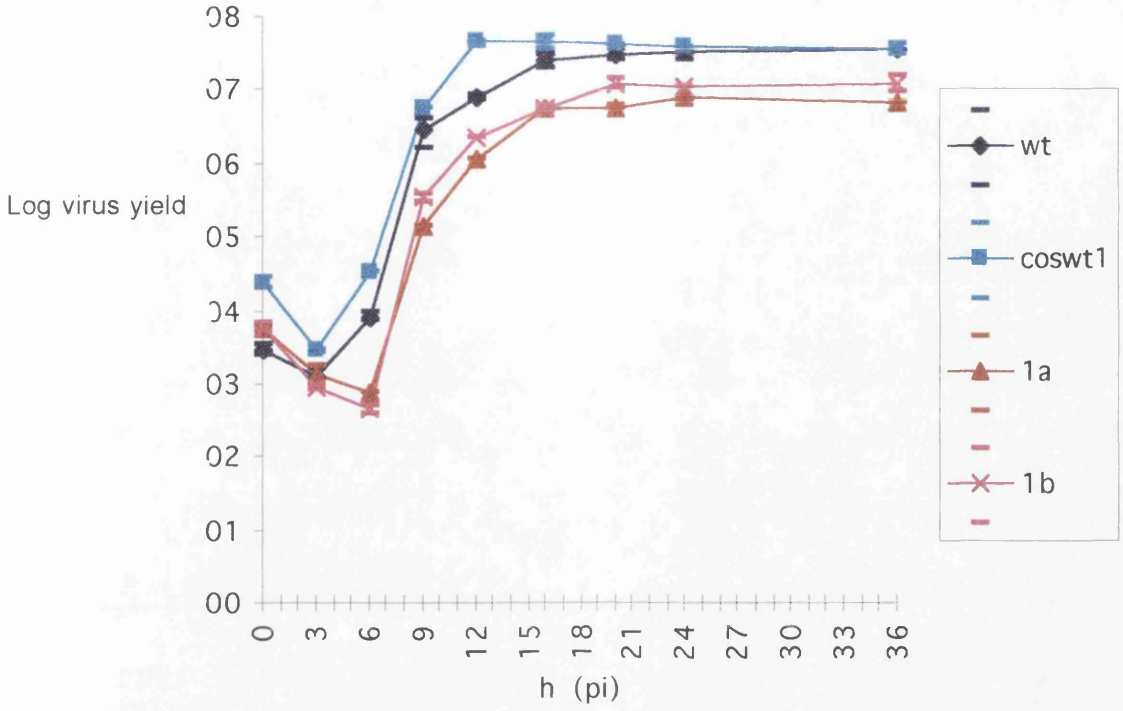
Mutants 1a and 1b, cos*wt*1 and *wt* were tested in a single experiment for their ability to grow on cell lines other than BHK C13 and MeWo. The cell lines tested are shown in Table 17a. Cells on 35mm 6-well plates were infected with virus (1 x 10^4 pfu/plate), incubated at 37° for 3 days, scraped into the medium, sonicated and titrated on BHK C13 cells. The virus yields are shown in Table 17b.

Table 17. Growth on different cell lines

(a) Cell types investigated

Cell line	Type	Reference
HeLa	Human epithelial	Scherer *et al.*, 1953
HFL/2002	Human fibroblastoid	Breul *et al.*, 1980
BSC-1	Monkey epithelial	Hopps *et al.*, 1963
CV1	Monkey fibroblastoid	Kit *et al.*, 1965
MDCK	Canine epithelial	Madin and Darby, 1958
BHK C13	Hamster fibroblastoid	McPherson and Stoker, 1962

(a)



(b)

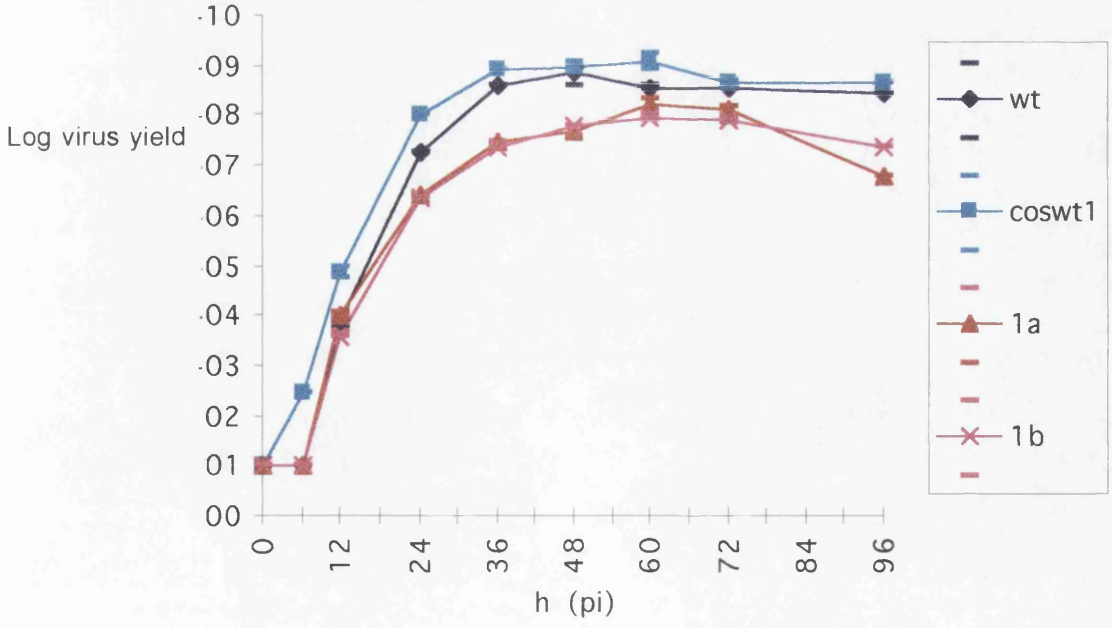
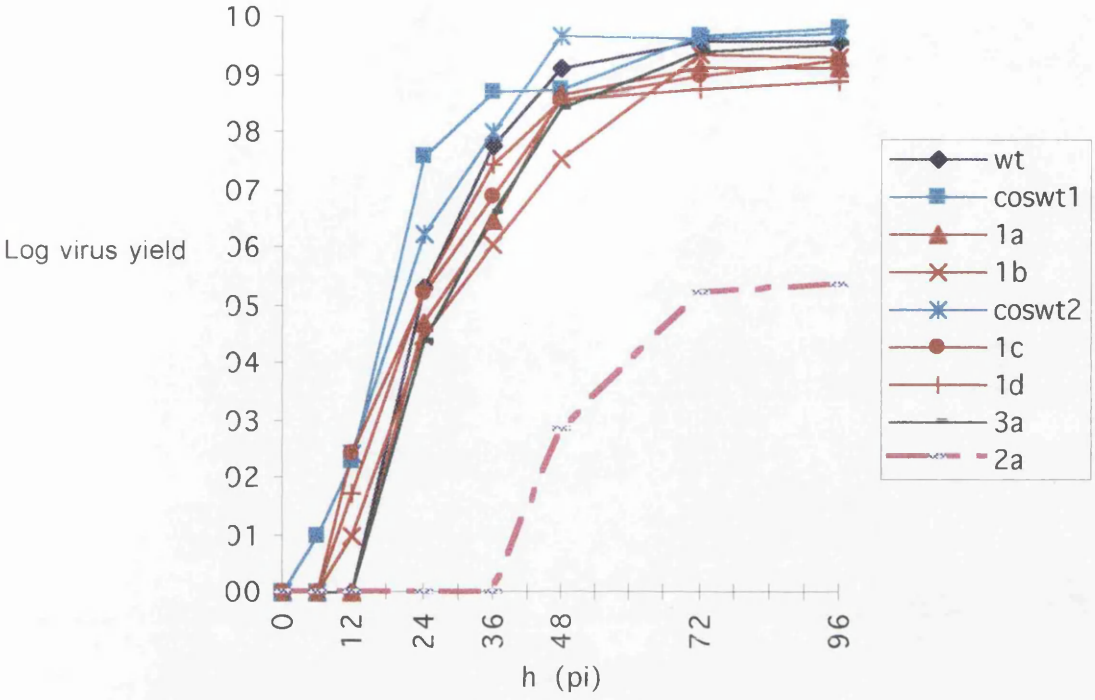


Fig. 34. High and low multiplicity growth curves of *wt* and mutant viruses

(a) BHK C13 cells were infected with virus at 10 pfu/cell and maintained at 37°C for time points of 0-36h pi, or (b) BHK C13 cells were infected with virus at 0.01 pfu/cell and maintained at 37°C for a period of 0-96h pi. Virus yield was titrated on BHK C13 cells. The standard deviation is shown as an appropriately coloured bar above and below the value at each time point.



Virus	Final titre (96h pi) ±SD
wt	$4.0 \pm 0.03 \times 10^9$
cos*wt*1	$6.8 \pm 0.017 \times 10^9$
1a	$1.3 \pm 0.3 \times 10^9$
1b	$2.1 \pm 0.051 \times 10^9$
cos*wt*2	$5.4 \pm 0.13 \times 10^9$
1c	$1.8 \pm 0.088 \times 10^9$
1d	$7.4 \pm 0.029 \times 10^8$
3a	$3.6 \pm 0.2 \times 10^9$
2a	$2.3 \pm 0.26 \times 10^5$

Fig. 35. Low multiplicity growth curve of *wt* and mutant viruses

BHK C13 cells were infected with virus at 0.01 pfu/cell and maintained at 37°C for periods of 0-96 h pi. Virus was titrated on BHK C13 cells for 2-3 days before the plates were stained and the plaques counted. The table shows the titre at the last time point ±standard deviation.

(b) Yield of virus

Cell type	Titre of *wt*	Titre of cos*wt*1	Titre of 1a	Titre of 1b
HeLa	$5x10^6$	$7.5x10^6$ (150)[a]	$7x10^5$ (14)	$6.5x10^5$ (13)
HFL/2002	$1x10^9$	$1x10^9$ (100)	$7.2x10^7$ (7)	$8.7x10^7$ (9)
BSC-1	$1.8x10^8$	$3x10^8$ (166)	$3.5x10^7$ (19)	$2.4x10^7$ (13)
CV1	$1.4x10^8$	$1.4x10^8$ (100)	$2.7x10^7$ (19)	$1.9x10^7$ (14)
MDCK	$5.5x10^6$	$5.5x10^6$ (100)	$4x10^5$ (7)	$2.5x10^5$ (5)
BHK C13	$5x10^7$	$9x10^7$ (180)	$8x10^6$ (16)	$8.4x10^6$ (17)

[a] = The number in parenthesis is the yield as a percentage of the *wt* yield

These results show that the mutants grew on four of the cell lines to about 13-19% of *wt*. Yields were somewhat lower on HFL and MDCK cells (5-9%).

4.2.2.4. Particle:pfu ratio

Mutant 1a, mutant 1b, cos*wt*1 and *wt* virus were harvested late in infection (2-3 d pi) from single roller bottle stocks of infected BHK C13 cells. Cell-released particles were counted by Mr J. Aitken and virus yields were titrated on BHK C13 cells. In the first experiment, the particle:pfu ratio of the *wt* viruses was found to be within the normal range observed for *wt* HSV-1, while those of the mutants was 30-37 (1a) or 8-10 (1b) times greater. In a second experiment, the *wt* ratio was lower but still within expected *wt* values, whereas the mutant values were 4-5 fold higher (Table 18).

Table 18. Particle:pfu ratio

	Experiment 1				Experiment 2			
	wt	cos*wt*1	1a	1b	*wt*	cos*wt*1	1a	1b
pfu	$1.4x10^{10}$	$2x10^{10}$	$2.4x10^8$	$1.1x10^9$	$3.2x10^{10}$	$2.1x10^{11}$	$8.2x10^9$	$6.6x10^9$
pc	$2.5x10^{11}$	$2.9x10^{11}$	$1.3x10^{11}$	$1.6x10^{11}$	$2x10^{11}$	$1.5x10^{11}$	$2x10^{11}$	$2x10^{11}$
ratio	**17.8**	**14.5**	**541**	**145**	**6**	**7**	**24**	**30**

4.2.2.5. EM analysis

The ability of the mutants to produce the normal range of viral particles was investigated by EM. BHK C13 cells were infected with mutant 1a, mutant 1b, cos*wt*1 or *wt* virus at high moi and fixed at 9 or 16 h pi. The mutants produced all three types of capsid in

normal ratios (data not shown). It was noted, however, that mutant-infected cells contained fewer virions. The results in Section 4.2.2.4, however, indicate that the mutants produced the same number of particles, although the number of infectious particles was less. This may be explained by the different samples used (CR virus for particle counts and CA virus for EM) and the times in infection when the samples were examined (2 days pi for particle counts and 9-16 h pi for EM). The mutants may be slower to produce viral particles initially but reach *wt* levels later in infection.

4.2.2.6. Temperature sensitivity

A single experiment was carried out to investigate the ability of the mutants to grow at different temperatures. BHK C13 cells were infected at 10 pfu/cell with mutant 1a, mutant 1b, cos*wt*1 and *wt* virus at 31°, 37° or 39.5° for 16 h and then titrated on BHK C13 cells at 37°. The results in Table 19 show that the titre of mutants 1a and 1b was 12-25 fold less than *wt* at 37°, 4-10 fold less at 31° and 5-fold less than *wt* at 39.5°, and indicate that the mutants are no more temperature sensitive than *wt*.

Table 19. Viral yields at three different temperatures on BHK C13 cells

Virus	31°	37°	39.5°
wt	$1.4x10^8$	$2.8x10^8$	$1.7x10^7$
cos*wt*1	$7.8x10^7$	$1.5x10^8$	$8.6x10^6$
1a	$1.3x10^7$	$1.2x10^7$	$1.7x10^6$
1b	$2x10^7$	$1.1x10^7$	$3.1x10^6$

4.2.2.7. Efficiency of adsorption and penetration

Experiments were carried out to ascertain whether the mutants are able to adsorb to and penetrate cells as efficiently as *wt*. Mutants 1a and 1b, cos*wt*1 and *wt* were allowed to adsorb to cells at 4° for 30-60 min, or at 37° for 0-120 min, then non-adsorbed virus was removed by washing the cells with PBS A. Infected cells were incubated at 37° for 2-3 days pi before staining and counting plaques.

To investigate penetration, virus was adsorbed for 10 min at 4° and unbound virus was removed by washing with PBS A. Cells were maintained at 37° for a period of 5-60 min and washed with PBS A to remove unbound virus or with a low pH buffer to remove non-penetrated and unbound virus. Infected cells were incubated at 37° for 2 days before staining and counting plaques.

The adsorption results are shown in Fig. 36a and b and the penetration results in Fig. 37. Adsorption of the mutants appears to be as efficient as *wt* and penetration is slightly reduced from *wt*.

4.2.2.8. Binding of virus to cells

Confluent BHK C13 cells were infected with radiolabelled virions and maintained at 37° for periods of 0-2 h. The percentage of virus bound to the cells at each time point was calculated using the equation described in Section 3.12. These results indicate that there may be a very slight impairment of the ability of mutant 1b to bind to cells (Fig. 38).

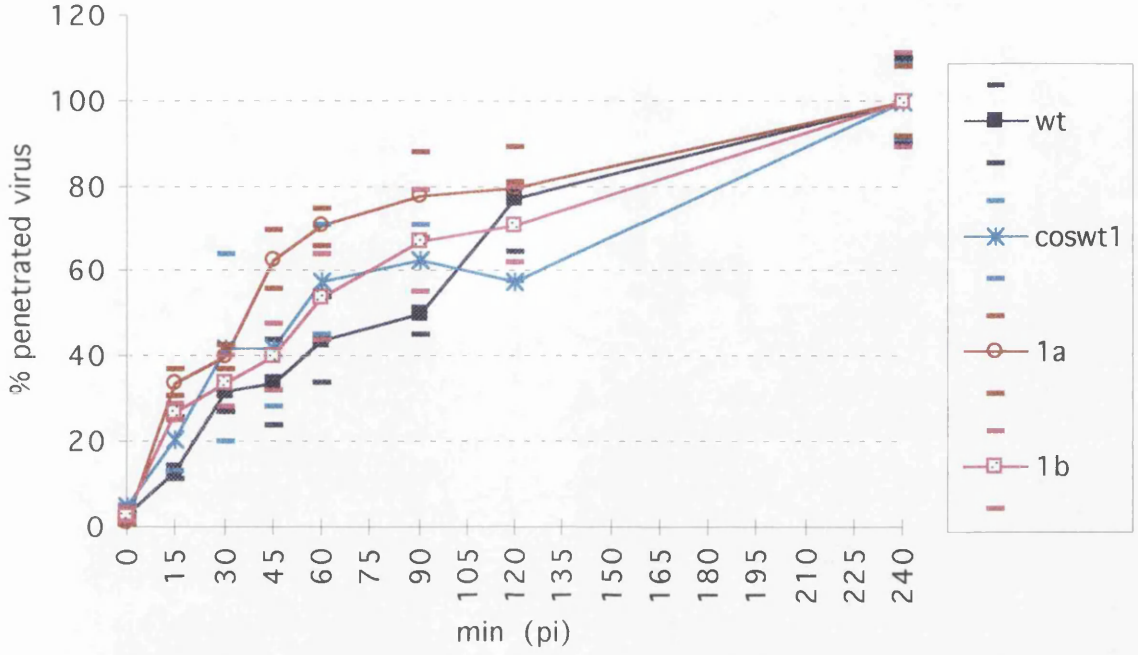
4.2.2.9. Effect of interferon treatment on virus yield

Su *et al.* (1993) mapped a region of the HSV-1 genome conferring resistance to interferon α and β (IFN) to UL14, UL15 and UL16. Therefore, the UL16 mutants were examined to see whether they are more sensitive to IFN than *wt*. IFN-sensitive HFL cells were pretreated overnight with 1U/ml IFN-α, washed twice with medium and then infected at 5 pfu/cell. Untreated HFL cells were also infected as controls. At 24 h pi the cells were scraped into 2 ml of medium and viral yields determined by titration on untreated BHK C13 cells (Table 20).

Table 20. Virus yields from cells pretreated with interferon

Virus	Expt 1			Expt 2		
	Untreated	IFN-treated	% of untreated	Untreated	IFN-treated	% of untreated
wt	$2x10^{10}$	$6x10^{9}$	30	$1.4x10^{10}$	$1x10^{10}$	71
cos*wt*1	$4.2x10^{10}$	$3x10^{10}$	71	$3x10^{10}$	$1.6x10^{10}$	53
cos*wt*2	$4.5x10^{10}$	$1.1x10^{10}$	24	$2.4x10^{10}$	$1.4x10^{10}$	58
1a	$9.2x10^{9}$	$2.3x10^{9}$	25	$8.1x10^{9}$	$8x10^{9}$	99
1b	$3x10^{9}$	$2.8x10^{9}$	93	$4.8x10^{9}$	$3.1x10^{9}$	65
1c	$1.6x10^{10}$	$9.2x10^{9}$	58	$2.9x10^{9}$	$1.7x10^{9}$	59
1d	$2.6x10^{9}$	$6.8x10^{8}$	26	$4.4x10^{9}$	$3.7x10^{9}$	84
2a	$2.7x10^{7}$	$4.6x10^{6}$	17	$2.3x10^{7}$	$1x10^{7}$	43
3a	$2.7x10^{10}$	$2x10^{9}$	7	$1x10^{10}$	$8.4x10^{9}$	84

(a)



(b)

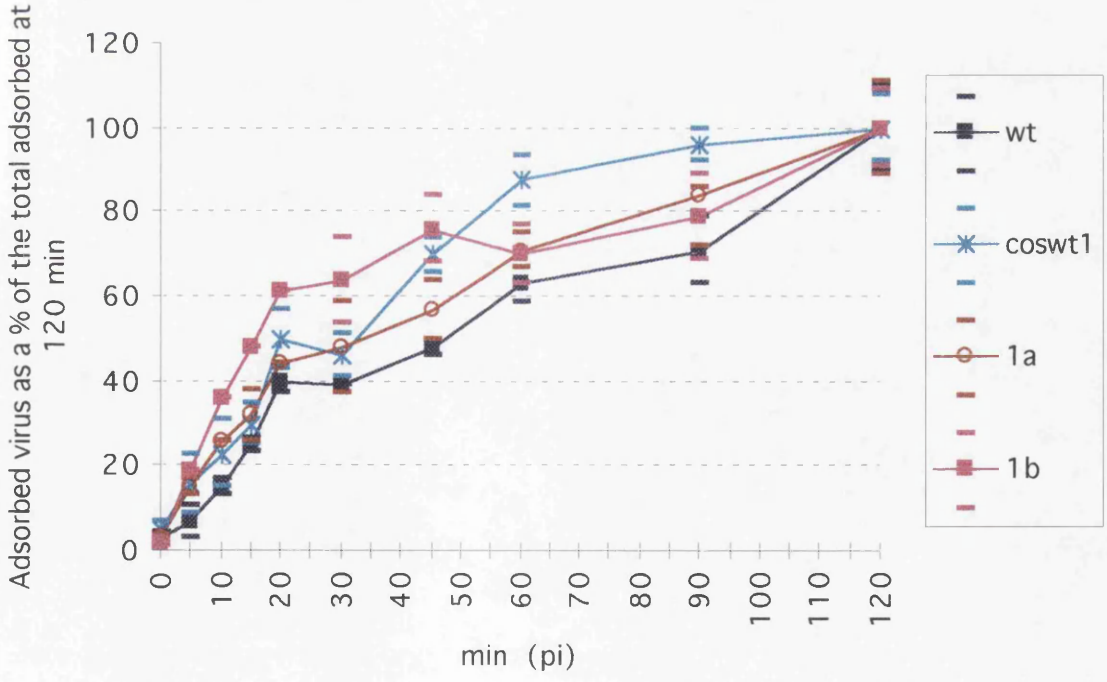


Fig. 36. Virus adsorption

BHK C13 cells were infected at 200 pfu/plate at 4° for 10 min then non-adsorbed virus was removed. The plates were maintained (a) at 4° or (b) 37°, then the cells were washed at various time points to remove non-adsorbed virus. The cells were maintained in medium for 2-3 days, then the plates were stained and plaques counted. The standard deviation is shown as an appropriately coloured bar above and below the value at each time point. It must be noted that in some cases the SD bar is found behind another bar or value marker and cannot therefore be clearly observed.

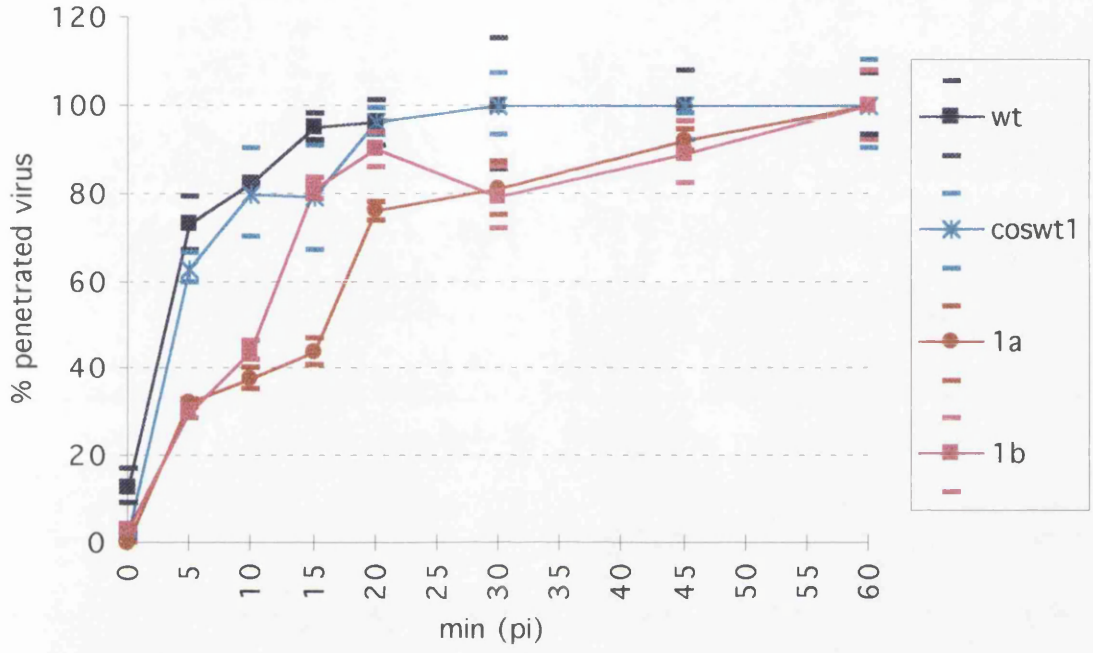


Fig. 37. Virus penetration

BHK C13 cells were infected at 200 pfu/plate at 4°C for 10 min then non-adsorbed virus was removed. The cells were incubated at 37°C and then washed at various time points with either PBS A or low pH buffer. The cells were incubated for 2-3 days, then the monolayers were stained and plaques counted. The standard deviation is shown as an appropriately coloured bar above and below the value at each time point.

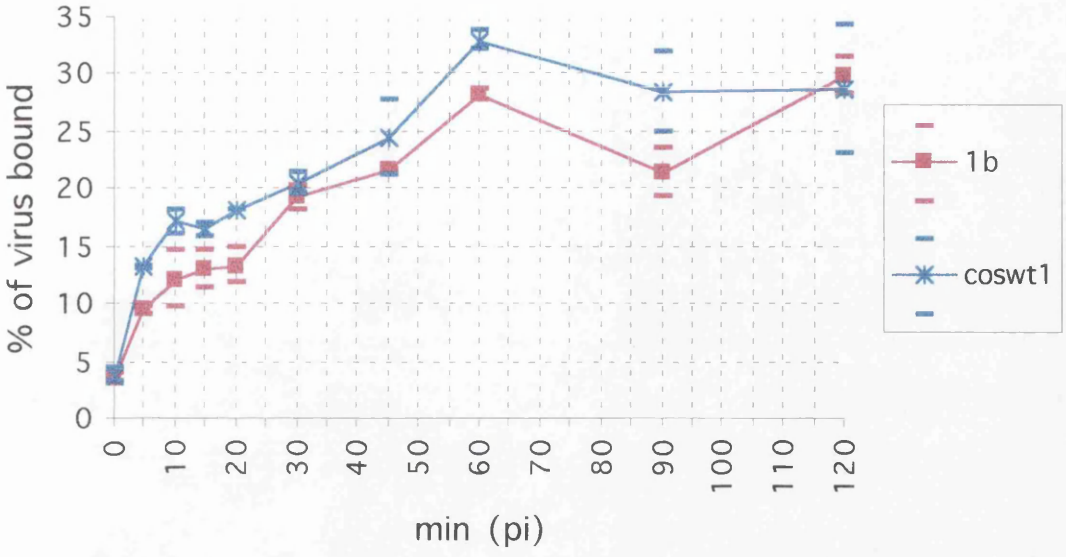


Fig. 38. Binding of virus to cells

BHK C13 cells were infected with 10^5 particles/cell and 10^4-10^5 cpm of [^{35}S]-methionone-labelled virions. At various time points, the supernatant was removed, and the cells were washed and scraped into medium. Radioactivity in these samples was counted in a scintillation counter and the results expressed as percentage binding. The standard deviation is shown as an appropriately coloured bar above and below the value at each time point.

The yield of *wt* virus on treated cells was expected to be reduced to 10-50 % of the untreated value, depending on the amount of interferon used and the cell type used (Munoz and Carrasco, 1984; DeStasio and Taylor, 1990; Chatterjee *et al.*, 1995). Therefore if the mutation of gene UL16 caused a significant difference in interferon resistance, the mutants would fail to replicate on interferon treated cells. Results from these experiments indicate that there is substantial variation in the reduction in yield caused by IFN treatment and that UL16 does not play a role in conferring interferon resistance.

4.2.3. IDENTIFICATION OF THE UL16 PROTEIN

Several techniques were employed in an attempt to identify the UL16 protein, which has a predicted mass of 40444.2 Da, and is produced late in infection.

4.2.3.1. Western blotting and Immune precipitation

Two β-galactosidase fusion protein rabbit antisera potentially directed against the UL16 protein (residues 309 to 373) were available in the laboratory, but had not been tested previously, so an attempt was made to identify the UL16 protein using these antisera.

Mutant 1b and *wt* virions were fractionated into envelope and capsid/tegument fractions, which were electrophoresed, blotted and then incubated with a potential UL16 antibody (16Ab/1) (1/200 dilution of stock). No significant difference was observed between the results obtained using pre-immune and immune sera (Fig. 39A). A second UL16 antibody (16Ab/2) (1/200 dilution of stock) also failed to identify a convincing candidate protein in infected cells (Fig. 39B). Various conditions of Western blotting were assessed in order to reduce non-specific binding, including longer washing periods, more frequent washes and increased amounts of detergent in the wash buffer, but these did not significantly improve the results. These antibodies may not recognise the UL16 protein, or may not be sensitive enough to detect the protein if it is made in small amounts.

The UL16 antisera were also used in immune precipitation reactions utilizing extracts of infected BHK C13 cells pulse labelled with [^{35}S]-methionine or [^{35}S]-cystine for periods of 2 h to 12 h pi or throughout infection (4-24 h pi). Attempts to detect a specific reaction yielded no obvious difference between mutant and *wt* (data not shown). The extraction and reaction buffers were varied in an attempt to improve the results but this was of no benefit.

In summary, use of antisera potentially recognising the UL16 gene product did not lead to identification of the protein.

4.2.3.2. Pulse labelled infected cells

BHK C13 cells were infected with virus and labelled with [^{35}S]-methionine for 2 or 4 h intervals up to 12 h pi or throughout infection (4-24 h pi). Analysis of electrophoretically separated proteins revealed differences in the banding pattern between the 1a and 1b mutants and cos*wt*1 and *wt* virus (band a; 42 kDa) (Fig. 40). Close examination revealed that mutant 1a and cos*wt*1 share a band in common and mutant 1b and *wt* virus share a band in common which is lower in mobility. This difference was consistently

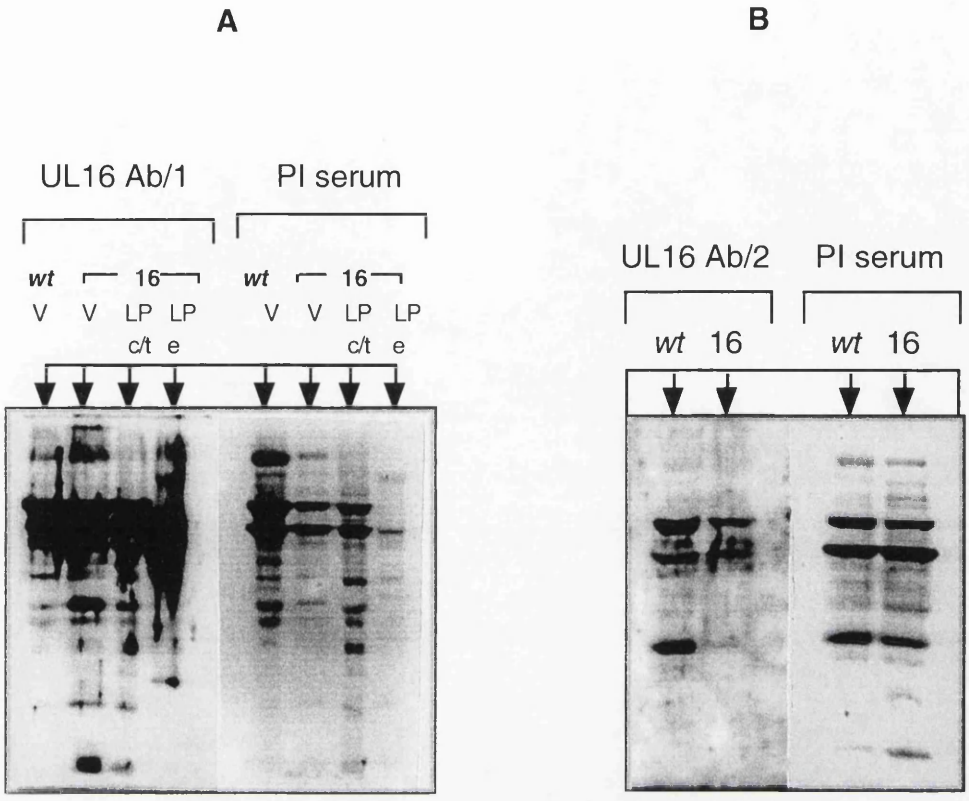


Fig 39. Western blot of *wt* and UL16$^-$ virions and virus infected cells

An autoradiograph of a Western blot in which virion fractions (A) or infected cell extracts (B) were electrophoresed on a 9% acrylamide gel, electroblotted onto a membrane and incubated with a candidate UL16 antibody (UL16Ab/1 or 2) or pre-immune serum. PI is pre-immune serum, *wt* is *wt* virus, 16 is UL16$^-$ virus, V=virions, LP=L-particles, c/t=capsid/tegument fraction, e=envelope fraction. Size markers were not included on the acrylamide gels.

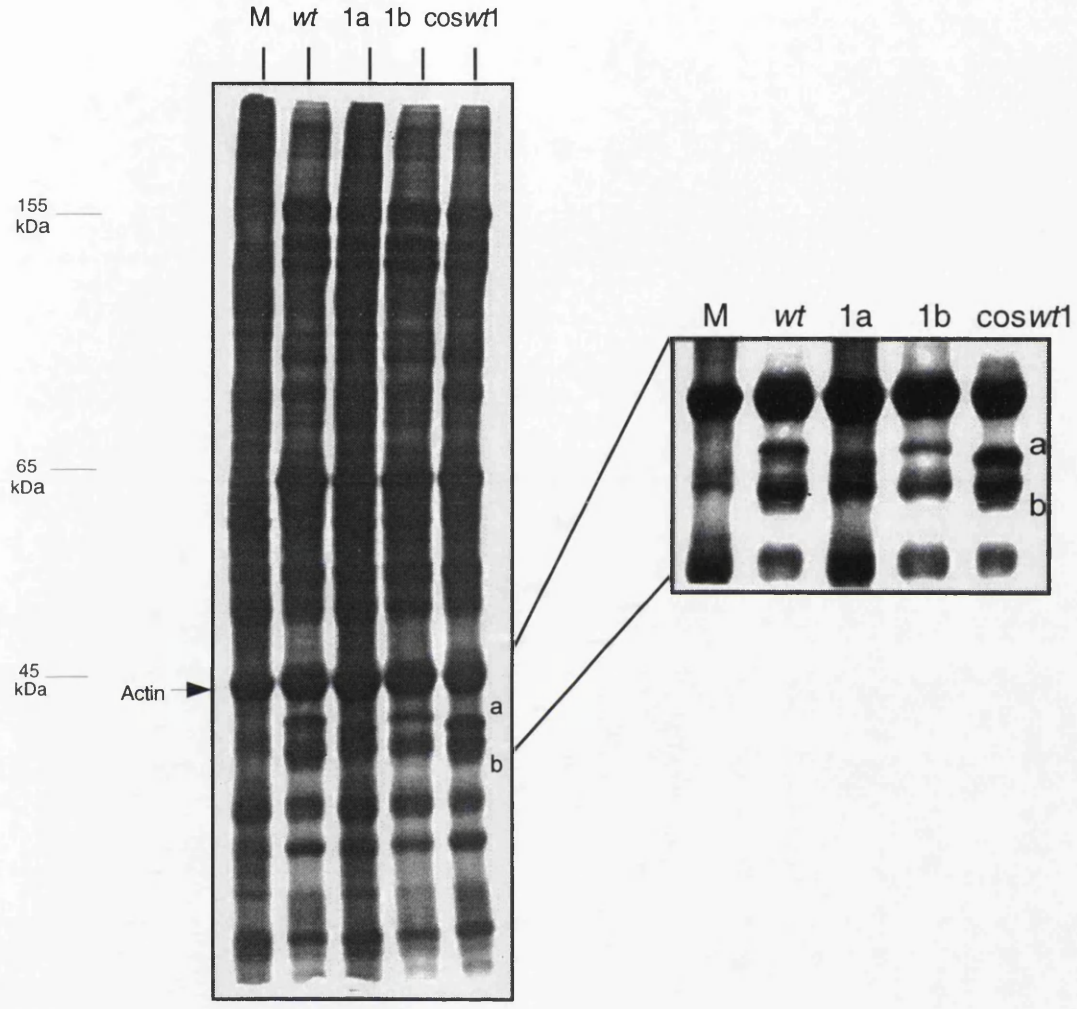


Fig. 40. Candidate UL16 protein in *wt*, cos*wt*1, mutant 1a and 1b infected cells (labelled with [^{35}S]-methionine)

An autoradiograph of a 12% polyacrylamide gel in which pulse labelled infected cells were subjected to SDS-PAGE. The region of the gel containing the 4-24 h labelled cells is shown. The bands refered to in the text (a ~42 kDa and b ~40 kDa) are indicated on the right. Actin is denoted by an arrow and molecular weights are given on the left in kDa. M is mock infected.

observed. On the same autoradiograph, another difference in the same area was observed: a band appeared to be present in both *wt* samples but not the mutants (band b; 40 kDa) (Fig. 40). This band represents a candidate UL16 protein. Further attempts to resolve this region of the gel were made using acrylamide/DATD gels, but failed to provide additional information (data not shown).

4.2.3.3. *In vitro* transciption and translation

The UL16 protein was produced *in vitro* in order to help identify the protein in radiolabelled extracts of infected cells.

*Kpn*I q was digested with *Bsu*36I and *Bss*SI to excise the fragment containing UL16 and 457 bp at the 5' end and 134 bp at the 3' end (29717-31427 bp in the HSV-1 sequence). This fragment was cloned in both orientations into the *Sma*I site of pGEM1 and pGEM2 under control of the T7 and SP6 promoters, giving rise to four UL16-containing plasmids: p1 (UL16 inserted into pGEM1 under control of the T7 promoter), p2 (pGEM1/sp6), p3 (pGEM2/T7) and p4 (pGEM2/sp6). The plasmids were transcribed and translated *in vitro* and the resulting proteins, labelled with [^{35}S]-methionine or [^{35}S]-cystine, were subjected to SDS-PAGE. Fig. 41 and 42 show that the UL16 protein was produced from all four plasmids (approximately 39 kDa). In both cases the UL16 protein was produced in greater amounts from the T7 promoter. The UL10 protein was produced as a control for the the procedure. A plasmid containing UL20 was also used but it did not produce a protein.

In order to confirm that the 39 kDa protein is the product of UL16, p1 was cleaved within the UL16 sequence. Digestion with *Bam*HI and *Cla*I results in cleavage of the 3' terminal 395 bp of UL16 (t1), and digestion with *Pst*I removes the 3' terminal 197 bp of UL16 (t2). Fig. 43 shows that a truncated UL16 protein was not detected using the template cleaved with *Bam*HI and *Cla*I (t1) (the protein would have electrophoresed from the bottom of the gel). Digestion with *Pst*I (t2) produced two bands, one of 39 kDa and one of approximately 35 kDa. The production of both the *wt* protein and truncated protein was due to partial digestion. Controls for the procedure were as described above.

The UL16 protein produced *in vitro* (39 kDa) does not correspond in mobility to any viral protein detected in methionine- or cystine-labelled infected cells, including the candidate UL16 protein identified in Section 4.2.3.2, which has a lower mobility (band b; 40 kDa) (see Fig. 41 and 42). This may be due to differences in modification between the viral UL16 protein and the protein produced *in vitro* from *E. coli*.

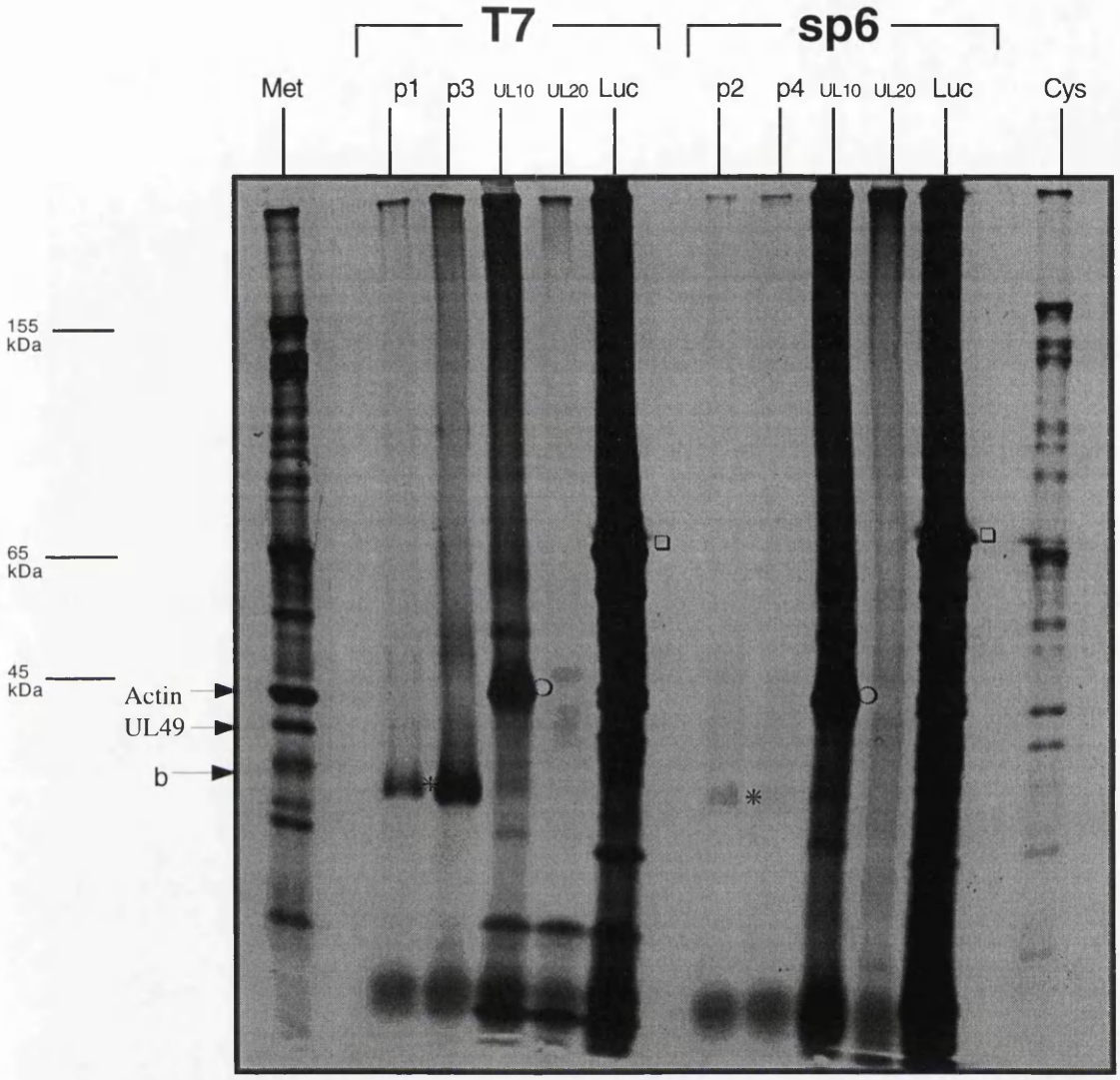


Fig. 41. *In vitro* transcription and translation of plasmids containing UL16 (labelled with [^{35}S]-methionine)

An autoradiograph of a 12% polyacrylamide gel in which [^{35}S]-methionine labelled proteins were separated. The products of plasmids p1, p2, p3, p4 (see text for details) (✳), UL10 (○) and luciferase (❑) are indicated. [^{35}S]-methionone and [^{35}S]-cystine labelled infected cells were used as markers. The candidate UL16 protein (b; 40 kDa) is shown by an arrow on the left. Actin and UL49 proteins are denoted by arrows and molecular weights are given in kDa on the left.

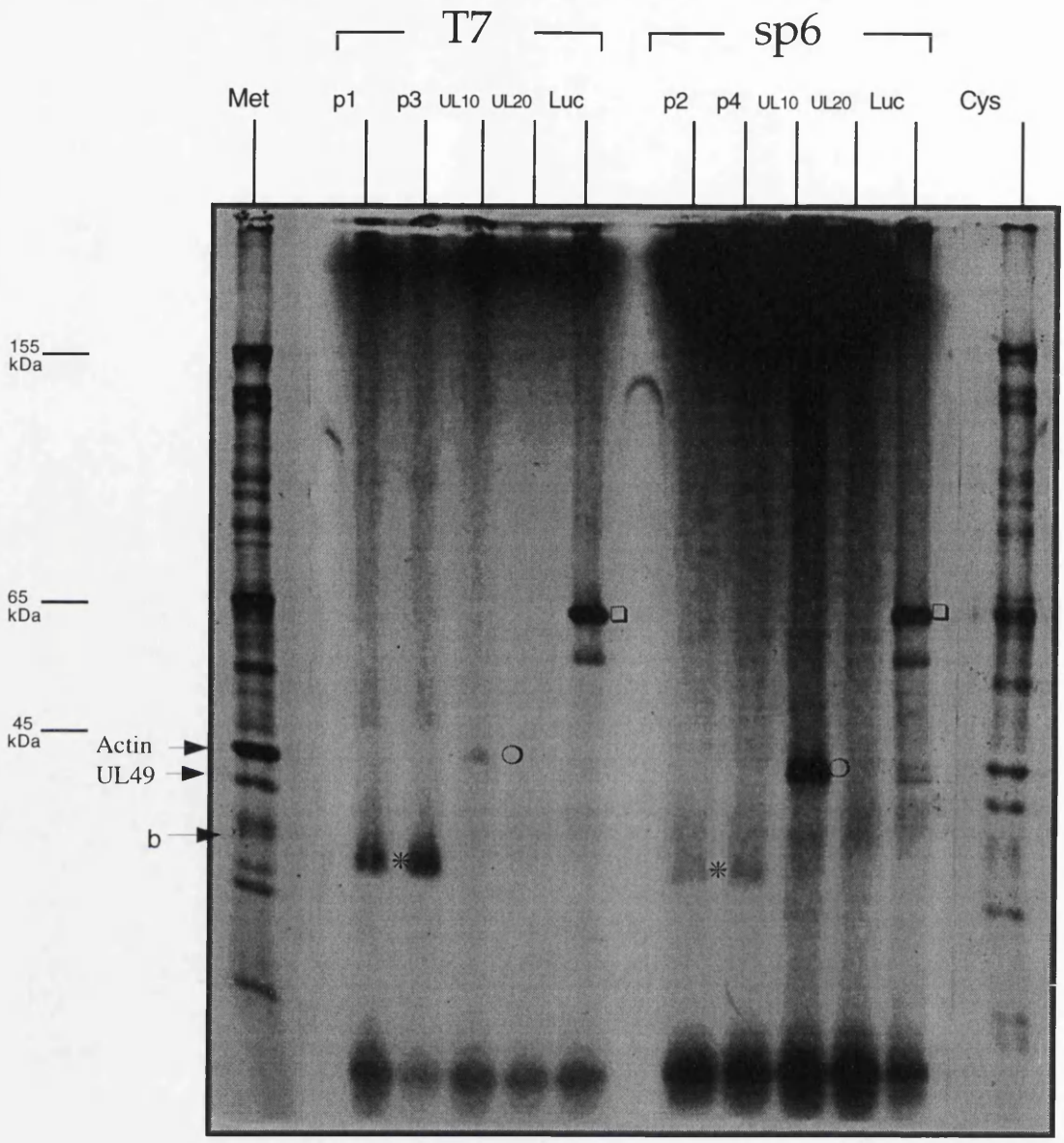


Fig. 42. *In vitro* transcription and translation of plasmids containing UL16 (labelled with [^{35}S]-cystine)

An autoradiograph of a 12% polyacrylamide gel in which [^{35}S]-cystine lalelled samples were separated. The products of plasmids p1, p2, p3, p4 (see text for details) (✳), UL10 (○) and luciferase (❑) are shown. [^{35}S]-methionone and cystine labelled infected cells were used as markers. The candidate UL16 protein (b; 40 kDa) is identified in the methionine labelled sample by an arrow. The actin and UL49 proteins are denoted by arrows and molecular weights are given in kDa on the left.

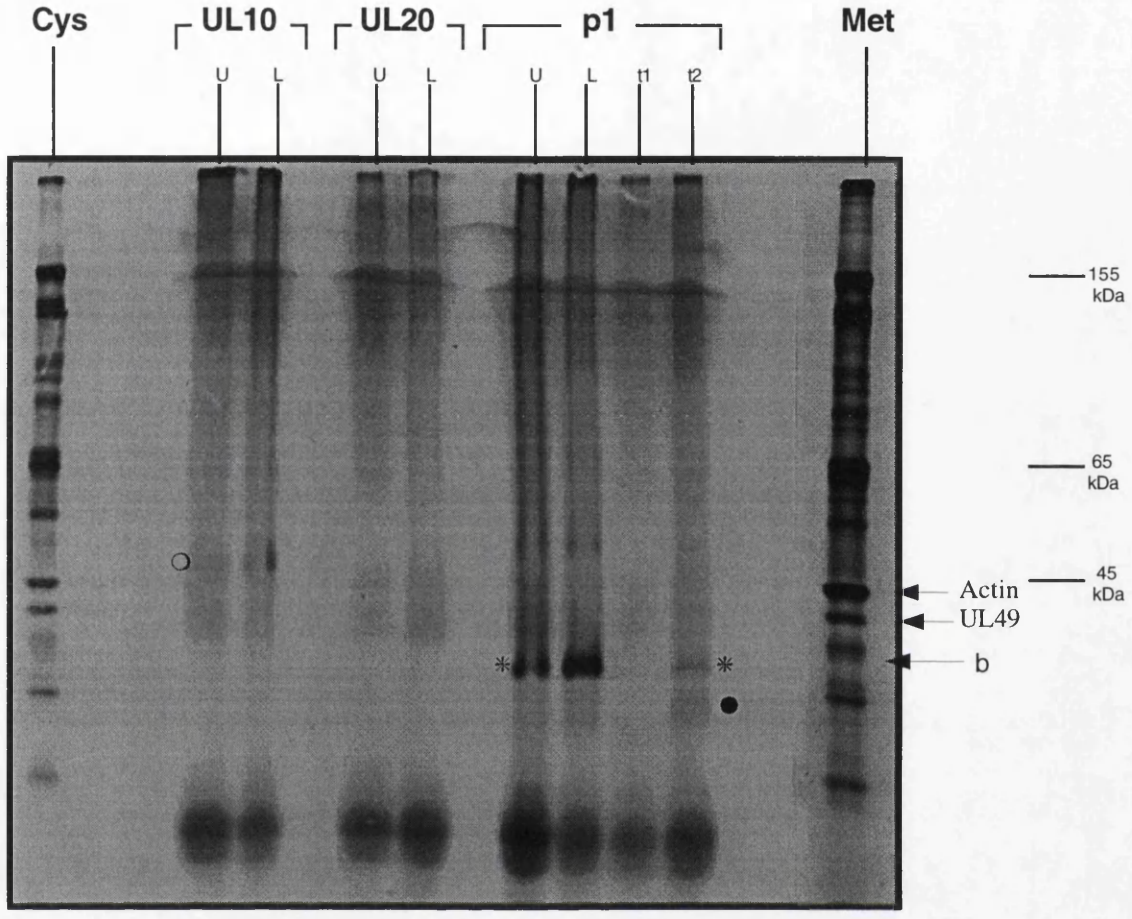


Fig. 43. Confirmation of the *in vitro* transcription and translation product of plasmid p1 by truncation of the gene

An autoradiograph of a 12% polyacrylamide gel in which [^{35}S]-cystine labelled samples were separated. Plasmid p1 was either uncut (U), linearised (L) or digested with *BamHI*/*ClaI* (t1) or *PstI* (t2). UL10 and UL20 were expressed from the T7 promotor as controls. The products of plasmids p1 (✳),truncation t2 (●) and control UL10 (○) are shown. [^{35}S]-methionone and cystine labelled infected cells were run as markers. The candidate UL16 protein (b; 40 kDa) is denoted by an arrow on the right. The actin and UL49 proteins are denoted by arrows and molecular weights are given in kDa on the right.

It was noted that neither the candidate band (band b; 40 kDa) nor any other protein in the same region was prominent in the cystine-labelled infected cell extracts (see Figs. 41 and 42). Since the predicted UL16 protein contains 20 cysteine residues in the coding sequence and only three methionine residues, it was expected that the UL16 protein should be relatively easier to identify in [^{35}S]-cystine labelled cells.

Taking into account the relative intensities of actin (which contains 17 methionine residues and six cysteine residues) and the UL49 protein (which contains four methionine residues and two cysteine residues) in [^{35}S]-methionine and [^{35}S]-cystine labelled infected cell extracts (see Figs. 41 and 42; Met and Cys marker tracks), results indicate that neither the candidate band nor other bands in this region of the gel exhibit relative intensities consistant with their identity as the UL16 protein.

In summary, failure to identify the UL16 protein indicates that it may be a very minor protein, may comigrate with a more abundant protein, or may be processed such that it migrates to a different region of the gel.

4.2.3.4. Mass spectrometric analysis

On Coomassie-blue stained polyacrylamide gels, there was no obvious difference between the protein profiles of mutant 1b and *wt* virions (data not shown). However, since the protein encoded by the UL16 counterpart was identified in the virions of EHV-1 virus by mass spectrometric analysis (43kDa) (A.J. Davison, personal communication) (see Section 4.1.7), a number of faint protein bands in the 40 kDa region were subjected to laser desorption mass spectrometry during analysis of the UL17 and UL14 proteins (details in Section 4.1.7.), but the UL16 protein was not identified despite extensive analysis (data not shown).

4.2.4. *IN VIVO* ANALYSIS OF UL16⁻ MUTANTS

UL16 is not essential for virus growth in cell culture, although mutants 1a-d yielded approximately five to ten fold less virus than *wt* and have a smaller plaque size compared to *wt*. Since a number of genes which are non-essential in cell culture have been shown to have vital roles *in vivo* (see Section 1.9 for details), cos*wt*1 and the UL16⁻ mutants were tested for their pathogenicity and ability to replicate and establish and reactivate from latent infection *in vivo* using the mouse model.

4.2.4.1. Neurovirulence

Two experiments were carried out at different times in which cos*wt*1, UL16⁻ mutants and revertants were injected into mice intracerebrally (IC). The inoculations were performed by Dr A. MacLean. The virus stocks were titrated on the day of use to confirm the amount of virus injected. Mice were injected with a range of doses and their clinical state was monitored for 14 days pi. LD_{50} values were calculated by the formula of Reed and Muench (1938), and the detailed results are given in Tables 21 and 22, and summarised Table 23.

Table 21. Fate of IC injected mice - experiment 1

Virus/titre	mouse 1	mouse 2	mouse 3	mouse 4	mouse 5	Total	LD_{50}
cos*wt*1 10^2	K[a] 4d	S[b]	S	S	-[c]	1/4	$5x10^2$
cos*wt*1 10^3	K 5d	S	K 5d	S	-	3/4	
1a 10^2	S	S	S	S	-	0/4	$>3.16x10^5$
1a 10^3	S	S	S	S	S	0/5	
1a 10^4	S	S	S	S	-	0/4	
1a 10^5	S	S	S	S	-	0/4	
1b 10^2	S	S	S	S	-	0/4	$7x10^3$
1b 10^3	S	S	S	S	S	0/5	
1b 10^4	K 5d	K 5d	K 7d	S	-	3/4	
1b 10^5	K 5d	K 5d	K 5d	S	-	3/4	

[a] Killed on day stated pi.
[b] Survived.
[c] Not done.

Table 22. Fate of IC injected mice - experiment 2

Virus	mouse 1	mouse 2	mouse 3	mouse 4	Total	LD_{50}
cos*wt*1 10^2	D[d] 8d	D 8d	S[b]	S	2/4	10^2
cos*wt*1 10^3	K[a] 8d	D 8d	K 8d	K 8d	4/4	
1a 10^3	S	S	S	S	0/4	10^5
1a 10^4	S	S	S	S	0/4	
1a 10^5	K 3d	K 3d	S	S	2/4	
1b 10^2	S	S	S	S	0/4	$5x10^4$
1b 10^3	K 8d	S	S	S	1/4	
1b 10^4	K 8d	K 8d	K 8d	S	3/4	
1d 10^2	S	S	S	S	0/4	$7x10^4$
1d 10^3	K 6d	S	S	S	1/4	
1d 10^4	K 5d	K 5d	S	S	2/4	
1b rev 10^2	K 4d	K 4d	K 4d	K 4d	4/4	$<6x10^1$
1b rev 10^3	K3d	K 3d	K 4d	K 4d	4/4	

[a] Killed on days stated pi.
[b] Survived.
[d] Died on date stated.

Table 23. Summary of LD_{50} values from two experiments

Virus	Expt 1 - LD_{50}	Expt 2 - LD_{50}
cos*wt*1	5×10^2	10^2
1a	$>3.16 \times 10^5$	10^5
1b	7×10^3	5×10^4
1d	-[a]	7×10^4
1b rev	-	$<6 \times 10^1$

[a] Not done.

It is evident that there is substantial variation between the two experiments, probably due to the age and state of health of the animals. This is discussed further in Chapter 5.

In each of the experiments cos*wt*1 produced an LD_{50} value within the *wt* range as defined by MacLean *et al.* (1991) (Dr A. MacLean, personal communication). Mutants 1a and 1b, which should be genetically identical, appeared in experiment 1 to be attenuated to different extents (10^3 and 10^1 fold respectively). However, this difference was not repeated in experiment 2; mutants 1a and 1b were attenuated to a similar degree (5×10^2 - and 10^3 fold respectively). These results firmly support the conclusion that mutants 1a and 1b are attenuated, but experimental factors may be too variable to support the conclusion that they are attenuated to different degrees. Mutant 1d, which has a different insertion at the same site as in 1a and 1b (see Fig. 28), was attenuated to a degree similar to 1a and 1b (7×10^2) in experiment 2. A revertant of 1b (1b rev) was virulent in experiment 2, and this indicates that the attenuation of the mutants is due to the UL16 mutation.

The genotype of virus replicating in the brains of mice inoculated with mutant 1b was tested. Virus was recovered by homogenising the brains and titrating the homogenate on BHK C13 cells. Small viral stocks were made and probing of *KpnI/BglII* digests of infected cell DNA with a plasmid containing *Kpn*I q showed that all of the recovered viruses were mutant (Fig. 44). The presence of the 4 bp insertion in 1b should result in the generation of a novel *Cla*I site (AGATCGATCT), and probing of *BamHI/Cla*I digested infected cell DNA with a plasmid containing *Kpn*I q confirmed the presence of this site (Fig. 45).

These experiments show that cos*wt*1 and revertant 1b are as virulent as *wt* and that UL16$^-$ mutants with a lesion at position 31096 bp exhibit significantly reduced neurovirulence, although they can replicate in the brain.

4.2.4.2. Latency

The ability of the cos*wt*1 and mutants 1a and 1b to establish and be reactivated from latency was examined by inoculation of 4 week old BALB/c mice by a peripheral route, the footpad (FP). In accordance with the observation that inoculation of HSV-1 by the peripheral route results in a high LD_{50} (MacLean *et al.*, 1991; McKie *et al.*, 1994; Dr A. MacLean, personal communication), the LD_{50} of the mutants 1a and 1b and cos*wt*1 were high. The mutants exhibited higher LD_{50} values than *wt*, but, the difference was not accurately quantified (Table 24).

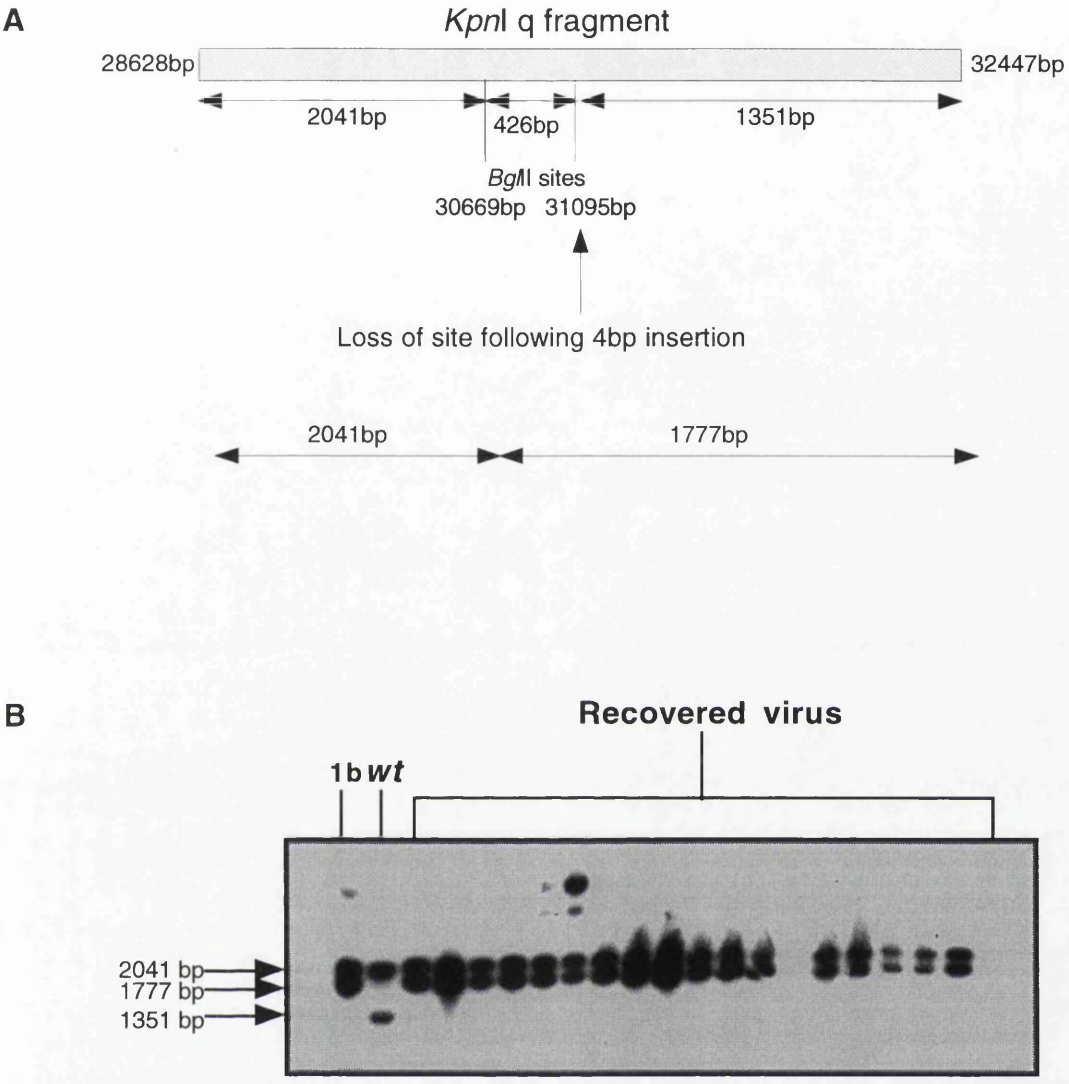


Fig. 44. Virus recovered from the brains of mice infected with mutant 1b

A Map showing the RE profile of 1b mutants following digestion with *KpnI*/*BglII* (not drawn to scale).

B An autoradiograph of a Southern blot in which *KpnI*/*BglII* digested infected cell DNA was transferred from a 0.8% (w/v) agarose gel and probed with a plasmid containing *KpnI* q. The sizes of detected fragments are shown.

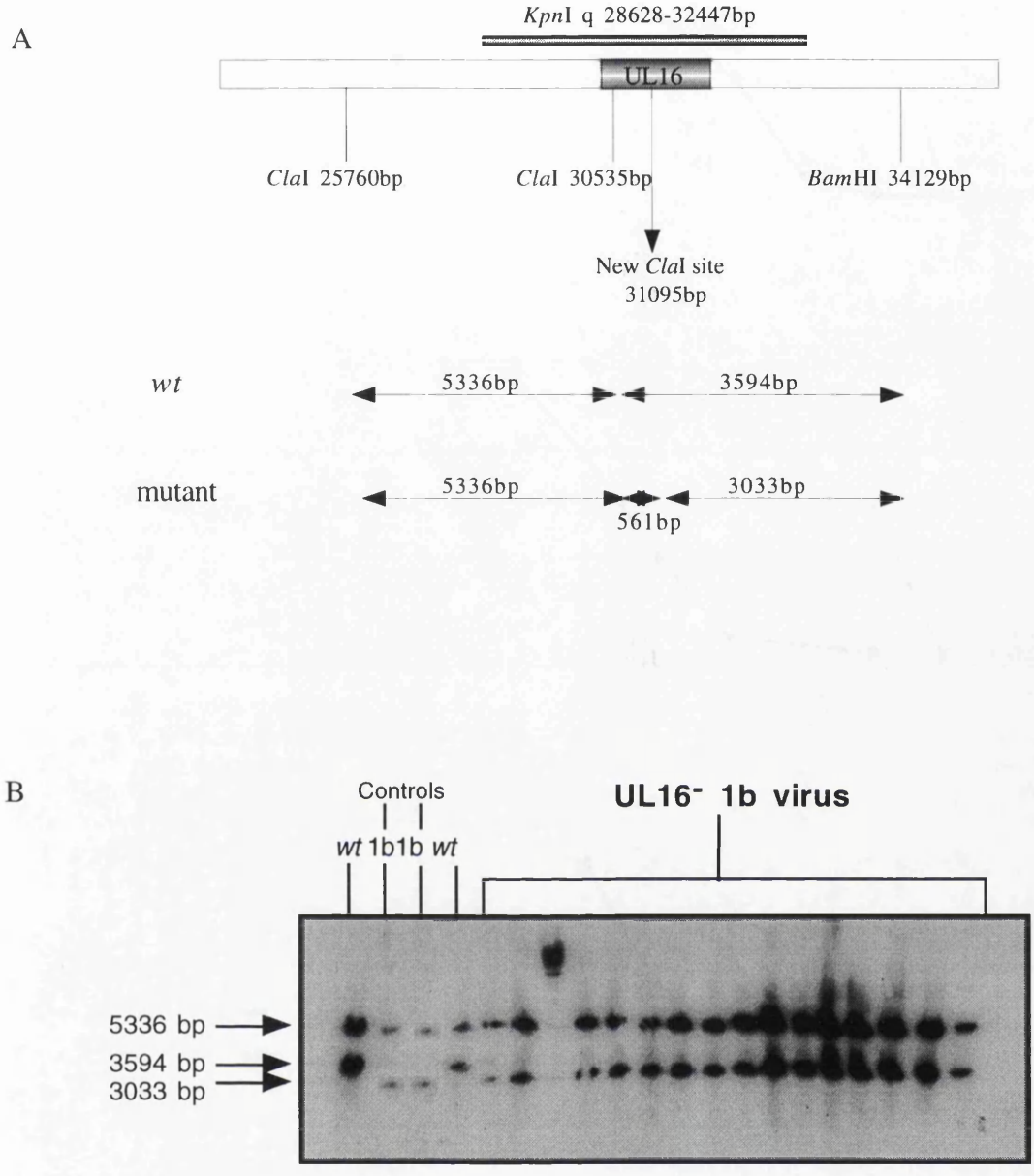


Fig 45. Confirmation of a novel *Cla*I site in mutant 1b

A Map of the RE profile of *wt* and 1b mutant following digestion with *Cla*I/*Bam*HI (not drawn to scale). The position of the sites and the UL16 coding region are shown. *Kpn*I q is shown above the figure.

B An autoradiograph of a Southern blot in which *Bam* HI/*Cla*I digested infected cell DNA was transferred from a 0.8% (w/v) agarose gel and probed with a plasmid containing *Kpn*I q. The sizes of detected bands are shown.

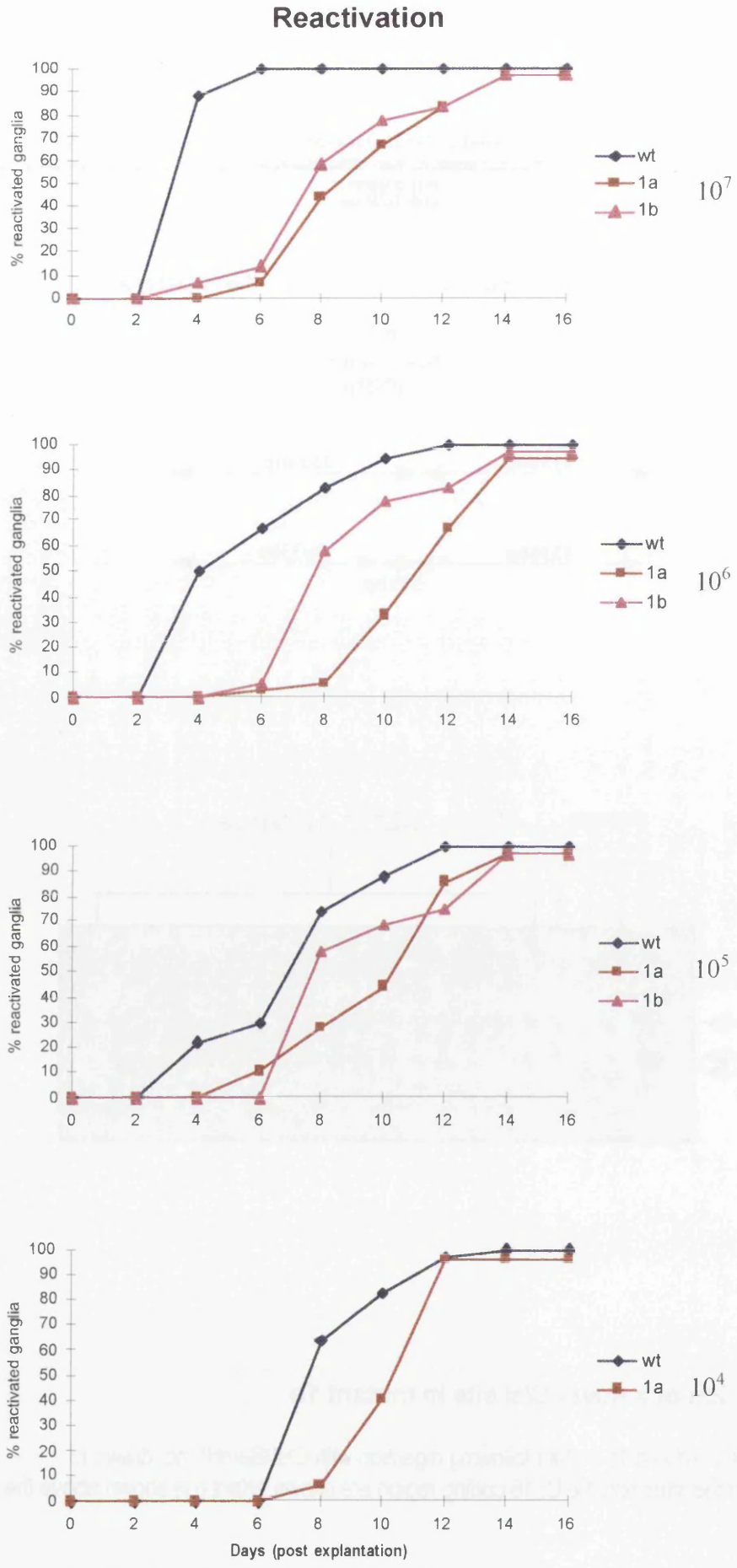


Fig. 46. Reactivation of viruses from latency
Mice were injected with virus into the footpad, ganglia were removed at six weeks pi and cultured for 16 days. The rate of reactivation is expressed as the percentage of total ganglia tested at each concentration of virus.

Table 24. Fate of FP injected mice

Virus	mouse 1	mouse 2	mouse 3	mouse 4	Total	LD_{50}
cos*wt*1 10^4	S[b]	S	S	S	0/4	$7x10^6$
cos*wt*1 10^5	S	K[a] 10d	S	S	1/4	
cos*wt*1 10^6	S	S	S	S	0/4	
cos*wt*1 10^7	K 10d	K 9d	S	-[c]	2/3	
1a 10^4	S	S	S	-	0/3	$>3.16x10^7$
1a 10^5	S	S	S	S	0/4	
1a 10^6	S	S	S	S	0/4	
1a 10^7	S	S	S	S	0/4	
1b 10^4	S	S	S	-	0/3	$>3.16x10^7$
1b 10^5	S	S	S	S	0/4	
1b 10^6	S	S	S	S	0/4	
1b 10^7	S	S	S	S	0/4	

[a] Killed on day stated pi.
[b] Survived.
[c] Not done.

Reactivation of virus was investigated by removing ganglia from infected mice at 6 weeks pi and culturing them in ETF10 for up to 21 days. Virus reactivated from all of the cos*wt*1 ganglia tested by 6 d post explantation. Mutants 1a and 1b reactivated more slowly than cos*wt*1 but reached 90-95 % reactivation by day 16 (Fig. 46). A selection of reactivated plaques were picked at 12 d post explantation, infected cell DNA was produced, and *Kpn*I/*Bgl*II digests were probed with a plasmid containing *Kpn*I q. All of the recovered viruses from mutant 1a or 1b infected mice were *wt* except for one which was a mixture of mutant and *wt* (Fig. 47).

This experiment was repeated using all of the UL16$^-$ mutants and an additional cos*wt* virus. Cos*wt*1 reactivated much more slowly than previously, and did not reach 100% reactivation. Cos*wt*2 reactivated less well and only reached 59% reactivation by day 20, a value still within the normal range for a *wt* virus (MacLean *et al.*, 1991b; Dr A. Maclean, personal communication). The six mutants reactivated very slowly and only 10-38 % of ganglia had reactivated after 20 days (Fig. 48).

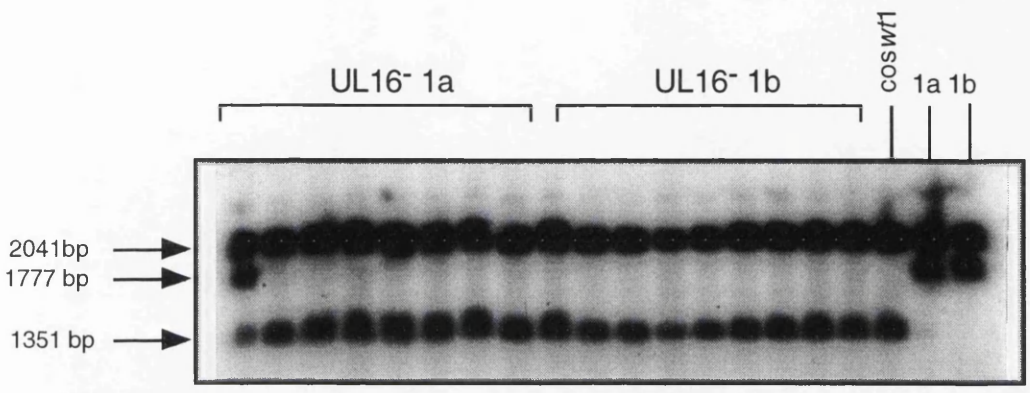


Fig. 47. Genotypes of reactivated viruses from ganglia of mice infected with UL16- 1a or 1b

An autoradiograph of a Southern blot in which *Kpn*I/*Bgl*II digested infected cell DNA was probed with a plasmid containing *Kpn*I q. Infected cell DNA was produced from plaques picked at 12 days post explantation. The positions of detected fragments are shown (see Fig. 44 for additional details).

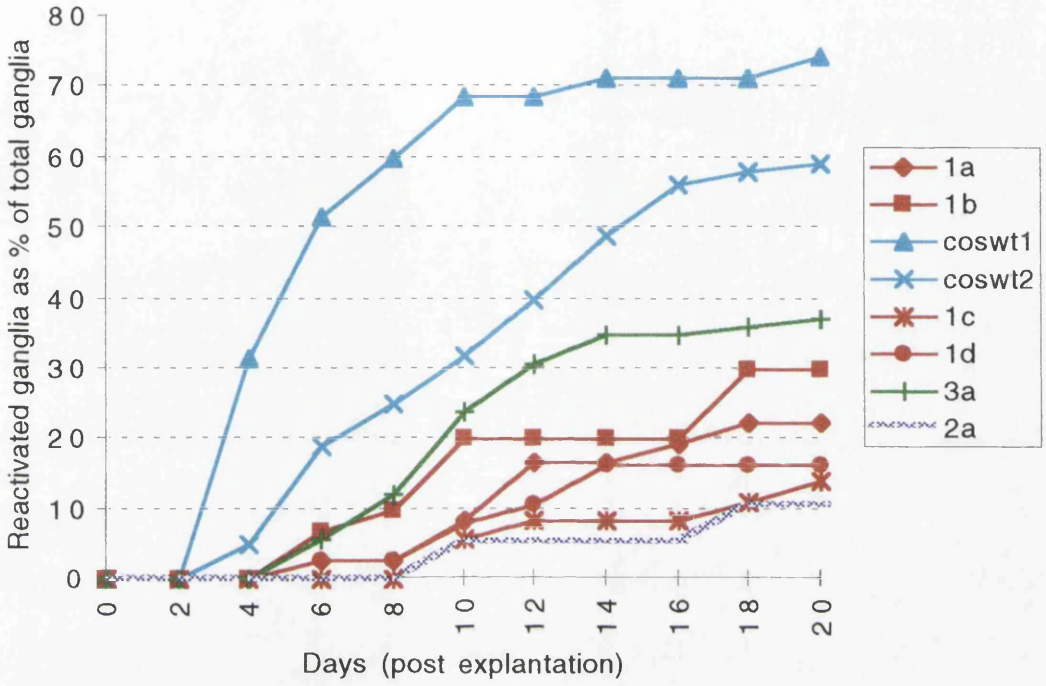


Fig. 48. Reactivation of viruses from latency.

Mice were injected with virus into the footpad, and ganglia were removed at six weeks pi and cultured for 21 days. The rate of reactivation is expressed as the percentage of total ganglia tested.

Infected cell DNA was made from a selection of reactivated viral plaques picked at 4-6 d post explantation, digested with the appropriate enzymes and probed with a plasmid containing *Kpn*I q. Fig. 49 shows that reactivated viruses from mutants 1b, 1c, 1d, 2a, and 3a were *wt*. The virus recovered from mice infected with mutant 1a was mutant. The genotypes of 1a plaques picked later during reactivation (day 10-12) proved to be *wt* (2 plaques), mutant (1 plaque) and a mixture of *wt* and mutant (1 plaque) (Fig. 50). Thus, as reactivation progressed, *wt* virus was recovered from 1a-infected ganglia.

These results suggest that the mutants establish and reactivate from latency less efficiently than *wt* and that there is a strong selection pressure in favour of *wt* virus arising by reversion.

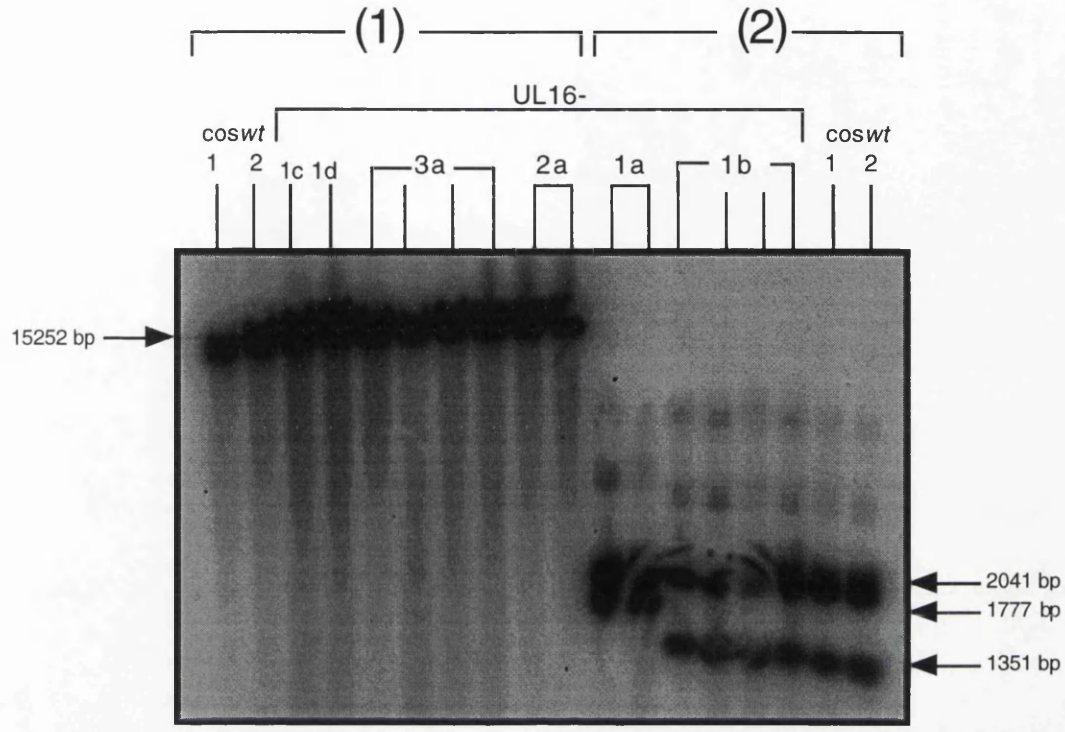


Fig. 49. Genotype of virus reactivated from ganglia of mice infected with UL16⁻ mutants

An autoradiograph of a Southern blot in which *Xba*I/*Eco*RI (1) or *Kpn*I/*Bgl*II (2) digested infected cell DNA was probed with a plasmid containing *Kpn*I q. Virus was recovered at 4-6 days post explantation. The position of the detected fragments are shown (see Figs. 29 and 30 for details).

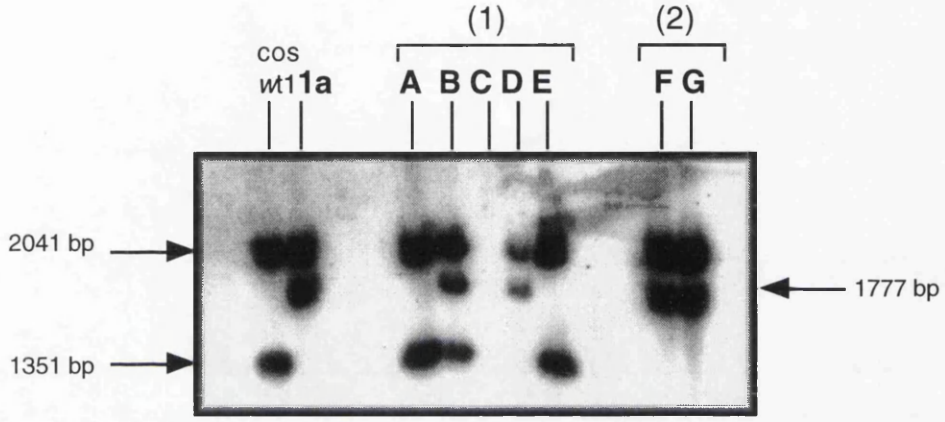


Fig. 50. Genotype of reactivated virus from the ganglia of mice infected with UL16⁻ 1a mutant

An autoradiograph of a Southern blot in which *Kpn*I/*Bgl*II digested infected cell DNA was probed with a plasmid containing *Kpn*I q. Virus was recovered at 10-12 days (1) or 4-6 days (2) post explantation. The position of the detected fragments are shown (see Fig. 29 for details).

Chapter 5

Discussion

5.1. Background

HSV-1 UL14, UL15, UL16 and UL17 are part of a block of genes (UL10 to UL19) which is conserved among the alpha-, beta- and gammaherpesviruses (Davison, 1993). They are expressed as late mRNAs (McGeoch and Schaffer, 1992).

UL15 is the only spliced gene in U_L (McGeoch *et al.*, 1988). UL15 homologues in the three herpesvirus subfamilies have an equivalent structure with two exons, and a variable number of genes (2-4) in the intron (Dolan *et al.*, 1991). In contrast, the proposed counterpart in CCV has three exons, and the boundary between the first and second exons corresponds precisely to that in other herpesviruses (Davison, 1992). Construction of an HSV-1 mutant expressing UL15 without the intron has shown that separation of the two exons is not essential for replication of HSV-1 in cell culture (Baines and Roizman, 1992b). The effect of the absence of the intron in an animal model has not been reported.

The UL15 protein contains potential ATP-binding motifs (Dolan *et al.*, 1991), and a possible function was suggested by Davison (1992) who noted that the UL15 protein may be related to a protein encoded by T4 bacteriophage gene 17. This protein is part of the phage terminase, or DNA packaging protein complex, and mutations result in the arrest of phage production during DNA packaging, leading to an accumulation of empty proheads and uncleaved concatemeric DNA (Bhattachorgga and Rao, 1993; Powell *et al.*, 1990; Carrascose and Kellenberge, 1978).

During the course of this work, Poon and Roizman (1993) described a *ts* mutant with a lesion in UL15. The mutant produced viral DNA at the NPT, but failed to package it into capsids. In a subsequent paper, Baines *et al.* (1994) showed that the UL15 gene encodes two proteins (one is a truncated form of the full length UL15 protein, similar to the situation with UL26 and UL26.5, see Section 1.8.2). The full length protein is necessary for cleavage and packaging of viral DNA. The published data on UL15 are therefore consistent with the view that UL15 is a subunit of the herpesvirus terminase.

No mutants have been described for UL14 or UL17, and the roles of the protein products of these genes are not known. The status of both genes in cell culture is also unknown, although Baines and Roizman (1991) suggested from their inability to obtain a mutant that UL17 may be essential.

The function of the UL16 protein is unknown although it has been shown to be non-essential in cell culture (Baines and Roizman, 1991). Growth curves of a mutant lacking

988 bp of UL16 (1119 bp) indicated that yields of UL16$^-$ virus in Vero or BHK cells were reduced from those of *wt* (10- or 3-fold respectively) (Baines and Roizman, 1991).

An alignment of the predicted amino acid sequence of the UL16 protein with counterparts in other herpesviruses is shown in Fig. 51. Of the 20 cysteine residues in HSV-1 UL16, 6 are conserved in all three subfamilies and a seventh (in parentheses in Fig. 51) is conserved in all but HSV-2 where it is replaced by a glycine. Indeed, two thirds of the total conserved residues are cysteines, and are likely to have an important role, possibly in forming intra- or inter-molecular disulphide bonds.

5.2. Cosmid-based mutagenesis of UL14, UL15 and UL17

The manipulation of large fragments of HSV-1 DNA is difficult, but in principle the cosmid system should permit easier manipulation and production of mutants. Mutants produced using a mutant cosmid set can be isolated in the absence of a background of *wt* virus, and even viruses which have multiple mutations or are at a severe growth disadvantage to *wt* can be isolated. However, Cunningham and Davison (1993) reported that transfections with mutant cosmids containing 4 bp insertions resulted in the generation of revertants (*wt* progeny) on non-complementing cell lines when the mutation was in an essential gene, owing to recombination events occuring during generation of the virus. This aspect of the cosmid system in fact provides a positive indication of the importance of target genes to growth in cell culture: a failed transfection yields no plaques, mutation of a non-essential gene yields only mutants and mutation of an essential gene yields only *wt* viruses. Also, genes which are neither completely essential or non-essential but disruption of which confers a severe growth disadvantage will produce mutants and a background of revertant virus (*wt*). In other systems, particularly those utilising plasmid recombination, failure to obtain a mutant has been taken as an indication of the essential nature of the target gene, whereas this may actually have been due to inefficient transfection. Another major advantage of the cosmid system is the ability to introduce minimal lesions, for example, the insertion or deletion of only a few bp, so that adjacent genes are less likely to be disrupted than they are in mutants in which sizeable insertions or deletions are made.

Despite the potential advantages of the cosmid system, there are still a number of difficulties. Manipulation of large fragments of DNA derived from cosmids is not easy, and obtaining optimal conditions for linearisation of the cos24 in the presence of EtBr proved to be critical in the experiments: if conditions were not correct large deletions or *wt* cosmids predominated. Also, transformation of bacterial cells with the cosmids

Fig 51. Alignment of the amino acid sequence of the HSV-1 UL16 protein and its counterparts in the three herpesvirus subfamilies

The alignment was produced using the Pileup program from Genetics Computer Group (1994). Padding characters are indicated by dots. Residues conserved between the three subfamilies and, non-conserved residues are given in the CON line. The 20 cysteine residues in the HSV-1 sequence are denoted by asterisks. HSV-1 (UL16) (McGeoch *et al.*, 1985, 1986, 1988), D.J. McGeoch kindly provided the HSV-2 sequence (UL16): it should be noted that this sequence begins with a leucine (CTG) rather than a methionine (ATG) codon, EHV-1 (gene 46) (Telford *et al.,* 1992), EHV-4 (gene 46) (kindly provided by E. Telford and A.J. Davison), BHV-1 (UL16) (Vlcek *et al.*, 1995), VZV (gene 44) (Davison and Scott, 1986), HCMV (UL94) (Chee *et al.*, 1990), HHV-6 (U65) (Gompels *et al.,* 1995), EBV ((BGLF2) Baer et al., 1984), HVS (ORF33) (Albrecht *et al.*, 1992), EHV-2 (ORF33) (Telford *et al.*, 1995), HHV8 (ORF33) (Moore *et al.*, 1996). An error in the published HHV-8 sequence by which the insertion of an additional residue causes a frameshift was noted. The putative corrected sequence is underlined.

```
                                                                                 *        *
HSV-1   MAQLGPRRPLAPPGPPGTLPRPDSRAGARGTRDRVDDLGTDVDSIARIVNSVFVWRVVRADERLKIFRCLTVLTEPLCQVALP
HSV-2   LAQRALWRPQATPGPPGAAAPPGHRGAPPDARAP..DPGPEADLVARIANSVFVWRVVRGDERLKIFRCLTVLTEPLCQVALP
EHV-1   MMAAASDSCLSLWEGSASSPNRQLTPEAVNCLTEALTEDVAVLRLIRSDPRVKIFMAVSVLTPRLARFAPP
EHV-4   MAADLNSYSSIWEGSSLSPNRQLTIEAANCLTEALTEDIAVLRLIRSDPRVKIFMAVSVLTPRLARFAPP
BHV-1   MAEDPAAAGALLARALTEELGCLHLVRTDSRVKIYVAVATLGRLLARLVSP
VZV     MELQRIFPLYTATGAARKLTPEAVQRLCDALTLDMGLWKSILTDPRVKIMRSTAFITLRIAPFIPL
HCMV    MAWRSGLCETDSRTLKQFLQEECMWKLVGKSRKH.REYRAVACRSTIFSPE...
HHV-6   MAISTFSIGDLGYLRNFLQNECNWFRICKKTFY.REYRSVATSSPTFSLN...
EBV     MASAANSSREQLRKFLNKECLWVLSDASTPQMKVYTATTAVSAVY...VPQ
HVS     MDDFRNKLRNFLNNECLWVKNVACTSFTKVYCATTAVSPFFKP.ISP
EHV-2   MASGGVGGTRELFRQFLNKECIWKKSPNSSPYLKIYVATTAISPVFKPDVGG
HHV-8   MASRRRKLRNFLNKECIWTVNPMSGDHIKVFNACTSISPVYDPELVT
CON     ---------------------------------------------------------------------------------

                *                                     *                  *
HSV-1   NPD.PGRALFCEIFLYLTRPKALRLPPNTFFALFFFNRERRYCAIVHLRSVTHPLTPLLCTLTFARIRAATPPEETPDPTTEQ
HSV-2   DPD.PERALFCEIFLYLTRPKALRLPSNTFFAIFFFNRERRYCATVHLRSVTHPRTPLLCTLAFGHLEAASPPEETPDPAAEQ
EHV-1   PPKLTHTAKCAVIMIYLTRPKALALQPKQFHMLVTFNKASVYSLVVRVKTKPFPVGTQRFRAVFQDPEFIGLPSDIPDPAAEN
EHV-4   QSKLTHTAKCAVIMIYLTRPKALALQPKQFHVLVTFSKSSVYSLVMRVKTKPFPISPQRFCGVFQDPEPIGLPSDVPNPATEN
BHV-1   EDASPGAA..VRVTLYITRPRSLELPPRHFHVLVLFGGAVARACVAGVRTRALVPGSTRVRAVFRDAVAVPAPADLPDPSAEV
VZV     QTDTTNIA.VVVATIYITRPRQMNLPPKTFHVIVNFNYEVSYAMTATLRIYPVENIDHVFGATFKNPIAYPLPTSIPDPRADP
HCMV    ..DDSS..CILCQLLLLY.RDG.....EWIICFCCNGRYQGHYGVNHVHRRRRRICHLPTLYQLSFGGPLGPASIDFLPSF.S
HHV-6   ..NKPKKFCMHCEIVIFK.RSE.....EFMFSLAVNGIHFGQFLTGKMKFNKKAVPEGLYYYILELGS.ITPIDLGFIPRYNS
EBV     IAGPPKTY.MNVTLIVLKPKKK.....PTYVTVYINGTLATVARPEVLFTKAVQGPHSLTLMYFGVFSDAVGEAVPVEIRGNP
HVS     QGVPDKHY.INVTLIILKPKKS.....HPYITVYINDLAVDCCSTEILQVKPVPCSH.FSLIYFGPL...IAPPHNVQIPANL
EHV-2   RGRPGSAHAINVTALFMKPKGR.....RTCAAFYVNGLLLEACVPEVIFTKVVPGVLGLFLIYFGPFAE...PRRPFPIPTEP
HHV-8   .SYALSVPAYNVSVAILLHKVM.....GPCVAVGINGEMIMYVVSQCVSVRPVPGRDGMALIYFGQF...LEEASGLRFPYIA
CON     ----------------------------------------------------------------------------------

                                                                   **          *          *
HSV-1   ......................LAEEPVVGELDGAYLVPAKTPPEPGACCALGPGAWWHLPSGQIYCWAMDSDLGSLCPPGSR
HSV-2   ......................LADEPVAHELDGAYLVPTDTAPESGACCALGPGAWWHLPGGRIYCWAMDDDLGSLCPPGSR
EHV-1   ......................IPTEINDRLDVSNFATPAQPPKDKYDCCVLAPGVWWSNANKAIYFLQMDVALLALCPAGWK
EHV-4   ......................IPTEINDRLDVSNFATQTQPPKDKYDCCVLAPGVWWYKAQKAIYFLQMDEALLALCPAGWK
BHV-1   ......................VPPAPAEHVDPFAFTAFARPPRDAADCFQLAPGVWWSYADRRLYLVQMDEALLALCPAGWR
VZV     ......................TPADLTPTPNLSNYLQPPRLPKNPYACKVISPGVWWSDERRRLYVLAMEPNLIGLCPAGWH
HCMV    QVTSSMTCDGITPDVIYEVCMLVPQDEAKRILVKGHGAMDLTCQKAVTLGGAGAWLLPRPEGYTLFFYILCYDLFTSCGNRCD
HHV-6   DCVTNMRC..VTPEVIYENCSIVCPEEANRLTVKGSGDNKLT.....PLGGCGAWCLKNGGDLYIYTFALAYDLFLTCYDKST
EBV     VVTC...TD.LTTAHVFTTSTAVKTVEE.........LQDITPSEIIPLGRGGAWY....AEGALYMFFVNMDMLMCCPNMPT
HVS     SIKASKKSH.LTKNQVIFTSKVIHPERL.........PDGYKSASLIG...ACAWY....SEGAIFQHFLSTDYMSLCPAFKE
EHV-2   AISAPQNVQLLNRMEMLDTSTHIALSDL.........GEAVAGREFTSVGKL.VWW....DGEAFFFYYLSMEYMMCCPTISE
HHV-8   PPPSREHVPDLTRQELVHTSQVVRRGDL.........TNCTMGLEFRNVNPF.VWL....GGGSVWLLFLGVDYMAFCPGVDG
CON     ----------------------------------------------------------------------------C-----

                   *  *                             * *   *       *          *
HSV-1   ARHLGWLLARIT.NHPGGCESCAPPP.HIDSANAL..WLSSVVTESCPCVAPCLWAKMAQCTLAVQGDASLCPLLFGHPVDT.
HSV-2   ARHLGWLLSRIT.DPPGGGGACAPTA.HIDSANAL..WRAPAVAEACPCVAPCMWSNMAQRTLAVRGDASLCQLLFGHPVDA.
EHV-1   ARGLGIILGRLL.NHQEGCATCRFTE.HSDPLNAT..ADSVATPESCLCWAPCLWRKAHQRELTVEGDRYLFRVLFMDAVER.
EHV-4   ARGLGIILGRLL.NHQEGCSTCRFTE.HSDPLNAT..ADSVATPESCLCWAPCLWRKSRQRELKVEGDRYLFRVLFMDAVER.
BHV-1   SRSLGGVLGRLL.SHPGGCAACTAKW.HIDALNAA..PEPGAC.DVCPCAAPCLWKKARRQDVPVAGDSSLFRVLFTDAVAR.
VZV     ARILGSVLNRLL.SHADGCDECNHRV.HVGALYAL..PHVTNHAEGCVCWAPCMWRKAGQRELKVEVDIGATQVLFVDVTTC.
HCMV    IPSMTRLMAAATACGQAGCSFCTDHEGHVDPTGNYVGCTPDM..GRCLCYVPC..GPMTQSLIH...NEEPATFFCESDDAKY
HHV-6   FPSLAKIIFDMIACESEDCVFCKDHNKHVSQAGQIVGCVSNQ..ETCFCYTSC..KKKMAN.IN...NPELISLLCDQEINK.
EBV     FPSLTHFINLLTRCDNGECVTCYGAGAHVNILRGWTEDDSPGTSGTCPCLLPC..TALNNDYVPITGHRALLGLMFKPEDAPF
HVS     FPSLSRILSLLTRCDDLSCVPCYGEKIHVNCQSGYTDSDCDGKSNSCPCITSC..TALKKDIVPITGHRNLLSLLFDATIQHN
EHV-2   HTTLSRFITLLTQCDDRGCVPCHGRKIHANVAGGYTDPGSDGVSTTCLCTLSC..AALEKELVPVTKNKSLLSLMFGPMTYQQ
HHV-8   MPSLARVAALLTRCDHPDCVHCHGLRGHVNVFRGYCSAQSPGLSNICPCIKSC..GT.GNGVTRVTGNRNFLGLLFDPIVQSR
CON     -----------------(C)-C-----H------------------C-C---C-----------------------------

                  *              *                *              *
HSV-1   .VTLLQAPRRPCITDRLQEVVGGR.CGADN.IPPTSAGWRLCVFSSYISRLFATSCPTVARAVARASSSDPE
HSV-2   .VILRQATRRPRITAHLHEVVVGR.DGAESVIRPTSAGWRLCVLSSYTSRLFATSCPAVARAVARASSSDYK
EHV-1   .VRLTGLRRSPKITANLADLVVGI.GPHGQQIPVNNAGWKLVALDADISRLIVCGCYALRYICPPTNSKHQPSSPDEYA
EHV-4   .VRLTGLRRSPKITADLADLVVGI.GSHGQQIPVNSAGWKLVALDANISKLIVCGCYSLRYLCPSTDCKTQQLSTSEDA
BHV-1   .VRILGSRRRPRITGALPELLAGV.GPRGEAVPVNGAGWQLRVMDDDVTRLLASSCRAMRSCAAARGSPWG
VZV     .IRITST.KNPRITANLGDVIAGT.NASGLSVPVNSSGWQLYMFGETLSRAIINGCGLLQRICFPETQRLSGEPEPTTT
HCMV    LCAVGSKTAAQVTLGDGLDYHIGVKDSEGRWLPVKTDVWDLVKVEEPVSRMIVCSCPVLKNLVH
HHV-6   IDIMYPKIKASLSLDIN.SYAHGYFGDDP..YALKCVNWIPVRISAALSRLIVLSCPVCKRVVMD
EBV     VVGL..RFNPPKMHPDMSRVLQGVLA.NGKEVPCTAQPWTLLRFSDLYSRAMLYNCQVLKRQVLHSY
HVS     ITSI..KFFSPQTPTTVNNVFCGVLD.TGETVECTCEAWNLLMFSDFISRQMIYNCQIMKRFCLRSC
EHV-2   VTHL..RFTPSTRPARIQDIISGVTE.TGDRVDVRDGTWNLLKMSSLYSRCTLYECQILKRQCLRSY
HHV-8   VTAL..KITSHPTPTHVENVLTGVLD.DGTLVPSVQGTWVLLRMSDYFSRLLIYECKKLKALGLRSY
CON     ----------------------G---------------W---------------C---------------------
```

occurs at a low efficiency. As an indication of the experimental difficulties, in one experiment 200 colonies were screened in order to obtain a single mutant cosmid.

Having obtained cosmids with a 4 bp insertion in UL6, UL7, UL14, UL15 and UL17, attempts were made to produce mutant viruses by cotransfection of cosmid sets into BHK C13 cells. As described before, it was anticipated that, although only mutated DNA was transfected into cells, *wt* progeny would be produced if the mutant was severely disabled. Indeed, transfection of BHK C13 cells with mutant cosmid sets produced *wt* virus. The results obtained indicate that UL7, UL14, UL15 and UL17 are important for viral replication in cell culture, since only *wt* progeny was produced.

Candidate complementing cell lines which would facilitate growth of mutants with lesions in essential genes were produced by three different strategies. These were generated using either cos24 (containing genes UL1 to UL21), a fragment containing genes UL14 to UL18, or fragments containing UL14, UL15 or UL17 individually. Overall, 122 candidate cell lines were produced, and 40 were screened by transfection with the appropriate mutant cosmid sets. All of the progeny generated from these cell lines were *wt*. This indicates that the cell lines tested did not complement the defects. It could be that there was a low copy number of expressing plasmids per cell, such that only a small amount of protein was produced, not sufficient amounts to complement the defect, or that part of the plasmid may have been lost from the cells. It would have been beneficial to check for the presence of HSV-1 DNA by PCR in the possible complementing cell lines before transfecting them with the mutant cosmids sets, especially given the large number of cell lines.

Recently, a cell line expressing the UL15 protein was obtained by C. Cunningham from Dr J. Baines (Cornell University, New York) and used to generate several UL15$^-$ mutants. These mutants synthesise but do not cleave or package viral DNA. The availability of UL15$^-$ mutants will facilitate screening of the remainder of the cell lines produced during this work.

The UL15 expressing cell line (which may also express UL14) and the parental rabbit skin cell line have been tested for the ability to support the growth of virus generated from cosmids containing a UL14$^-$ mutation (C. Cunningham, personal communication). The UL14$^-$ virus produced extremely small plaques on both cell lines, suggesting that UL14 is not an essential gene but that mutants are severely disabled and that UL14$^-$ virus is not complemented by the UL15 expressing cell line. The mutant can be isolated from

these cells because monolayers survive in culture for extensive periods, whereas BHK C13 monolayers do not survive long enough to support the growth of the mutant.

It is now known that UL15 is an essential gene which requires a complementing cell line for isolation of mutants. Although UL14 is not essential, mutants are severely disabled and require cell lines which survive for long periods in cell culture for the isolation of mutants. UL17$^-$ viruses have not been isolated from any cell lines tested so far (including rabbit skin cells) and is probably essential. The situation with UL14 indicates that although some genes are strictly essential or non-essential in cell culture, some fall into neither category in that mutants affect growth properties in cell culture. For example, a UL51$^-$ mutant does not spread efficiently on confluent Vero cells but can spread, form plaques and destroy subconfluent cultures infected shortly after passage (Barker and Roizman, 1990). Also, Goldstein and Weller (1988) found that a UL39$^-$ mutant was more severely compromised in serum starved cells and suggested that UL39 is required in non-dividing cells. As another example, a UL20$^-$ mutant grows on human *tk*$^-$ cells but fails to produce plaques on Vero cells (Baines *et al.*, 1991).

5.3. Identification of proteins

Antibodies potentially recognising the UL17 or UL16 proteins were available in the laboratory and were used in Western blotting and immune precipitation experiments under a range of conditions. None reacted with convincing candidate proteins in infected cells or in virions.

Since the counterparts of the HSV-1 UL16 and UL17 proteins have been identified as minor components of EHV-1 virions (A.J. Davison, personal communication), mass spectrometric analysis of HSV-1 virions was carried out. Use of a UL47$^-$ virus revealed at least two faint bands usually obscured by this major tegument protein on polyacrylamide gels. The data suggested that these bands contain the UL17 and UL46 proteins. The use of a mutant lacking UL47 and UL46 may facilitate the conclusive identification of UL17 as a virion component. During the mass spectrometric work, analysis of a minor virion protein in the 20-40 kDa region revealed evidence for the presence of the UL14 protein plus the UL49 protein. The UL16 protein was not identified, despite extensive mass spectrometric analysis of virion proteins, and no difference was observed between the protein profiles of *wt* and UL16$^-$ virus on polyacrylamide gels.

Results from various rounds of Massmap analysis from a number of different experiments imply that UL17 and UL14 are virion components, but they are present in minor amounts and the analysis was carried out at the limits of detection. Confirmation of these proteins could be obtained by the use of appropriate antibodies.

Experiments involving pulse labelling of infected cells revealed differences between *wt* and mutants, and indicated a candidate UL16 protein. The UL16 protein produced *in vitro* did not correspond in mobility to a protein in infected cells labelled with [^{35}S]-cysteine or [^{35}S]-methionine, including the candidate protein which migrated a little more slowly. It is possible that the UL16 protein is modified differently during virus infection than in the *in vitro* system used, which utilised *E.coli* extracts. However, the presence of 20 cystine residues and only three methionine residues in the UL16 protein implies that the UL16 protein should be relatively more prominent in cystine-labelled cells than in methionine labelled cells. None of the proteins migrating in the vicinity of the candidate protein, including the candidate protein itself, exhibited labelling properties supporting its identity as the UL16 protein. Thus, the UL16 protein was not convincingly identified, suggesting that it is minor or comigrates with a major protein encoded by a different gene.

Since the completion of this work Dr J. Baines used an antibody to identify the UL16 protein as a virion component which comigrates with the UL49 protein (a major tegument protein) and is produced late in infection (personal communication). In future work, use of a UL49$^-$ mutant (if viable) may be of benefit in identifying the UL16 protein using mass spectrometric analysis. Recently, Wing *et al.* (1996) reported that the counterpart of UL16 in HCMV (UL94) encodes a capsid- or tegument-associated virion protein that is expressed with true late kinetics, by the use of a monoclonal antibody.

5.4. *In vitro* characterisation of UL16$^-$ virus

Six UL16$^-$ viruses (1a-d, 2a and 3a), two revertants (1b rev and 2a rev), *wt* viruses derived from the parental cosmid set (cos*wt*1 and 2) and *wt* HSV-1 were used in *in vitro* experiments.

The production of three to five-fold less infectious virus by mutants 1a-d on BHK C13 cells at high and low moi is consistent with the results of Baines and Roizman (1991), who reported a ten-fold reduction in the yield of a UL16 deletion mutant on Vero cells and a three-fold reduction on BHK C13 cells. Mutant 2a was 15,000-fold reduced in yield compared with *wt*, while mutant 3a, which contains all but the last two codons of

UL16, was indistinguishable from *wt*. Mutants 1a and 1b grew to about 13-19% of the titre of *wt* on four cell lines, confirming that the yield reduction is not specific to BHK C13 cells. The yield was somewhat less (5-9%) on two other cell lines. The extremely low yield of 2a is surprising, since the truncated protein contains a larger portion of the UL16 protein (208 residues of the 373 residue full length protein) than mutants 1a-d (66 residues). It is possible that the truncated protein interferes with the normal function of UL16 or that the mutant has a secondary mutation.

Consistent with reduced viral yields, most UL16$^-$ mutants produced smaller plaques than *wt* virus on BHK C13 cells. Mutants 1a-d produced plaques which were 20-27% the size of *wt* plaques, 2a was 4% the size of *wt* while 3a was the same size as *wt*. On MeWo cells, 1a and 1b plaques were 50 % the size of *wt* plaques. The small plaque size was shown to be linked to the mutations introduced into UL16 by the analysis of several distinct mutants with lesions at the same site and by the use of revertants, which produced plaques of *wt* size.

Particle counts (Section 4.2.2.4) showed that cells infected with mutants 1a and 1b released a similar number of particles to *wt*, but that fewer were infectious (i.e. they displayed a high particle:pfu ratio). The mutants appeared to be slower to produce virions early in infection (Section 4.2.2.5) but produced as many particles as *wt* virus later in infection. This is consistent with the growth curves where the mutants are slower to initiate growth at the first stages of infection.

Mutants 1a and 1b did not bind to cells as well as *wt*, which may explain the small plaques and lag in growth, but were able to adsorb and penetrate cells as efficiently as *wt* (no significant differance) and are not significantly temperature sensitive or cell type-specific. There was no evidence that UL16 is involved in inducing IFN resistance, despite the report by Su *et al.* (1993) who mapped a region of the HSV-1 genome conferring IFN resistance to UL14, UL15 and UL16. It should be noted that the work of Su *et al.* (1993) involved the use of intertypic recombinants between HSV-1 and HSV-2, and thus it is possible that the effect they observed was due to secondary mutations. Also, it is possible that IFN resistance may be multifactorial (i.e. other genes may be involved).

Although UL16 is non-essential for virus growth in cell culture, the mutants yield less infectious virus and produce smaller plaques than *wt*. HSV-1 mutants with a small plaque phenotype have been described, including, the Vmw65 mutant *in*1814 (Ace *et al.*, 1989), which has a high particle to pfu ratio and is avirulent but can establish and

reactivate from latency, an RL1$^-$ mutant which produces smaller plaques on BHK C13 cells and is avirulent (Brown *et al.*, 1994), and a UL39$^-$ mutant which yields 4-5 fold less virus and is avirulent (Goldstein and Weller, 1988c).

The function of the UL16 protein is unknown. Mutants produce the same number of virus particles as *wt* but fewer are infectious, they yield less infectious virus and produce smaller plaques. It is possible that the UL16 protein is involved at a number of stages in the replicative cycle. It may act early in infection since although mutants are able to adsorb as efficiently as *wt* virus, they bind and penetrate cells slightly less well. It may also have a role in the later stages of infection during the formation of the mature virions, since the UL16 protein has been identified as a virion component (Dr J. Baines).

5.5. *In vivo* characterisation of UL16$^-$ virus

There is considerable interest in the use of mutants to identify virulence factors. However, there are inherent difficulties in interpreting results from animal models since variation may occur due to differences in the age and general state of health of the animals, factors which may influence host immune responses. There are several important criteria which should be met when analysing mutants in animal models. These include the use of independent mutants and revertants, repetition of experiments, and the determination of the genotype of viruses replicating or reactivated from ganglia.

5.5.1. Neurovirulence

A number of workers have linked small plaque size to avirulence (see above), and some genes which have been shown to be non-essential in cell culture are important in neurovirulence and latency *in vivo* (see Table 12 for details).

In neurovirulence experiments, cos*wt*1 was as virulent as *wt* virus, and a firm indication was obtained that mutants with a lesion at position 31096 bp in UL16 have reduced neurovirulence. This has been directly linked to the mutation in UL16 by the use of a revertant which is as virulent as cos*wt*1. Genotypic analysis revealed that, although they were less virulent, mutants were able to replicate in the brains of mice.

In a pilot experiment mutants 1a and 1b were found to be less virulent that *wt* but to different extents (1000- and 10-fold less than *wt* respectively). Since these mutants have a 4 bp insertion at the same site in UL16 it was expected that they would behave in a similar manner. In a second experiment, however, this difference was not observed and

the mutants (1a, 1b, 1d) were all attenuated to a similar extent (500-1000-fold), indicating that there was a degree of variation in these experiments. The variation may have been due to factors such as the age and general health of the animal, variation in the inoculation proceedure and the conditions in which the animals are maintained. Also, the scoring of disease, which influences when animals are killed, is subjective.

5.5.2. Latency

Mutants 1a-d, 2a and 3a and 1b established and reactivated from latency less efficiently than *wt* virus following FP injection and explantation and cocultivation of ganglia.

In an initial experiment, all of the *wt* viruses reactivated by 6 days post explantation. Mutants 1a and 1b reactivated much more slowly than *wt* but reached 90-95% reactivation. The genotypes of a selection of reactivated viruses picked at a late stage of reactivation (10-12 days) were determined, and all but one were found to be revertants to *wt*. The other was a mixture of mutant and revertant virus. These results suggest that UL16 has a role in efficient establishment of or reactivation from latency.

In a second experiment all of the mutants were tested. Cos*wt*1 and cos*wt*2 reactivated within accepted levels for *wt* and all of the mutants reactivated slowly and reached a lower final level of reactivation. The genotypes of reactivating viruses arising early during reactivation (4-6 days) were *wt* except for mutant 1a. Plaques from 1a at later stages of reactivation (10-12 days) were found to consist of revertants (2 plaques), mutant (1plaque) and a mixture of revertant and mutant (1 plaque). These results suggest that although mutant 1a is able to establish latent infections and reactivate, it does so at a lower efficiency than *wt*. The slower kinetics of reactivation of the UL16$^-$ mutants indicates that the majority of reversion occurred during reactivation and that the UL16$^-$ mutants must be at a selective disadvantage to *wt*.

Detection of virus directly in the ganglia by PCR would aid further investigation of the block in establishment and reactivation of latent infection, as would the use of a mutant with a small deletion in UL16 since it would be unable to revert to *wt*.

5.6. Analysis of mutants using animal models

Many of the published data do not meet one or more of the important criteria required to minimise variation (see Section 5.5), and they also differ from each other in the type of animal model used, the site of inoculation, strain of virus, type of mutant and the titre of virus inoculated (see Table 10 for details). These factors combine to make it difficult to interpret results.

The work by Pyles and Thompson (1994) investigating UL2$^-$ mutants is the most comprehensive *in vivo* study in recent literature. They used two insertion mutants and a single revertant and they determined the genotype of reactivated viruses. In a contrasting study, Balan *et al.* (1994) produced a single insertion mutant in US7 and another in US8 but did not produce revertants, and Efstathiou *et al.* (1989) investigated UL23 using a single deletion mutant without a revertant. Although deletion mutants cannot revert to *wt*, they are not without potential problems. For example, the UL50$^-$ deletion mutant tested by Pyles *et al.* (1992) had also lost the UL49A promoter and so the phenotype attributed to loss of UL50 may have been affected by loss of UL49A expression.

In other studies, UL39$^-$ and UL40$^-$ mutants were tested in three systems using mice or guinea pigs. In one, a revertant was produced confirming the avirulence of the mutant (Cameron *et al.*, 1988), but in a second, although the mutant was less virulent than *wt*, the revertant was still reduced in virulence compared to *wt* virus (Jacobson *et al.*, 1989a), while in a third study using guinea pigs, no impairment in virulence was reported (Turk *et al.*, 1989). Thus different conclusions can be drawn from different animal models.

Intertypic recombinants or drug resistant mutants have been used in animal models, but may be less reliable than insertion or deletion mutants since they may contain secondary mutations. It is of particular importance to produce revertants in these cases. Intertypic and drug resistant UL30$^-$ mutants have been used in the mouse eye model (Oakes *et al.*, 1986; Day *et al.*, 1987a, b) but revertants were not produced. An intertypic recombinant used in a mouse model indicated that UL56 plays a role in neurovirulence (Becker *et al.*, 1986; Rosen *et al.*, 1986), but, a UL56$^-$ insertion mutant tested in the mouse showed that this gene is not necessary for intraperitoneal virulence or for the establishment and reactivation from latency (Nash and Spivak, 1994). This again indicates the variable results which may be obtained by the use of different kinds of mutants and of different sites of inoculation.

Since many workers do not comply with the basic criteria required to minimise variation in animal experiments, results in the literature must be viewed cautiously. In this work,

an attempt has been made to carry out the analysis of the UL16$^-$ mutants *in vivo* using more than one mutant in more than one experiment, producing a revertant and by analysing the genotype of reactivated viruses. The results show that UL16 plays a role in neurovirulence and in latency.

References

Abbotts, A.P and Stow, N.D., (1995). The origin-binding domain of the herpes simplex virus type 1 UL9 protein is not required for DNA helicase activity. *J. Gen. Virol.* **76**, 3125-3130.

Abgharis, S.Z. and Stulting, R.D. (1988). Recovery of herpes simplex virus from ocular tissues of latently infected inbred mice. *Invest. Opthamol. Visual Sci.* **29**, 239-243.

Ackermann, M., Braun, D.K., Pereira, L. and Roizman, B. (1984). Characterisation of herpes simplex virus type 1 α proteins 0, 4 and 27 with monoclonal antibodies. *J. Virol.* **52**, 108-118.

Ace, C.I., McKee, T.A., Ryan, J.M., Cameron, J.M. and Preston, C.M. (1989). Construction and characterisation of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. *J. Virol.* **63**, 2260-2269.

Addison, C., Rixon, F.J., Palfreyman, J.W., O'Hara, M. and Preston, V.G. (1984). Characterisation of a herpes simplex virus type 1 mutant which has a temperature-sensitive defect in penetration of cells and assembly of capsids. *Virology* **138**, 246-255.

Addison, C., Rixon, F.J. and Preston. V.G. (1990). Herpes simplex virus type 1 UL28 gene product is important for the formation of mature capsids. *J. Gen. Virol.* **71**, 2377-2384.

Ahn, K., Meyer, T.H., Uebel, S., Sempe, P., Djaballah, H., Yang, Y., Peterson, P.A., Fruh, K. and Tampe, R. (1996). Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus protein ICP47. *EMBO. J.* **15**, 3247-3255

Albrecht, J.C., Nicholas, J., Biller, D., Cameron, K.R., Biesinger, B., Newman, C., Witmann, S., Craxton, M.A., Coleman, H., Kleckenstein, B. and Honess, R.W. (1992). Primary structure of the herpesvirus saimiri genome. *J. Virol.* **66**, 5047-5058.

Alford, C.A. and Britt, W.J. (1990). Cytomegalovirus. In: *Virology* (2nd edn), pp 1981-2010. B.N. Fields, D.M. Knipe, R.M. Chanode, M.S. Hirsch, J.L. Melnick, T.P. Monath and B. Roizman (eds), Raven press New York.

Ali, M.A., Forghani, B. And Cantin, E.M. (1996). Characterisation of an essential HSV-1 protein encoded by the UL25 gene reported to be involved in virus penetration and capsid assembly. *Virology* **216**, 278-283.

Al-Kobaisi, F.M., Rixon, F.J., McDougall, I. and Preston, V.G. (1991). The herpes simplex virus UL33 gene product is required for the assembly of full capsids. *Virology* **180**, 380-388.

Ananvoranich, S., Misra, V., Trudel, M. and Simard, C. (1995). The generation of a cosmid-based system for the construction of mutants of bovine herpesvirus type 1. Abstract 6, 20th International Herpesvirus Workshop, Groningen, The Netherlands.

Arbuckle, M.I. and Stow, N.D. (1993). A mutational analysis of the DNA-binding domain of the herpes simplex virus type 1 UL9 protein. *J. Gen. Virol.* **74**, 1349-1355.

Asher, Y. Heller, M. and Becker, Y. (1969). Incorporation of lipids into herpes simplex virus particles. *J. Gen. Virol.* **4**, 65-76.

Aurelian, L and Roizman, B. (1964). The host range of herpes smplex virus. Interferon, viral DNA, and antigen synthesis in abortive infection of dog kidney cells. *Virology* **22**, 452-461.

Baer, R., Bankier, A., Biggin, M., Deininger, P.,Farrell, P., Gibson, T., Hatfull, G., Hudson, G., Satchwell, S., Sequin, C., Tuffnell, P and Barrell, B. (1984). DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature* **310**, 207-211.

Baines, J.D. and Roizman, B. (1991). The open reading frames UL3, UL4, UL10 and UL16 are dispensible for the replication of herpes simplex virus 1 in cell culture. *J. Virol.* **65**, 938-944.

Baines, J.D. and Roizman, B. (1992a). The cDNA of U_L15, a highly conserved herpes simplex virus 1 gene, effectively replaces the two exons of the wild-type virus. *J. Virol.* **66**, 5621-5626.

Baines, J.D. and Roizman, B. (1992b). The UL11 gene of herpes simplex virus 1 encodes a function that facilitates nucleocapsid envelopment and egress from cells. *J. Virol.* **66**, 5160-5174.

Baines, J.D., Koyama, A.H., Huang, T.M. and Roizman, B. (1994). The U(L)21 gene-products of herpes simplex virus type 1 are dispensible for growth in cultured cells. *J. Virol.* **68**, 2929-2936.

Baines, J.D., Poon, A.P.W., Rovnak, J. and Roizman, B. (1994). The herpes simplex virus 1 UL15 gene encodes 2 proteins and is required for cleavage of genomic viral DNA. *J. Virol.* **68**, 8118-8124.

Baines, J.D., Ward, P.L., Campdelli-Fiume, G. and Roizman, B. (1991). The U_L20 gene of herpes simplex virus 1 encodes a function necessary for viral egress. *J. Virol.* **65**, 6414-6424.

Balan, P., Davis-Poynter, N., Bells, S., Atkinson, H., Browne, H. and Minson, A. (1994). An analysis of the *in vitro* and *in vivo* phenotypes of mutants of herpes simplex virus type 1 lacking glycoproteins gG, gE, gI, or the putative gJ. *J. Gen. Virol.* **75**, 1245-1258.

Banatvala, J.E., Best, J.M. and Waller, D.K. (1972). Epstein-Barr virus-specific IgM in infectious mononucleosis, Burkitt lymphoma anfd nasopharyngeal carcinoma. *Lancet* **1** 1205-1208.

Barker, D.E. and Roixman, B. (1990). Identification of 3 genes non-essential for growth in cell culture near the right terminus of the unique sequence of the long component of herpes simplex virus 1. *Virology* **177**, 684-691.

Barker, D.E. and Roizman, B. (1992). The unique sequence of the herpes simplex virus 1 L component contains an additional translated open reading frame designated UL49.5. *J. Virol.* **66**, 562-566.

Barnett, B.C., Dolan, A., Telford, E.A., Davison, A.J. and McGeoch, D.J. (1992). A novel herpes simplex virus gene (UL49A) encodes a putative membrane protein with counterparts in other herpesviruses. *J. Gen. Virol.* **73**, 2167-2171.

Batchelor, A.H. and O'Hare, P. (1990). Regulation and cell-type-specific activity of a promoter located upstream of the latency-associated transcript of herpes simplex virus type 1. *J. Virol.* **64**, 3269-3279.

Batterson, W., Furlong, D. and Roizman, B. (1983). Molecular genetics of herpes simplex virus. VIII: Further characterisation of a temperature sensitive mutant defective in release of viral DNA and in other stages of the viral replicative cycle. *J. Virol.* **45**, 397-407.

Bayliss, G.J., Marsden, H.S. and Hay, J. (1975). Herpes simplex virus proteins. DNA binding proteins in infected cells and in the virus structure. *Virology* **68**, 124-134.

Bean, M.A., Bloom, B.R., Heberman, R.B., Old, L.J., Oettgen, A.F., Klein, G. And Terry, W.D. (1975). Cell-mediated cytotoxicity for bladder carcinoma: Evaluation of a workshop. *Cancer Res.* **33**, 2902-2913.

Becker, Y., Hadar, J., Tabor, E., Ben-Hur, T., Raibstein, I., Rosen, A. and Darai, G. (1986). A sequence in *Hpa*I-P fragment of herpes simplex virus-1 DNA determines intraperitonal virulence in mice. *Virology* **149**, 255-259.

Beers, D.R., Henkel, J.S. and Stroop, W.G. (1994). Herpes simplex viruses. In: *Handbook of neurovirology*, Chr 17. Edited by R.R. McKendall, W.G. Stroop and Dekker.

Ben-Hur, T., Asher, Y., Tabor, E., Darai, G. and Becker, Y. (1987). HSV-1 virulence for mice by the intracerebral route is encoded by the *Bam*HI-L DNA fragment containing the cell fusion gene. *Arch. Virol.* **96**, 117-122.

Ben-Hur, T., Hadar, J., Shtram, Y., Gilden, D.H. and Becker, Y. (1983). Neurovirulence of herpes simplex virus type 1 depends on age of mice and thymidine kinase expression. *Arch. Virol.* **78**, 303-308.

Ben-Porat, T., Rixon, F.J. and Blakenship, M.L. (1979). Analysis of the structure of the pseudorabies virus. *Virology* **95**, 285-294.

Berneman, Z.N., Saxinger, C., Hall, L., Gallo, R.C. and Reitz, M.S. (1995). Sequence-analysis and mapping of the genome of human herpesvirus 7 (HHV-7). *Aids Research and human retroviruses* **11**, 8101.

Berneman, Z.N., Ablashi, D.V., Li, G., Eger-Fletcher, M., Reitz Jr, M.S., Hung, C.-L., Brus, I., Komaroff, A.L. and Gallo, R.C. (1992). Human herpesvirus 7 is a T-lymphotropic virus and is related to, but significantly different from, human herpesvirus 6 and human cytomegalovirus. *Proc. Natl. Acad. Sci.* USA **89**, 10552-10556.

Bhattachorgga, S.P. and Rao, V.B. (1993). A novel terminase activity associated with the DNA packaging protein gp17 of bacteriophage T4. *Virology* **196**, 34-44.

Block, T., Jordan, R., Farkas, D.H. and Hughes, R.G. (1991). Inhibition of transient gene expression with plasmids encoding herpes simplex virus type 1 UL55 and alpha genes. *J. Gen. Virol.* **72**, 131-141.

Block, T.M., Spivack, J.G., Steiner, I., Deshmane, S., McIntosh, M.T., Lirette, R.P. and Fraser, N.W. (1990). A herpes simplex virus type 1 latency-associated transcript mutant reactivates with normal kinetics from latent infection. *J. Virol.* **64**, 3417-3426.

Bloom, D.C. and Stevens, J.C. (1994). Neuron-specific restriction of a herpes simplex virus recombinant maps to the UL5 gene. *J. Virol.* **68**, 3761-3772.

Bolovan, C.A., Sawtell, N.M. and Thompson, R.L. (1994). ICP34.5 mutants of herpes simplex virus type 1 strain 17syn+ are attenuated for neurovirulence in mice and for replication in confluent primary mouse embryo cell cultures. *J. Virol.* **68**, 48-55.

Booy, F.P., Newcomb, W.W., Trus, B.L., Brown, J.C., Baker, T.S. and Steven, A.C. (1991). Liquid-crystalline, phage-like packing of encapsidated DNA in herpes simplex virus. *Cell* **64**, 1007-1015.

Booy, F.P. Trus, B.L., Davison, A.J. and Steven, A.C. (1996). The capsid archetecture of Channel catfish virus, an evolutionarily distinct herpesvirus, is largely conserved in the absence of discernible sequence homology with herpes simplex virus. *Virology* **251**, 134-141.

Bornkamm, G.W., Delius, H., Leckenstein, F., Werner, F-J. and Mulder, C. (1976). Structure of herpesvirus saimiri genomes: Arrangement of heavy and light sequences in the M genome. *J. Virol.* **19**, 154-161.

Branca, A.A. and Baglioni, C. (1981). Evidence that types I and II interferons have different receptors. *Nature* **249**, 768-770.

Braun, D.K., Batterson, W. and Roizman, B. (1984). Identification and genetic mapping of a herpes simplex virus capsid protein that binds DNA. *J. Virol.* **50**, 645-648.

Breul, S.D., Bradley, K.H., Hance, A.J., Schaffer, M.P., Berg, R.A. and Crystal, R.G. (1980). Control of collagen production by human diploid lung fibroblasts. *J. Biol. Chem.* **255**, 5250-5260.

Brown, S.M. and Harland, J. (1987). Three mutants of herpes simplex virus type 2: one lacking the genes US 10, 11 and 12 and two in which RS has been extended by 6 kb to 0.91 map units with a loss of US sequences between 0.94 and the US/TRS junction. *J. Gen. Virol.* **68**, 1-18.

Brown, S.M., MacLean, A.R., Atken, J.D. and Harland, J. (1994). ICP34.5 influences herpes simplex virus type 1 maturation and egress from infected cells *in vitro*. *J. Gen. Virol.* **75**, 3679-3686.

Browne, H., Bell, S., Minson, T. and Wilson, D.W. (1996). An endoplasmic reticulum retained herpes simplex virus glycoprotein H is absent from secreted virions: evidence for re-envelopment during egress. *J. Virol.* **70**, 4311-4316.

Buckmaster, E.A., Scott, S.D., Saunderson, M.J., Bourshell, S.M. and Ross, N.L.J. (1988). Gene sequence mapping data from Mareck's disease virus and herpesvirus of turkey's: Implications for herpesvirus classification. *J. Gen.Virol.* **69**, 2033-2042.

Cai, W., Astor, T.L., Liptak, L.M., Cho, C., Coen, D.M. and Schaffer, P.A. (1993). The herpes simplex virus type 1 regulatory protein ICP0 enhances viral replication during acute infection and reactivation from latency. *J. Virol.* **67**, 7501-7512.

Cai, W., Gu, B. and Person, S. (1988). Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. *J. Virol.* **62**, 2596-2604.

Cai, W., Gu, B. and Schaffer, P.A. (1989). Herpes simplex virus type 1 ICP0 plays a critical role in the *de novo* synthesis of infectious virus following transfection of viral DNA. *J. Virol.* **63**, 4579-4589.

Cai, W., Gu, B. and Schaffer, P.A. (1992). Herpes simplex virus type 1 ICP0 regulates expression of immediate early, early and late genes in productively infected cells. *J. Virol.* **66**, 2904-2915.

Cai, W, Person, S., Warner, S.C., Zhou, J. and DeLuca, N.A. (1987). Linker-insertion nonsense and restriction-site deletion mutants of the gB glycoprotein gene of herpes simplex virus type 1. *J. Gen. Virol.* **61**, 714-721.

Calder, J.M. and Stow, N.D. (1990). Herpes simplex virus helicase-primase: The UL8 protein is not required for DNA dependent ATPase and DNA helicase activities. *Nucl. Acids Res.* **18**, 3573-3578.

Calder, J.M., Stow, E.C. and Stow, N.D. (1992). On the cellular localisation of the components of the herpes simplex virus type 1 helicase-primase complex and the viral origin-binding protein. *J. Gen. Virol.* **73**, 531-538.

Cameron, J.M., McDougall, I., Marsden, H.S., Preston, V.G., Ryan, M.D. and Subak-Sharpe, J.H. (1988). Ribonucleotide reductase encoded by herpes simplex virus is a determinant of the pathogenicity of the virus in mice and a valid antiviral target. *J. Gen. Virol.* **69**, 2607-2612.

Campadelli-Fiume, G. (1994). Herpes simplex viruses: Viral glycoproteins. In: *Encyclopedia of virology*, vol 2. Edited by R.G. Websrer and A. Granoff. Academic press.

Campadelli-Fiume, G., Avitabile, E., Fini, S., Stirpe, D., Arsenakis, M. and Roizman, B. (1988). Herpes simplex virus glycoprotein D is sufficient to induce spontaneous pH-independent fusion in a cell line that constitutively express the glycoprotein. *Virology* **166**, 598-602.

Campione-Piccardo, J., Rawls, W.E. and Bacchetti, S. (1979). Selective assay for herpes simplex virus expressing thymidine kinase. *J. Virol.* **31**, 281-287.

Caradonna, S.J. and Cheung, Y.C. (1981). Induction of uracil-DNA glucosylase and dUTP nucleotidyltransferase activity in herpes simplex virus infected human cells. *J. Biol. Chem.* **256**, 9834-9837.

Caradonna, S., Worrad, D. and Lirette, R. (1987). Isolation of a herpes simplex virus cDNA encoding the DNA repair enzyme uracil-DNA glycosylase. *J. Virol.* **61**, 3040-3047.

Carmichael, E.P. and Weller, S.K. (1989). Herpes simplex virus type-1 DNA-synthesis requires the product of the UL8 gene - isolation and characterisation of an ICP6-*LacZ* insertion mutant. *J. Virol.* **63**, 591-599.

Carrascose, J.L. and Kellenberge, R E. (1978). Head maturation pathwayof bacteriophage T4 and T2. III. Isolation and characterization of particles produced by mutants in gene 17. *J. Virol.* **25**, 831-844.

Cesarman, E., Chang, Y.A., Moore, P.S., Said, J.W. and Knowles, D.M. (1995). Kaposis scrcoma-associated herpesvirus-like DNA-sequences in AIDS-related body-cavity-based lymphomas. *New Eng. J. Med.* **332**, 1186-1191.

Chang, Y., Cesarman, E., Knowles, D. and Moore, P. (1995). Identification and partial characterisation of a new human herpesvirus associated with Kaposis-sarcoma. *Aids Res. Human Retroviruses.* **11**, s68.

Chang, Y., Cesarman, E., Pessin, M.S., Lee, F., Culpepper, J., Knowles, D.M. and Moore, P.S. (1994). Identification of herpesvirus-like DNA-sequences in AIDS-associated Kaposis-sarcoma. *Science* **266**, 1865-1869.

Chartrand, P., Crumpacker, C.S., Schaffer, P.A. and Wilkie, N.M. (1980). Physical and genetic analysis of the herpes simplex virus DNA polymerase locus. *Virology* **103**, 311-326.

Chatterjee, S., Burns, P. and Koga, J. (1995). Effect of protein kinase C inhibitors on the antiviral activity of human alpha interferon on herpes simplex virus-infected human neiroblastoma cells. *J. Virol.* **69**, 1315-1318.

Chee, M.S., Bankier, A.T., Beck, S., Bohni, R., Brown, C.M., Cerng, R., Horsnell, T., Hutchison, C.A., Kouzaires, T., Martignetti, A.J., Preddie, E., Satchwell, S.C., Tomlinson, P., Weston, K.M. and Barrell, B.G. (1990). Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. In: *Current topics in microbiology and immunology*. **154**, 125-169. J.K. McDougall (ed). Springer-Verlog, Berlin/Heidelberg.

Chen, S., Mills, L., Perry, P., Riddel, S., Wobig, R., Lown, R. and Millette, R.L. (1992). Transactivation of the major capsid protein gene of herpes simplex virus type 1 requires a cellular transcription factor. *J. Virol.* **66**, 4304-4314.

Chen, J., Zhu, X. and Silverstein, S. (1991). Mutational analysis of the sequence encoding ICP0 from herpes simplex virus type 1. *Virology* **180**, 207-220.

Chou, J. and Roizman, B. (1985). Isomerisation of herpes simplex virus 1 genome: identification of *cis*-acting and recombination sites within the domain of the *a* sequence. *Cell* **41**, 803-811.

Chou, J. and Roizman, B. (1989). Characterisation of a DNA sequence-common and sequence-specific proteins binding to *cis*-acting sites for cleavage of the terminal *a* sequence of the herpes simplex virus 1 genome. *J. Virol.* **63**, 1059-1068.

Chou, J. and Roizman, B. (1990). The herpes simplex virus 1 gene for ICP34.5 which maps in the inverted repeats is conserved in limited-passage isolates but not in strain 17 syn. *J. Virol.* **64**, 1014-1020.

Chou, J. and Roizman, B. (1992). The α1 34.5 gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells. *Proc. Natl. Acad. Sci. USA* **89**, 3264-3270.

Chou, J. and Roizman, B. (1994). Herpes simplex virus 1 α1 34.5 gene function, which blocks the host response to infection, maps in the homologous domain of the genes expressed during growth arrest and DNA damage. *Proc. Natl. Acad. Sci. USA* **91**, 5247-5252.

Chou, J., Kern, E.R., Whitley, R.J. and Roizman, B. (1990). Mapping of herpes simplex virus-1 neurovirulence to α1 34.5, a gene non-essential for growth in culture. *Science 250*, 1262-1265.

Chousterman, S., Lacasa, M and Sheldrick, P. (1979). Physical map of the channel catfish virus genome: Location of sites for restriction endonucleases *Eco*R I, *Hind* III, *Hpa* I and *Xba* I. *J. Virol.* **31**, 73-85.

Clements, J.B. and Stow, N.D. (1989). A herpes simplex virus type 1 mutant containing a deletion within immediate early gene 1 is latency-competent in mice. *J. Gen. Virol.* **70**, 2501-2506.

Clements, J.B. and Subak-Sharpe, J.H. (1988). Herpes simplex virus type 2 establishes latency in the mouse footpad. *J. Gen. Virol.* **89**, 375-383.

Clements, J.B., Watson, J.R. and Wilkie, N.M. (1977). Temporal regulation of herpes simplex virus type 1 transcription: location of transcripts on the viral genome. *Cell* **12**, 275-285.

Coen, D.M., Aschman, D.P., Gelep, P.T., Retando, M.J., Weller, S.K. and Schaffer, P.A. (1984). Fine mapping and molecular cloning of mutations in the herpes simplex virus DNA polymerase locus. *J. Virol.* **49**, 236-247.

Coen, D.M., Bogarel, C.L., Kosz-Vnenchak, M., Jacobson, J.G., Leib, D.A., Schaffer, P.A., Tyler, K.L. and Knipe, D.M. (1989). Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia, but do not reactivate. *Proc. Natl. Acad. Sci. USA* **86**, 4736-4740.

Coen, D.M., Furman, P.A., Gelep, P.T. and Schaffer, P.A. (1984). Mutations in the herpes simplex virus DNA polymerase gene can confer resistance to 9-β-D-arabinofuranosyladenine. *J. Virol.* **41**, 909-918.

Coen. D.M., Kosz-Vnenchak, M., Jacobson, J.G., Leib, D.A., Bogart, C.L., Schaffer, P.A., Tyler, K.L. and Knipe, D.M. (1989). Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia but do not reactivate. *Proc. Natl. Acad. Sci. USA* **86**, 4736-4740.

Coen, D.M., Winheimer, S.D. and McKnight, S.L. (1986). A genetic approach to promotor recognition during trans-induction of viral gene expression. *Science* **234**, 53-59.

Cohen, J.I. and Seidel, K.E. (1993). Generation of varicella-zoster virus (VZV) and viral mutants from cosmid DNAs: VZV thymidylate synthetase is not essential for replication *in vitro. Proc. Natl. Acad. Sci. USA* **90**, 7376-7380.

Comps, M. and Cochennec, N. (1993). A herpes-like virus from the European oyster ostrae-edulis L. *J. Invertebrate Pathology* **62**, 201-203.

Conley, A.F., Knipe, D.M., Jones, P.C. and Roizman, B. (1981). Molecular genetics of herpes simplex virus. VII. Characterisation of a temperature-sensitive mutant produced by *in vitro* mutagenesis and defective in DNA synthesis and accumulation of γ peptides. *J. Virol.* **37**, 191-206.

Cook, S.D. and Brown, S.M. (1987). Herpes simplex virus type 1 latency in rabbit corneal cells *in vitro*: reactivation and recombination following intratypic superinfection of long term cultures. *J. Gen. Virol.* **68**, 813-824.

Cook, S.D., Hill, J.M., Lynas, C. and Maitland, N.J. (1991b). Latency-associated transcripts in cornea and ganglia of HSV-1 infected rabbits. *Br. J. Ophthalmol.* **75**, 644-648.

Cook, S.D., Paveloff, M.J., Doucer, J.J., Cottingham, A.J., Sedarti, F. and Hill, J.M. (1991a). Ocular herpes simplex virus reactivation in mice latently infected with latency-associated transcript mutants. *Invest. Ophthalmol. Vis. Sci.* **32**, 1558-1561.

Costa, R.H., Draper, K.G., Devi-Rao, G., Thomson, R.L. and Wagner, E.K. (1985). Virus-induced modification of the host cell is required for expression of the bacterial chloramphenicol acetyltransferase gene controlled by a late herpes simplex virus protein (VP5). *J. Virol.* **56**, 19-30.

Coulter, L.J., Moss, H.W.M., Lang, J. And McGeoch, D.J. (1993). A mutant herpes simplex virus type 1 in which the UL13 protein-kinase gene is disrupted. *J. Gen. Virol.* **74**, 387-395.

Cousens, D.J., Greaves, R., Goding, C.R. and O'Hare, P. (1989). The C-terminal 79 amino acids of the herpes simplex virus regulatory protein, Vmw65, efficiently activate transcription in yeast and mammalian cells in chimeric DNA-binding proteins. *EMBO J.* **8**, 2337-2342.

Crute, J.J. and Lehman, I.R. (1989). Herpes simplex-1 DNA polymerase. Identification of an intrinsic 5'-3' exonuclease with ribonuclease H activity. *J. Biol. Chem.* **426**, 19266-19270.

Crute, J.J., Mocarski, E.S. and Lehman, I.R. (1988). The DNA helicase induced by herpes simplex virus type 1. *Nucl. Acids Res.* **16**, 6585-6596.

Crute, J.J., Tsurmi, T., Zhu, L, Weller, S.K. Olivo, P.D., Challberg, M.D., Mocarski, E.S. and Lehman, I.R. (1989). Herpes simplex virus type 1 helicase-primase. A complex of three herpes-encoded gene products. *Proc. Natl. Acad. Sci. USA* **86**, 2186-2189.

Cunningham, C., Davison, A.J., Dolan, A., Frame, D.J., McGeoch, D.J., Meredith, D.M., Moss, H.W.M. and Orr, A.C. (1992). The UL13 virion protein of herpes simplex virus type 1 is phosphorylated by a novel virus induced protein kinase. *J. Gen. Virol.* **73**, 303-311.

Cunningham, C. and Davison, A.J. (1993). A cosmid-based system for constructing mutants of herpes simplex virus type 1. *Virology* **197**, 116-124.

Curtin, K.D. and Knipe, D.M. (1993). Altered properties of the herpes simplex virus ICP8 DNA-binding protein in cells infected with ICP27 mutants. *Virology* **196**, 1-14.

Dambaugh, T., Beisel, C., Hummel, M., King, W., Fennewald, S., Cheung, A., Heller, M., Raab-Traub, N. and Kieff, E. (1980). Epstein -Barr virus (B95-8) DNA. VII: Molecular cloning and detailed mapping. *Proc. Natl. Acad. Sci. USA* 77, 2999-3003.

Dargan, D.J. and Suback-Sharpe, J.H. (1991). The difference in sensitivity to cycloxolone sodium between herpes simplex virus types 1 and 2 maps to the locations of genes UL22 (gH) and UL44, (gC). *J. Gen. Virol.* **72**, 377-384.

Davido, D.J. and Leib, D.H. (1996). Role of *cis*-acting sequences of the ICP0 promoter of herpes simplex virus type 1 in viral pathogenesis, latency and reactivation. *J. Gen. Virol.* 77, 1858-1863.

Davison, A.J. (1984). Structure of the genomic termini of varicella-zoster virus. *J. Gen. Virol.* **65**, 1969-1977.

Davison, A.J. (1992). Channel Catfish virus: A new type of herpesvirus. *Virology* **186**, 9-14.

Davison, A.J. (1993). Herpesvirus genes. *Rev. Med. Virol.* **3**, 237-244.

Davison, A.J. and Clements, J.B. (1996). Herpesviruses: General properties. In: *Topley and Wilsons, Microbiology and Microbial Infections*, 9th edition. Edited by L. Collier. In press.

Davison, A.J. and Davison, M.D. (1995). Identification of structural proteins of channel catfish virus by mass spectrometry. *Virology* **206**, 1035-1043.

Davison, A.J. and McGeoch, D.J. (1995). Herpesviridae. In: *Molecular evolution of viruses*. A.J. Gibbs, F. Garcia-Arenal, C. Callisher eds. Cambridge University press.

Davison, A.J. and Scott, J.E. (1986). The complete DNA sequence of Varicella-zoster virus. *J. Gen. Virol.* **67**, 1754-1816.

Davison, A.J. and Wilkie, N.M. (1981). Nucleotide sequence of the joint between the L and S segments of herpes simplex virus types 1 and 2. *J. Virol.* **55**, 315-331.

Davison, A.J. and Wilkie, N.M. (1983). Inversion of the 2 segments of the herpes simplex virus genome in intertypic recombinants. *J. Gen. Virol.* **64**, 1-18.

Day, S.P., Lausch, R.N. and Oakes, J.E. (1987a). Nucleotide sequences important in DNA replication are responsible for differnces in capacity of two herpes simplex virus strains to spread from the cornea to central nervous system. *Curr. Eye Res.* **6**, 19-26.

Day, S.P., Lausch, R.N. and Oakes, J.E. (1987b). Evidence that the gene for herpes simplex virus type 1 DNA polymerase accounts for the capacity of an intertypic recombinant to spread from eye to central nervous system. *Virology* **163**, 166-173.

Deatly, A.M., Spivack, J.G., Lavi, E, O'Boyle, D.R. and Fraser, N.W. (1988). Latent herpes simplex virus type 1 transcripts in peripheral and central nervous system tissue of mice map to similar regions of the viral genome. *J. Virol.* **62**, 749-756.

Deatly, A.M., Spivack, J.G., Lavi, E. and Fraser, N.W. (1987). RNA from an immediate early region of the type 1 herpes simplex virus genome is present in the trigeminal ganglia of latently infected mice. *Proc. Natl. Acad. Sci. USA* **84**, 3204-3208.

Deiss, L.P., Chou, J. and Frenkel, N. (1986). Functional domains within the *a* sequence involved in the cleavage-packaging of herpes simplex virus DNA. *J. Virol.* **59**, 605-615.

DeLuca, N.A. and Schaffer, P.A. (1988). Physical and functional domains of herpes simplex virus transcriptional regulatory protein ICP4. *J. Virol.* **62**, 767-776.

Delius, H. and Clements, J.B. (1976). A partial denaturation map of herpes simplex virus type 1: Evidence for inversion in the unique DNA regions. *J. Gen. Virol.* **33**, 125-133.

Desai, P., DeLuca, N.A., Glorioso, J.C. and Person, S. (1993). Mutations in herpes simplex virus type 1 genes encoding VP5 and VP23 abrogate formation and cleavage of replicated DNA. *J. Virol.* **67**, 1357-1364.

Desai, P.J., Shaffer, P.A. and Minson, A.C. (1988). Excretion of non infectious virus particles lacking glycoprotein H by a temperature-sensitive mutant of herpes simplex virus type 1: evidence that gH is essential for virion infectivity. *J. Gen. Virol.* **69**, 1147-1156.

Deshmane, S.L., Nicosia, M., Valyi-Nagy, T., Feldman, T.L., Dillner, A. and Fraser, N.W. (1993). An HSV-1 mutant lacking the LAT TATA element reactivates normally in explant cocultivation. *Virology* **196**, 868-872.

Deshmane, S.L. and Fraser, N.W. (1989). During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. *J. Virol.* **63**, 943-947.

DeStasio, P.R. and Taylor, M.W. (1990). Specific effect of interferon on the herpes simplex virus transactivation event. *J. Virol.* **64**, 2588-2593.

Devi-Rao, G.B., Goodart, S.A., Hecht, L.M., Rochford, R., Rice, M.K. and Wagner, E.K. (1991). Relationship between polyadenylated and nonpolyadenylated herpes simplex virus type 1 latency-associated transcripts. *J. Virol.* **65**, 2179-2190.

Dewhurst, S., McIntyre, K., Schnabel, K. and Hall, C.B. (1993). Human herpesvirus 6 (HHV6) varient B accounts for the majority of symptomatic primary HHV6 infections in a population of US infants. *J. Clinical Microbiol.* **31**, 416-418.

Dick, J.W. and Rosenthal, K.S. (1995). A block in glycoprotein processing correlates with small plaque morphology and virion targeting to cell-cell junctions for an oral and anal strain of herpes-simplex virus type-1. *Arch. Virol.* **140**, 2163-2181.

Digard, P, Bebrin, W.R., Weisshart, K. and Coen, D.M. (1993). The extreem C-terminus of the herpes simplex virus DNA polymerase is crucial for functional interaction with the processivity factor UL42 and for viral replication. *J. Virol.* **67**, 398-406.

Dix, R.D., McKendall, R.R. and Baringer, J.R. (1983). Comparative neurovirulence of herpes simplex virus type 1 strains after peripheral or intracerebral inoculation of BALB/c mice. *Infect. Immun.* **40**, 103-112.

Dodson, M.S., Crute, J.J., Bruckner, R.C. and Lehman, I.R. (1989). Over expression and assembly of herpes simplex virus type 1 helicase-primase in insect cells. *J. Biol. Chem.* **264**, 20835-20838.

Dodson, M.S. and Lehman, I.R. (1991). Association of the DNA helicase and primase activities with a subassembly of the herpes simplex virus 1 helicase-primase composed of the UL5 and UL52 gene products. *Proc. Natl. Acad. Sci. USA* **88**, 1105-1109.

Dodson, M.S. and Lehman, I.R. (1992). The herpes-simplex virus type-1 origin binding-protein-DNA dependent nucleoside triphosphatase-activity. *J. Biol. Chem.* **268**, 1213-1219.

Doerig, C., Pizer, L.I. and Wilcox, C.L. (1991a). An antigen encoded by the latency-associated transcript in neuronal cell cultures latently infected with herpes simplex virus type 1. *J. Virol.* **65**, 2724-2727.

Doerig, C., Pizer, L.I. and Wilcox, C.L. (1991b). Detection of the latency-associated transcript in neuronal cultures during the latent infection with herpes simplex virus type 1. *Virology* **183**, 423-426.

Dolan, A., Arbuckle, M. and McGeoch, D.J. (1991). Sequence analysis of the splice junction in the transcript of herpes simplex virus type 1 gene U_L15. *Virus Res.* **20**, 97-104.

Dolan, A., McKee, E., MacLean, A.R. and McGeoch, D.J. (1992). Status of the ICP34.5 gene in herpes simplex virus type 1 strain 17. *J. Gen. Virol.* **73**, 971-973.

Dubbs, D.R. and Kit, S. (1964). Mutant strains of herpes simplex virus deficient in thymidine kinase induced activity. *Virology* **22**, 513-519.

Dutia, B.M. (1983). Ribonucleotide reductase induced by herpes simplex virus has a specific constituent. *J. Gen. Virol.* **64**, 513-521.

deBruyn-Kops, A. and Knipe, D.M. (1988). Formation of DNA replication structures in the herpes virus-induced cells requires a viral DNA binding protein. *Cell* **55**, 857-868.

deWind, N., Peeters, B.P.H., Zijderveld, A., Gielkens, A.L.J., Berns, A.J.M. and Kimman, T.G. (1994). Mutagenesis and characterisation of a 41-kilobase-pair region of the pseudorabies virus genome - transcription map, search for virulence genes, and comparison with homologs of herpes-simplex virus type-1. *Virology* **200**, 784-790.

deWind, N., Wagenaar, F., Pol, J., Kimman, T. and Berns, A. (1992). The pseudorabies virus homologue of the herpes simplex virus UL21 gene product is a capsid protein which is involved in capsid maturation. *J. Virol.* **66**, 7096-7103.

deWind, N., Zijderveld, A., Glazenberg, K., Gielkins, A. and Berns, A. (1990). Linker insertion mutagenesis of herpesviruses: Inactivation of single genes within the Us region of pseudorabies virus. *J. Virol.* **64**, 4691-4696.

Efstathiou, S., Kemp, S., Darby., G. and Minson, A.C. (1989). The role of herpes simplex virus type 1 thymidine kinase in pathogenesis. *J. Gen. Virol.* **70**, 869-879.

Efstathiou, S., Lawrence, G.L., Brown, C.M. and Barrell, B.G. (1992). Identification of homologues of the Human cytomegalovirus US22 gene family in human herpes virus 6. *J. Gen. Virol.* **73**, 1661-1671.

Efstathiou, S., Minson, A.C., Field, H.J., Anderson, J.R. and Wildy,P. (1986). Detection of herpes simplex virus-specific DNA sequences in latently infected mice and in humans. *J. Virol.* **57**, 446-455.

Eisenberg, S.P., Coen, D.M. and McKnight, S.L. (1985). Promotor domains required for expression of plasmid bourne copies of the herpes simplex virus thymidine kinase gene in the virus infected mouse fibroblasts and microinjected frog oocytes. *Mol. Cell Biol.* **5**, 1940-1947.

Ejercito, P.M., Kieff, E.D. and Roizman, B. (1968). Characterization of herpes simplex virus strains differing in their effects on social behaviour of infected cells. *J. Gen. Virol.* **2**, 357-364.

El-Kareh, A., Murphy, A.G.M., Fichter, T., Efstratiadis, A. and Silverstein, S. (1985). "Transactivation" control signals in the promotor of the herpes simplex virus thymidine kinase gene. *Proc. Natl. Acad. Sci. USA* **82**. 1002-1006.

Elias, P., Gutaffssam, C.M. and Hammarsten, O. (1990). The origin binding protein of herpes simplex virus type 1 binds cooperatively to the viral origin of replication ori_S. *J. Biol. Chem.* **265**, 17167-17173.

Elliott, G.D. and Meredith, D.M. (1992). The herpes simplex virus type 1 tegument protein VP22 is encoded by gene UL49. *J. Gen. Virol.* **73**, 723-726.

Ellis, R.J. (1990). Molecular chaperones. *Annu. Rev. Biochem.* **60**, 321-347.

Emery, V.C. and Griffiths, P.D. (1990). Current status review molecular biology of cytomegalovirus. *Int. J. Exp. Path.* **71**, 905-918..

Epstein, M.A., Achong, B.G. and Barr, Y.M. (1964). Virus particles in cultured lymphoblasts from Burkitt's lymphoma. *Lancet* **1**, 702-703.

Evens, A.S. and Neiderman, J.C. (1991). Epstein-Barr virus. In: *Viral infections of humans, epidemiology and control.* Chr 10, 265-292. 3rd Edn. Edited by A.S. Evans.

Everett, R.D. (1983). DNA sequence elements required for regulated expression of the HSV-1 glycoprotein D gene lies within 83 bp of the RNA cap site. *Nucl. Acids Res.* **11**, 6647-6666.

Everett, R.D. (1984a). A detailed analysis of an HSV-1 early promotor: sequences involved in trans-activation by viral immediate-early gene products are not immediate early gene specific. *Nucl. Acids Res.* **12**, 3037-3056.

Everett, R.D. (1984b). Transactivation of transcription by herpes virus products: requirement for two HSV-1 immediate-early polypeptides for maximum activity. *EMBO J.* **3**, 3135-3141.

Everett, R.D. (1986). The products of herpes simplex virus type 1 (HSV-1) immediate early genes 1, 2 and 3 can activate HSV-1 gene expression in *trans*. *J. Gen. Virol.* **67**, 2507-2513.

Everett, R.D. (1987a). A detailed mutational analysis of Vmw110, a *trans*-acting transcriptional activator encoded by herpes simplex virus type 1. *EMBO J.* **6**, 2069-2076.

Everett, R.D. (1987b). The regulation of transcription of viral and cellular genes by herpesvirus immediate-early gene products (review). *Anticancer Res.* **7**, 584-604.

Everett, R.D. (1988a). Analysis of functionl domains of herpes simplex virus type 1 immediate early polypeptide Vmw110. *J. Mol. Biol.* **202**, 87-96.

Everett, R.D. (1989). Construction and characterisation of herpes-simplex virus type-1 mutants with defined lesions in immediate early gene-1. *J. Gen. Virol.* **70**. 1185-1202.

Everett, R.D., Orr, A. and Elliott, M. (1991). High level expression and purification of herpes simplex virus type 1 immediate early polypeptide Vmw110. *Nucl. Acids Res.* **19**, 6155-6161.

Faber, S.W. and Wilcox, K.W. (1986). Association of the herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences in DNA. *Nucl. Acids Res.* **14**, 6067-6083.

Faber, S.W. and Wilcox, K.W. (1988). Association of herpes simplex virus regulatory protein ICP4 with sequences spanning the ICP4 gene transcription initiation site. *Nucl. Acids Res.* **16**, 555-570.

Farrell, M.J., Dobson, A.T. and Feldman, L.T. (1991). Herpes simplex virus latency-associated transcript is a stable intron. *Proc. Natl. Acad. Sci. USA* **88**, 790-794.

Farrell, P., Breoeze, R. and Lengyel, P. (1979). Accumulation of a mRNA and protein in interferon-treated Ehrlich ascites tumour cells. *Nature* **279**, 523-525.

Fawl, R.L. and Roizman, B. (1993). Induction of reactivation of herpes simplex virus in murine sensory ganglia *in vivo* by cadmium. *J. Virol.* **67**, 7025-7031.

Fellous, M., Nir, V., Walach, D., Merlin, G., Rubenstein, M. and Revel, M. (1982). Interferon-dependent induction of mRNA for the major histocompatability antigens in human fibroblasts and lymphoblastoid cells. *Proc. Natl. Acad. Sci. USA* **79**, 3082-3086.

Fenner, F., Bachmann, P.A., Murphy, F.A., Studdert, M.J. and White, D.O. (1987). Herpesviridae. In: *Veternary virology*, Chr 19, 339-373. Academic press.

Fenwick, M.L. (1984). The effects of herpesviruses on cellular macromolecular synthesis. In: *Comprehensive Virology* 19, pp 359-390. Edited by Fraenkel-Conrat, H. and Wagner, R.R., Plenum Press, New York.

Fenwick, M.L. and Clarke, J. (1982). Early and delayed shut-off of host protein synthesis in cells infected with herpes simplex virus. *J. Gen. Virol.* **61**, 121-125.

Fenwick, M.L. and Everett, R.D. (1990a). Inactivation of the shutoff gene (UL41) of HSV-1 and HSV-2. *J. Gen. Virol.* **71**. 2961-2967.

Fenwick, M.L. and Everett, R.D. (1990b). Transfer of UL41, the gene controlling virion-associated host cell shutoff between different strains of HSV. *J. Gen. Virol.* **71**. 411-418.

Fierer, D.S. and Challberg, M.D. (1992). Purification and characterisation of UL9, the herpes simplex virus type 1 origin-binding protein. *J. Virol.* **66**, 3986-3995.

Fisher, F.B. and Preston, V.G. (1986). Isolation and characterisation of herpes simplex virus type 1 mutants which fail to induce dUTPase activity. *Virology* **148**, 190-197.

Flanagan, W.M., Papavassiliou, A.G., Rice, M., Hecht, L.B., Silverstein, S. And Wagner, E.K. (1991). Analysis of the herpes simplex virus type 1 promoter controling the expression of UL38, a true late gene involved in capsid assembly. *J. Virol.* **65**, 769-786.

Frame, M.C., McClean, D.J., Rixon, F.J., Orr, A.C. and Marsden, H.S. (1986). The 10k virion phosphoprotein encoded by gene US9 from herpes simplex virus type 1. *Virology* **150**, 321-332.

Fraser, N.W., Block, T.M. and Spivack, J.G. (1992). The latency-associated transcripts of herpes simplex virus: RNA in search of function. *Virology* **191**, 1-8.

Fraser, N.W., Lawrence, N.C., Wroblewska, Z., Gilden, D.H. and Koprowski, H. (1981). Herpes simplex virus type 1 DNA in human brain tissue. *Proc. Natl. Acad. Sci. USA* **78**, 6461-6465.

Fraser, N.W., Spivack, J.G., Wroblewska, Z., Block, T., Deshmane, S.L., Valyi-Nagy, T., Natarajan, R. and Gesser, R.M. (1991). A review of the molecular mechanism of HSV-1 latency. *Curr. Eye Res.* **10**, 1-14.

Freeze E. (1963). Molecular mechanisms of mutations. In: *Molecular Genetics* Vol. 1. pp 207-209. Edited by J.H. Taylor. Academic press.

Frenkel, N., Locker, H., Batterson, W., Hayward, G. and Roizman, B. (1976). Defective DNA originates from the S component. *J. Virol.* **20**, 527-531.

Frenkel, N., Schirmer, E.C., Wyatt, L.S., Katsafanas, G., Roffman, E., Danovich, R.M. and June, C.H. (1990). Isolation of a new herpesvirus from human CD4 T cells. *Proc. Natl. Acad. Sci. USA* **87**, 748-752.

Friedman, A., Sclomai, J. and Becker, Y. (1977). Electron microscopy of herpes simplex virus DNA molecules isolated from infected cells by centrifugation in CsCl density gradients. *J. Gen. Virol.* **34**, 507-522.

Fuller, A.O. and Lee, W.C. (1992). Herpes simplex virus type 1 entry through a cascade of virus cell interactions requires different role of gD and gH in penetration. *J. Virol.* **66**, 5002-5012.

Fuller, A.O. and Spear, P.G. (1987). Anti-glycoprotein D antibodies that permit adsorption but block infection by herpes simplex virus type 1, prevent virion-cell fusion at the cell surface. *Proc. Natl. Acad. Sci. USA* **84**, 5454-5458.

Fuller, A.O., Santos, R.E. and Spear, P.G. (1989). Neutralising antibodies specific for glycoprotein H of herpes simplex virus permit viral attachment to cells but prevent penetration. *J. Virol.* **63**, 3435-3443.

Furlong, G.D., Swift, H. and Riozman, B. (1972). Arrangement of herpesvirus deoxyribonucleic acid in the core. *J. Virol.* **10**, 1071-1074.

Gage, P.J., Sauer, B., Levine, M. and Glorioso, J.C. (1992). A cell-free recombination system for site-specific integration of multigenic shuttle plasmids into the herpes simplex virus type 1 genome. *J. Virol.* **66**, 5509-5515.

Gallo, M.L., Dorsky, D.I., Crumpacker, C.S. and Parris, D.S. (1989). The essential 65-kilodalton DNA binding protein of herpes simplex virus stimulates the virus-encoded DNA polymerase. *J. Virol.* **63**, 5023-5029.

Gallo, M.L., Jackwood, D.H., Murphy, M., Marsden, H.S. and Parris, D.S. (1988). Purification of the herpes simplex virus type 1 65-kilodalton DNA-binding protein: properties of the protein and evidence of it's association with the virus-encoded DNA polymerase. *J. Virol.* **62**, 2874-2883.

Gao, M., Bouchey, J., Curtin, K. And Knipe, D.M. (1988). Genetic identification of a portion of the herpes simplex virus ICP8 protein required for DNA binding. *Virology* **163**, 319-329.

Gao, M. and Knipe, D.M. (1989). Genetic evidence for multiple functions of the herpes simplex virus ICP8 DNA-binding protein. *J. Virol.* **63**, 5258-5267.

Gelb, L.D. (1990). Varicella-zoster virus. In: *Virology* (2nd edn), pp 2011-2054. B.N. Fields, D.M. Knipe, R.M. Chanode, M.S. Hirsch, J.L. Melnick, T.P. Monath and B. Roizman (eds), Raven press New York.

Genetics Computer Group (1994). Program manual for the GCG package, version 8. Genetic computer group, Madison, Wis.

Georgopoulou, U., Michaelidou, A., Roizman, B. and Mavromara-Nazos, P.M. (1993). Identification of a new transcriptional unit that yields a gene product within the unique sequences of the short component of the herpes simplex virus 1 genome. *J. Virol.* **67**, 3961-3968.

Georgopoulou, U., Kakkanas, A., Miriagou, V., Michaelidou, A. and Mavromara, P. (1995). Characterisation of the US8.5 protein of herpes simplex virus. *Arch. Virol.* **140**, 2227-2241.

Gerster, T. and Roeder, R.G. (1988). A herpesvirus trans-activating protein interacts with transcription factor OFT-1 and other cellular proteins. *Proc. Natl. Acad. Sci. USA* **85**, 6347-6351.

Gibson, M.G. and Spear, P.G. (1983). Insertion mutants of herpes simplex virus have a duplication of the glycoprotein D gene and express two different forms of glycoprotein D. *J. Virol.* **48**, 396-404.

Gibson, W. and Roizman, B. (1972). Proteins specified by herpes simplex virus. VIII. Characterization and composition of multiple capsid forms of subtype 1 and 2. *J. Virol.* **10**, 1044-1052.

Goldstein, D.J. and Weller, S.K. (1988a). Herpes simplex virus type 1-induced ribonucleotide reductase is dispensible for virus growth and DNA synthesis: isolation and characterisation of an ICP6 *lacZ* insertion mutant. *J. Virol.* **62**, 196-205.

Goldstein, D.J. and Weller, S.K. (1988b). Factor (s) present in herpes simplex virus can compensate for the loss of the large sub-unit of the viral ribonucleotide reductase: Characterisation of an ICP6 deletion mutant. *Virology* **166**, 41-51.

Goldstein, D.J. and Weller, S.K. (1988c). An ICP6-LacZ insertional mutagen is used to demonstrate that the UL52 gene of herpes simplex virus type 1 is required for virus growth and DNA synthesis. *J. Virol.* **62**, 2970-2977.

Gompels, U.A., Nicholas, J. Martin, M.E.D., Lawrence, G., Thomson, B., Efstathiou, S., Teo, I., Craxton, M.A., Barrell, B. and Honess, R.W. (1992). The DNA sequence of human herpesvirus 6 strain U1102: a comparative analysis of gene organisation and coding content. Abstract, *17th International Herpesvirus Workshop.* 43.

Gompels, U.A., Nicholas, J., Lawrence, G. Jones, M., Thomson, B.J., Martin,M.E.D., Efstathiou, S., Craxton, M. And Macaulay, H.A. (1995). The DNA sequence of human herpesvirus 6: structure, coding content and genome evolution. *Virology* **209**, 29-51.

Goodpasture, E.W. (1929). Herpetic infections with special reference to involvement of nervous system. *Medicine* **8**, 223-243.

Gorbalenya, A.E. and Koonin, E.V. (1989). Viral proteins containing the purine NTP-binding sequence pattern. *Nucl. Acids Res.* **17**, 8413-8435.

Gorbalenya, A.E., Kooin, E.V., Donchenko, A.D. and Blinov, V.M. (1989). Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. *Nucl. Acids Res.* **17**, 4713-4730.

Gottleib, J. And Challberg, M.D. (1994). Interaction of herpes simplex virus type 1 DNA-polymerase and the UL42 accessory protein with a model primer template. *J. Virol.* **68**, 4837-4945.

Gottleib, J., Marcy, A.I., Coen, D.M. and Challberg, M.D. (1990). The herpes simplex virus type 1 UL42 gene product: a subunit of DNA polymerase that functions to increase processivity. *J. Virol.* **64**, 5976-5987.

Graves, B.J., Johnson, P.F. and McKnight, S.L. (1986). Homologous recognition of a promotor domain common to the MSV LTR and the HSV tk gene. *Cell* **44**, 565-576.

Greaves, R. and O'Hare, P. (1989). Separation of requirements for protein-DNA complex assembly from those for functional activity in the herpes simplex virus regulatory protein. *J. Virol.* **63**, 1641-1650.

Greaves, R. and O'Hare, P. (1990). Structural requirements in the herpes simplex virus type 1 transactivator Vmw65 for interaction with the cellular octamer-binding protein and target TAATGARAT sequences. *J. Virol.* **64**, 2716-2724.

Guzowski, J.F., Singh, J. And Wagner, E.K. (1994). Transcriptional activation of the herpes simplex virus type 1 U(L)38 promoter conferred by the cis-acting downstream activation sequence is mediated by a cellular transcription factor. *J. Virol.* **68**, 7774-7789.

Hardwick, M.A. and Sandri-Goldin, R.M. (1994). The herpes simplex virus regulatory protein ICP27 contributes to the decrease in cellular mRNA levels during infection. *J. Virol.* **68**, 4797-4810.

Hardwick, M.A. and Schaffer, P.A. (1995). Cloning and characterisation of herpes simplex virus type 1 ori_L: Comparison of replication and protein-DNA complex formation by ori_L and ori_S. *J. Virol.* **69**, 1377-1388.

Hardy, SG-RM. (1994). Herpes simplex virus inhibits host cell splicing and regulatory protein ICP27 is required for this effect. *J. Virol.* **68**, 7790-7799.

Harland, J. And Brown, S.M. (1992). An HSV-1 revertant (1720) generates 4 equimolar isomers despite a 9200-bp deletion from TRL and sequences between 9200 np and 97000 np in the inverted orientation being covalently bound to sequences 94000-120372 np. *Virus genes* **6**, 291-299.

Harnett, G.B., Farr, T.J., Pietroboni, G.R. and Bucens, M.R. (1990). Frequent shedding of human herpesvirus-6 in saliva. *J. Med. Virol.* **30**, 128-130.

Harris, R.A. and Preston, C.M. (1991). Establishment of latency *in vitro* by the herpes simplex virus type 1 mutant *in*1814. *J. Gen. Virol.* **72**, 907-913.

Harris, S.L., Everett, R.D., Zhu, X., Silverstein, S. and Preston, C.M. (1989). Herpes simplex virus type 1 immediate-early protein Vmw110 reactivates latent herpes simplex virus type 2 in an *in vitro* latency system. *J. Virol.* **63**, 3513-3515.

Haruta, Y., Rootman, D.S., Xie, L., Kiritoshi, A. and Hill, J.M. (1989). Recurrent HSV-1 corneal lesions in rabbits induced by cyclophosphamide. *Invest. Ophthalmol. Vis. Sci.* **30**, 371-376.

Hay, K.A., Gaydos, A. And Tenser, R.B. (1995). The role of herpes simplex thymidine kinase expression in neurovirulence and latency in newborn vs adult mice. *J. Neuroimmunol.* **61**, 41-52.

Hay, R.T. and Hay, J. (1980). Properties of herpes simplex virus-induced "immediate early" polypeptides. *Virology* **104**, 230-234.

Hayward, G.S., Kacob, R.J., Wadsworth, S.C and Roizman, B. (1975). Anatomy of herpes simplex virus DNA: Evidence for 4 populations that differ in the relative orientations of their long and short components. *Proc. Natl. Acad. Sci. USA* **72**, 4243-4247.

He, B., Chou, J., Liebermann, D.A., Hoffman, B. and Roizman, B. (1996). The carboxyl terminus of the murine MyD116 gene substitutes for the corresponding domain of the α1 34.5 gene of herpes simplex virus to preclude the premature shutoff of total protein synthesis in infected humancells. *J. Virol.* **70**, 84-90.

Heine, J.W., Honess, R.W., Cassai, E. and Roizman, B. (1974). Proteins specified by herpes simplex virus XII. The virion polypeptides of type 1 strains. *J. Virol.* **14**, 640-651.

Herold, B.C., WuDunn, D., Soltys, N. and Spear, P.G. (1991). Glycoprotein C of herpes simplex virus type 1 plays a principle role in the adsorption of virus to cells and in infectivity. *J. Virol.* **65**, 1090-1098.

Herr, W. (1992). Oct1 and Oct2: differential transcriptional regulation by proteins that bind to the same DNA sequence. In: *Transcriptional regulation*, Edited by S.L. McKnight and K.R. Yamamoto. Cold spring harbor laboratory press. NY.

Hill, T.J., Blyth, W.A. and Harbour, D.A. (1978). Trauma to the skin causes recurrence of herpes simplex virus in the mouse. *J. Gen. Virol.* **39**, 21-28.

Hill, T.J., Field, H.J. and Blyth, W.A. (1975). Acute and recurrent infection with herpes simplex virus in the mouse; a model for studying latency and recurrent disease. *J. Gen. Virol.* **28**, 341-353.

Hill, A., Jugovic, P., York, I., Russ, G., Bennink, J., Yewdell, J., Ploegh, H. and Johnson, D. (1995). Herpes-simplex virus turns off the TAP to evade host immunity. *Nature* **375**, 411-415.

Ho, M. (1991). In: *Cytomegalovirus biology and infection.* Second edition, Plenum medical book company, NY and London.

Hochberg, E and Becker, Y. (1968). Adsorption, penetration and uncoating of herpes simplex virus. *J. Gen. Virol.* **2**, 231-241.

Hodgeman, T.C. (1988). A new superfamily of replicative proteins. *Nature* **333**, 22-23.

Holland, L.E., Anderson, K.P., Shipman, C. and Wagner, E.K. (1980). Viral DNA synthesis is required for the efficient expression of specific herpes simplex virus type 1 mRNA species. *Virology* **101**, 10-24.

Holland, T.C., Marlin, S.D., Levine, M. and Glorioso, J.C. (1983). Antigenic variants of herpes simplex virus selected with glycoprotein-specific monoclonal antibodies. *J. Virol.* **45**, 672-682.

Homa, F.L., Otal, T.M., Glorioso, J.C. and Levine, M. (1986). Transcriptional control signals of a herpes simplex virus type 1 late (gamma 2) gene lie within bases -311 to.124 relative to the 5' terminus of the RNA. *Mol. Cell Biol.* **6**, 3652-3666.

Honess, R.W. and Roizman, B. (1975). Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *J. Virol.* **14**, 8-19.

Hopps, H.E., Bernheim, B.C., Nisalak, A., Hin-Tjio, J. and Smadel, J.E. (1963). Biologic characteristics of a continuous kidney cell line derived from the African Green Monkey. *J. Immunol.* **91**, 416-424.

Huang, C.J. and Wagner, E.K. (1994). The herpes simplex virus type 1 major capsid protein (VP5 - UL19) promoter contains 2 *cis*-acting elements influencing late expression. *J. Virol.* **68**, 5738-5747.

Hummeler, K., Tomassini, N., Zajac, T. (1969). Early events in herpes simplex virus infection: A radiographic study. *J. Gen. Virol.* **4**, 67-74.

Hutchison, L., Browne, H., Wargnet, V., Davis-Poynter, N., Primorac, S., Goldsmith, K., Minson, A.C. and Johnson, D.C. (1992a). A novel herpes simplex virus glycoprotein, gL, forms a complex with glycoprotein H (gH) and affects normal folding and surface expression of gH. *J. Virol.* **66**, 2240-2250.

Hutchison, L., Goldsmith, K., Snoddy, D., Ghash, G., Graham, F.L. and Johnson, D.C. (1992b). Identification and characterisation of a herpes simplex virus glycoprotein gK, involved in cell fusion. *J. Virol.* **66**, 5603-5609.

Idowa, A.D., Fraser-Smith, E.B., Poffenberger, K.L. and Herman, R.C. (1992). Deletion of the herpes simplex virus type 1 ribonucleotide reductase alters virulence and latency *in vivo*. *Antiviral Res.* **17**, 145-156.

Igarashi, K., Fawl, R., Roller, R.J. and Roizman, B. (1993). Construction and properties of a recombinant herpes simplex virus 1 lacking both S-componant origins of DNA synthesis. *J. Virol.* **67**, 2123-2132.

Imbalanzo, A.N., Shepard, A.A. and DeLuca, N.A. (1990). Functional relevance of specific interactions between herpes simplex virus type 1 ICP4 and sequences from the promotor-regulatory domain of the viral thymidine kinase gene. *J. Virol.* **64**, 2620-2631.

Ingram, A., Phelan, A., Dunlop, J. and Clements, J.B. (1996). Immediate early protein IE63 of herpes simplex virus type 1 binds RNA directly. *J. Gen. Virol.* **77**, 1847-1851.

Jacob, R.J., Morse, L.S. and Roizman, B. (1979). Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. *J. Virol.* **29**, 442-457.

Jacobson, J.G., Leib, D.A., Goldstein, D.J., Bogart, C.L., Schaffer, P.A., Weller, S.K. and Cohen, D.M. (1989a). A herpes simplex virus ribonucleotide reductase deletion mutant is defective for productive acute and reactivatable latent infections of mice and for replication in mouse cells. *Virology* **173**, 276-283.

Jacobson, J.G., Martin, S.L. and Coen, D.M. (1989b). A conserved open reading frame that overlaps the herpes simplex virus thymidine kinase gene is important for viral growth in cell culture. *J. Virol.* **63**, 1839-1843.

Jamieson, A.T. and Subak-Sharpe, J.H. (1974). Biochemical studies on the herpes simplex virus-specified deoxypyridine kinase activity. *J. Gen. Virol.* **24**, 481-492.

Jamieson, D.R.S., Robinson, L.H., Daksis, J.I., Nicholl, M.J. and Preston, C.M. (1995). Quiescent viral genomes in human fibroblacts after infection with herpes simplex virus type 1 Vmw65 mutants. *J. Gen. Virol.* **76**, 1417-1431.

Javier, R.T., Izumi, K.M. and Stevens, J.G. (1988). Localisation of a herpes simplex virus neurovirulence gene dissociated from high titre virus replication in the brain. *J. Virol.* **62**, 1381-1387.

Jenkins, F.J. and Roizman, B. (1986). Herpes simplex virus 1 recombinants with noninverting genomes frozen in different isomeric arrangements are capable of independent replication. *J. Virol.* **59**, 494-499.

Johnson, P.A. and Everett, R.D. (1986a). DNA replication is required for abundant expression of plasmid-borne late US11 gene of herpes simplex virus type 1. *Nucl. Acids Res.* **14**, 3609-3625.

Johnson, P.A. and Everett, R.D. (1986b). The control of herpes simplex virus late gene transcription: a "TATA-box"/cap site region is sufficient for fully efficient regulated activity. *Nucl. Acids Res.* 14, 8247-8264.

Johnson, D.C., Frame, M.C., Ligas, M.W., Cross, A.M. and Stow, N.D. (1988). Herpes simplex virus immunoglobulin G fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. *J. Virol.* **62**, 1347-1354.

Johnson, D.C. and Ligas, M.W. (1988). Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: Quantitative evidence for virus-specific cell surface receptors. *J. Virol.* **62**, 4605-4612.

Johnson, P.A., MacLean, C.A., Marsden, H.S., Dalziel, R.G. and Everett, R.J. (1986). The product of gene US11 of herpes simplex virus type 1 is expressed as a true late gene. *J. Virol.* **67**, 871-883.

Johnson, R.M. amd Spear, P.G. (1989). Herpes simplex virus glycoprotein D mediates interference with herpes simplex virus infection. *J. Virol.* **63**, 819-827.

Johnson, D.C., York, I.A., Graham, F.L., Tomazin, R. and Riddell, S.R. (1995). Inhibition of antigen presentation to CD8+ T-lymphocytes by herpes simplex virus (HSV) immediate early (IE) protein ICP47. *J. Cell. Biochem.* **S19A-51A**, 272.

Jones, K.A., Yamamoto, K.R. and Tjian, R. (1985). Two distinct transcription factors bound to the HSV thymidine kinase promotor *in vitro*. *Cell* **42**, 559-572.

Jones, P.C. and Riozman, B. (1979). Regulation of herpesvirus macromolecular synthesis. VIII. The transcription program consists of three phases during which extent of transcription and accumulation of RNA in the cytoplasm are regulated. *J. Virol.* **31**, 299-314.

Kaner, R.A., Baird, A., Mansukhani, A., Basilico, C., Summers, B., Florkiewicz, R. and Hajjar, D. (1990). Fibroblast growth factor receptor is a portal of cellular entry for herpes simplex virus type 1. *Science* **248**, 1410-1413.

Kaerner, H.C., Schroder, C.H., Ott-Hartmann, A., Kumel, G. and Kirchner, H. (1983). Genetic variability of herpes simplex virus: development of a pathogenic varient during passaging of a non pathogenic herpes simplex virus type 1 strain in mouse brain. *J. Virol.* **46**, 83-93.

Kehm, R., Rosen-Wolff, A. And Darai, G. (1996). Restitution of the UL56 gene expression of HSV-1 HFEM led to restoration of virulent phenotype - deletion of the amino acids 217 to amino acids 234 of the UL56 protein abrogates the virulent phenotype. *Virus Res.* **40**, 17-31.

Kemble, G., Duke, G., Winter, R. and Spaete, R. (1996). Defined large scale alteration of the human cytomegalovirus genome constructed by cotransfection of overlapping cosmids. *J. Virol.* **70**, 2044-2048.

Kit, S., Dubbs, D.R., DeTorres, R.A. and Melnick, J.L. (1965). Enhanced thymidine kinase activity following infection of Green Monkey Kidney cells by Simian Adenoviruses, Simian Popovavirus SV40 and an Adenovirus-SV40 "Hybrid". *Virology* **27**, 453-457.

Klinedinst, D.K., Challberg, M.D. (1994). Helicase-primase complex of herpes simplex virus type 1: a mutant in the UL52 subunit abolishes primase activity. *J. Virol.* **68**, 3693-3701.

Knipe, D.M. and Smith, J.L. (1986). A mutant herpesvirus protein leads to a block in nuclear-localisation of other viral-proteins. *Mol. Cel. Biol.* **6**, 2371-2381.

Koff, A., Schwedes, J.F. and Tegtmeyer, P. (1991). Herpes simplex virus origin-binding protein (UL9) loops and distorts the viral replication origin. *J. Virol.* **65**, 3284-3292.

Krause, P.R., Croen, K.D., Straus, S.E. and Ostrove, J.M. (1988). Detection and preliminary characterization of herpes simplex virus type 1 transcripts in latently infected human trigeminal ganglia. *J. Virol.* **62**, 4819-4823.

Kristie, T.M. and Roizman, B. (1987). Host cell proteins bind to *cis*-acting site required for virion-mediated induction of herpes simplex 1 α genes. *Proc. Natl. Acad. Sci. USA* **84**, 71-75.

Kuhn, J.E., Kramer, M.D., Willenbacher, W., Welland, H., Lorentzen, E.U. and Braun, W.R. (1990). Identification of herpes simplex virus type 1 glycoproteins interacting with the cell surface. *J. Virol.* **64**, 2491-2497.

Kwon, B.S., Gangarosa, L.P., Burch, K.D., deBack, J. and Hill, J.M. (1981). Induction of ocular herpes simplex virus shedding by iontophoresis of epinephrine into rabbit cornea. *Invest. Ophthalmol. Vis. Sci.* **21**, 442-449.

Lagunoff, M. and Roizman, B. (1994). Expression of a herpes simplex virus 1 open reading frame antisense to the γ1 34.5 gene and transcribed by an RNA 3 coterminal with the unspliced latency-associated transcript. *J. Virol.* **68**, 6021-6028.

Lagunoff, M. and Roizman, B. (1995). The regulation of synthesis and properties of the protein product of open reading frame P of the herpes simplex virus genome. *J. Virol.* **69**, 3615-3623.

Larder, B.A., Lisle, J.J. and Darby, G. (1986). Restoration of wild-type pathogenicity to an attenuated DNA polymerase mutant of herpes simplex virus type 1. *J. Gen. Virol.* **67**, 2501-2506.

Latchman, D.S., Partridge, J.F., Estridge, J.K. and Kemp, L.M. (1989). The different competative abilities of viral TAATGARAT elements and cellular octamer motifs, mediate the induction of viral immediate early genes and the repression of the histone H2B gene in herpes simplex virus infected cells. *Nucleic Acids Research* **21**, 8533-8542.

LaThangue, N.B., Shriver, K., Dawson, C. and Chan, W.I. (1984). Herpes simplex virus infection causes the accumulation of a heat shock protein. *EMBO J.* **3**, 267-277.

Lawrence, G.L., Chee, M., Craxton, M.A., Gompels, U.A., Honess, R.W. and Barrell, B.G. (1990). Human herpesvirus 6 is closely related to human cytomegalovirus. *J. Virol.* **64**, 287-299.

Leib, D.A., Bogard, C.L., Kosz-Vnenchak, M., Hicks, K.A., Coen, D.M., Knipe, D.M. and Schaffer, P.A. (1989b). A deletion mutant of the latency-associated transcript of herpes simplex virus type 1 reactivates from latent state with reduced frequency. *J. Virol.* **63**, 2893-2900.

Leib, D.A., Coen, D.M., Bogard, C.L., Hicks, K.A., Yager, D.R., Knipe, D.M., Tyler, K.L. and Schaffer, P.A. (1989a). Immediate-early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency. *J. Virol.* **63**, 759-768.

Leib, D.A., Nadeau, K.C., Rundle, S.A. and Schaffer, P.A. (1991). The promoter of the latency-associated transcripts of herpes simplex virus type 1 contains a functional cAMP-response element: Role of the latency-associated transcripts and cAMP in reactivation of viral latency. *Proc. Natl. Acad. Sci. USA* **88**, 48-52.

Lengyel, P. (1982). Biochemistry of interferons and their actions. *Annu. Rev. Biochem.* **51**, 251-282.

Leungtack, P., Audonnet, J.C. and Riviere, M. (1994). The complete DNA-sequence and the genetic organisation of the short unique region (U-S) of the bovine herpesvirus type-1 (ST strain). *Virology* **199**, 409-421.

Ligas, M.W. and Johnson, D.C. (1988). A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to but is unable to penetrate into cells. *J. Virol.* **62**, 1486-1494.

Lillycrop, K.A., Dent, C.L., Wheatley, S.C., Beech, N.M., Ninkina, N.N., Wood, N.J. and Latchman, D.S. (1991). The octamer-binding protein Oct-2 represses HSV immediate-early genes in cell lines derived from latently infectable sensory neurons. *Neuron* **7**, 381-390.

Lillycrop, K.A., Howard, M.K., Estridge, J.K. and Latchman, D.S. (1994). Inhibition of herpes simplex virus infection by ectopic expression of neuronal splice varients of the Oct-2 transcription factor. *Nuc. Acids Res.* **22**, 815-820.

Liu, F. and Roizman, B. (1991a). The promoter, transcriptional unit, and coding sequences of the herpes simplex virus 1 family 35 proteins are contained within and in frame with the UL26 open reading frame. *J. Virol.* **65**, 206-212.

Liu, F. and Roizman, B. (1991b). The herpes simplex virus 1 gene encoding a protease also contains within its coding domain the gene encoding the more abundant substrate. *J. Virol.* **65**, 5149-5156.

Lockshon, D. and Galloway, D.A. (1988). Sequence and structural requirements of a herpes simplex viral DNA replication origin. *Mol. Cell Biol.* **8**, 4018-4927.

Longnecker, R. and Roizman, B. (1986). Generation of an inverting herpes simplex virus type 1 mutant lacking the L-S junction *a* sequences, an origin of DNA synthesis, and several genes including those specifying glycoprotein E and the α 47 gene. *J. Virol.* **58**, 523-591.

Longnecker, R. and Roizman, B. (1987). Clustering of genes dispensible for growth in culture in the S component of the HSV-1 genome. *Science* **236**, 573-576.

Lopez, C. (1985). Natural resistance mechanisms in herpes simplex virus infections. In: *The Herpesviruses* vol 4 pp 1-36. B. Roizman and C. Lopez (ed's). Plenum Press, New York.

Lord, K.A., Hoffman-Liebermann, B. and Liebermann, D.A. (1990). Sequenced MyD116 cDNA: a novel myeloid differentiation primary response gene induced by IL 6. *Nucl. Acids Res. 18*, 2823.

Low, M., Hay, J. and Keir, H.M. (1969). DNA of herpes simplex virus is not a substrate for methylation *in vivo*. *J. Mol. Biol.* **46**, 205-207.

Lycke, E., Hamark, B., Johansson, M., Krotochwil, A., Lycke, J. and Svennerholm, B. (1988). Herpes simplex virus infection of the human sensory neurone. An electron microscopy study. *Arch. Virol.* **101**, 87-104.

Lycke, E., Kristensson, K., Svennerholm, B., Vahlne, A. and Ziegler, R. (1984). Uptake and transport of herpes simplex virus in neurites of rat dorsal root ganglia cells in culture. *J. Gen. Virol.* **65**, 55-64.

Mace, M. and Alberts, B.M. (1984). Characterisation of the stimulatory effect of T4 gene 45 protein and the gene 44/62 protein complex on DNA synthesis is by T4 DNA polymerase. *J. Mol. Biol.* **177**, 313-327.

Machtiger, N.A., Pancake, B.A., Eberle, R., Courtney, R.J., Tevethia, SS. and Schaffer, P.A. (1980). Herpes simplex virus glycoproteins: Isolation of mutants resistant to immune cytolosis. *J. Virol.* **34**, 336-346.

MacLean, C.A., Dolan, A., Jamieson, F.E. and McGeoch, D.J. (1992). The myristylated virion proteins of herpes simplex type 1: investigation of their role in the virus life cycle. *J. Gen. Virol.* **73**, 539-547.

MacLean, C.A., Clark, B. And McGeoch, D.J. (1989). The UL11 of herpes simplex virus type 1 encodes a virion protein which is myristylated. *J. Gen. Virol.* **70**, 3147-3157.

MacLean, C.A., Efstathiou, S., Elliot, M.L., Jamieson, F.E. and McGeoch, D.J. (1991a). Investigation of herpes simplex virus type 1 genes encoding multiply inserted membrane proteins. *J. Gen. Virol.* **72**, 897-906.

MacLean, C.A., Robertson, C.M. and Jamieson, F.E. (1993). Characterisation of the UL10 gene product of herpes simplex virus type 1 and investigation of it's role *in vivo*. *J. Gen. Virol.* **74**, 975-983.

MacLean, A.R., UL-Fareed, M., Robertson, L., Harland, J. And Brown, S.M. (1991b). Herpes simplex virus type 1 deletion varients 1714 and 1716 pinpoint neurovirulence-related sequences in Glasgow strain 17+ between immediate early gene 1 and the "a" sequence. *J. Gen. Virol.* **72**, 631-639.

Madin, S.H. and Darby Jr, N.B. (1958). Established kidney cell lines of normal adult bovine and ovine origin. *Proc. Soc. Exp. Biol.* **98**, 574-576.

Manservigi, R. (1974). Method for isolation and selection of temperature sensitive mutants of herpes simplex virus. *Appl. Microbiol.* **27**, 1034-1040.

Marsden, H.S. (1987). Herpes simplex virus glycoproteins and pathogenesis. In: *Molecular basis of virus disease*. Edited by W.J. Russell and G. Almond. Cambridge university press.

Marsden, H.S., Campbell, M.E.M., Haar, L., Frame, M.C., Parris, D.S., Murphy, M., Hope, R.G., Muller, M.T. and Preston, C.M. (1987). The 65,000 Mr DNA binding and trans-inducing proteins of herpes simplex virus type 1. *J. Virol.* **61**, 2428-2437.

Marsden, H.S., Haarr, L. and Preston, C.M. (1983). Processing of herpes simplex virus proteins and evidence that translation of thymidine kinase messenger-RNA is initiated at 3 separate AUG codons. *J. Virol.* **46**, 434-445.

Marsden, H.S., Long, J., Davison, A.J., Hope, R.G. and McDonald, D.M. (1982). Genomic location and lack of phosphorylation of the HSV immediate early polypeptide IE12. *J. Gen. Virol.* **62**, 17-27.

Martin, T.E., Barghusen, S.C., Leser, G.P. and Spear, P.G. (1987). Redistribution of nuclear ribonucleoprotein antigens during herpes simplex virus infection. *J. Cell. Biol.* **105**, 2069-2082.

Martin, M.E.D., Thomson, B.J., Honess, R.W., Craxton, M.A., Gompels, U.A., Liu, M.Y., Littler, E., Arrand, J.R., Teo, I. and Jones, M.D. (1991). The genome of human herpesvirus-6 - maps of unit-length and concatameric genomes for 9 restriction endonucleases. *J. Gen. Virol.* **72**, 157-168.

Martinez, R., Sarisky ,R.T., Weber, P.C. and Weller, S.K. (1996a). Herpes simplex virus type 1 alkaline nuclease is required for efficient processing of viral DNA replication intermediates. *J. Virol.* **70**, 2075-2085

Martinez, R., Shao, L., Bronstein, J.C., Weber, P.C. and Weller, S.K. (1996b). The product of a 1.9-kb messenger RNA which overlaps the HSV-1 alkaline nuclease gene (UL12) cannot relieve the growth defects of a null mutant. *Virology* **215**, 152-164.

Mavromara-Nazos, P. and Roizman, B. (1987). Activation of herpes simplex virus 1 gamma 2 genes by viral DNA replication. *Virology* **161**, 593-598.

McCarthy, A.M., McMahan, L. and Schaffer, P.A. (1989). Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and is DNA deficient. *J. Virol.* **63**, 18-27.

McFarlane, M., Daksis, J.I. and Preston, C.M. (1992). Hexamethylene bisacetamide stimulates herpes simplex virus immediate early gene expression in the absence of trans-induction by Vmw65. *J. Gen. Virol.* **73**, 285-292.

McGeoch, D.J. (1989). The genomes of the human herpesviruses: contents, relationships, and evolution. *Annu. Rev. Microbiol.* **43**, 235-265.

McGeoch, D.J. and Barnett, B.C. (1991). Neurovirulence factor. *Nature* **353**, 609.

McGeoch, D.J., Barnett, B.C. and MacLean, C.A. (1993). Emerging functions of alphaherpesvirus genes. *Seminars in Virology* **4**, 124-133.

McGeoch, D.J. and Cook, S. (1994). Molecular phylogeny of the alphaherpesvirinae subfamily and a proposed evolutionary timescale. *J. Mol. Biol.* **238**, 9-22.

McGeoch, D.J., Cook, S., Dolan, A., Jamieson, F.E. and Telford, E.A. (1995). Molecular phylogeny and evolutionary timescale for the family of mammalian herpesviruses. *J. Mol. Biol.* **247**, 443-458.

McGeoch, D.J., Cunningham, C., McIntyre, G. and Dolan, A. (1991). Comparative sequence analysis of the long repeat regions adjoining parts of the long unique regions in the genomes of herpes simplex virus types 1 and 2. *J. Gen. Virol.* **72**, 3067-3075.

McGeoch, D.J., Dalrymple, M.A., Davison, A.J., Dolan, A., Frame, M.C., McNa,b D., Perry, L.J., Scott, J.E. and Taylor, P. (1988). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *J. Gen. Virol.* **69**, 1531-1574.

McGeoch, D.J., Dolan, A., Donald, S. and Brauer, D.H. (1986). Sequence determination and genetic content of the short repeat region in the genome of herpes simplex virus type 1. *Nature* **14**, 1727-1745.

McGeoch, D.J., Dolan, A., Donald, S. and Rixon, F.J. (1985). DNA sequence of the short unique region in the genome of herpes simplex virus type 1. *J. Mol. Biol.* **181**, 1-13.

McGeoch, D.J., Moss, H.W., McNab, D. and Frame, M.C., (1987). DNA sequence and genetic content of the Hind III L region in the short unique component of the herpes simplex virus type 2 genome: Identification of the gene encoding glycoprotein G and evolutionary comparisons. *J. Gen. Virol.* **68**, 19-38.

McGeoch, D.J. and Schaffer, P.A. (1992). DNA viruses: herpes simplex virus. In: *Genetic maps. Locus maps of complex genomes* 1.147-1.156. Edited by S.J. O'Brien.

McKendall, R.R., Klasses, T. and Baringer, R. (1979). Host defences in herpes simplex virus infections of the nervous system: Effect of antibody on disease and viral spread. *Infect. Immun.* **23**, 305-311.

McKie, E.A.E, Hope, G.R., Brown, S.M. and MacLean, A.R. (1994). Characterisation of the herpes simplex virus type 1 strain 17+ neurovirulence gene RL1 and its expression in a bacterial system. *J. Gen. Virol.* **75**, 733-741.

McKnight, S., Kingsbury, R., Eisenberg, R., Turfaro, F, Weinheimer, S., Triezenberg, S., Johnson, P. and Graves, B. (1985). Studies on the promotor of the herpes simplex thymidine kinase gene. In: *Eucaryotic Transcription* pp 116-122. Y. Gluzman (ed). Cold Spring Harbor Laboratory, New York.

McLauchlan, J., Addison, C., Craigie, M.C. and Rixon, F.J. (1992). Non-infectious L-particles supply functions which can facilitate infection by HSV-1. *Virology* **190**, 682-688.

McLauchlan, J., Phelan, A.C., Loney, C., Sandri-Goldin, R.M. and Clements, J.B. (1992). Herpes simplex virus IE63 acts at the post-transcriptional level to stimulate viral mRNA 3' processin. *J. Virol.* **66**, 6039-6945.

McLauchlan, J. and Rixon, F.J. (1992). Characterisation of enveloped tegument structures (L particles) produced by alphaherpesviruses: integrity of the tegument does not depent on the presence of capsid or envelope. *J. Gen. Virol.* **73**, 269-276.

McLean, G.W., Rixon, F.J., Langeland, N., Haar, L. and Marsden, H.S. (1992). Identification and characterisation of the virion protein promotors of herpes simplex virus type 1 gene UL47. *J. Gen. Virol.* **71**, 2953-2960.

McMahan, L. and Schaffer, P.A. (1990). The repressing and enhancing functions of the herpes simplex virus immediate regulatory protein ICP27 maps to the C-terminal regions and are required to modulate viral gene expression very early in infection. *J. Virol.* **64**, 3471-3485.

McNabb, D.S. and Courtney, R.J. (1992a). Characterisation of the large tegument protein (ICP 1/2) of herpes simplex virus type 1. *Virology* **190**, 221-232.

McNabb, D.S. and Courtney, R.J. (1992b). Analysis of the UL36 open reading frame encoding the large tegument protein (ICP1/2) of herpes simplex virus type 1. *J. Virol.* **66**, 7581-7584.

McPherson, I. and Stoker, M. (1962). Polyoma transformation of hamster cell clones - an investigation of genetic factors affecting cell competence. *Virology* **16**, 147-151.

Mehta, A., Baggioncalda, J., Bagasra, O., Saikumari, P., Valy-Nagy, T., Fraser, N.W. and Block, T.M. (1995). *In situ* DNA PCR and RNA hybridisation detection of herpes simplex virus sequences in trigeminal ganglia of latently infected mice. *Virology* **206**, 633-644.

Meigner, B., Longnecker, R., Mavromara-Nazos, P., Sears, A. and Roizman. (1988). Virulence and establishment of latency by genetically engineered deletion mutants of herpes simplex virus 1. *Virology* **162**, 251-254.

Mellerick, D.M. and Fraser, N.W. (1987). Physical state of the latent herpes simplex virus genome in mouse model system. Evidence suggesting an episomal state. *Virology* **158**, 265-275.

Miller, G. (1990). Epstein-Barr virus: Biology, pathogenesis and medical aspects. In: *Virology* (2nd edn), pp 1921-1958. B.N. Fields, D.M. Knipe, R.M. Chanode, M.S. Hirsch, J.L. Melnick, T.P. Monath and B. Roizman (eds), Raven press New York.

Mills, L.K., Shi, Y. and Millette, R.L. (1994). YY1 is the cellular factor shown previously to bind to the regulatory regions of several leaky-late ($\beta\gamma$ γ1) genes of herpes simplex virus type 1. *J. Virol.* **68**, 1234-1238.

Mittnacht, S., Straub, P., Kirchner, H. and Jacobsen, H. (1988). Interferon treatment inhibits onset of herpes simplex virus immediate-early transcription. *Virology* **164**, 201-210.

Mocarski, E.S., Post, L.E. and Roizman, B. (1980). Molecular engineering of the herpes simplex virus genome: insertion of a second L-S junction into the genome causes additional genome reversions. *Cell* **22**, 243-255.

Mocarski, E.S. and Roizman, B. (1981). Site-specific inversion sequences of the herpes simplex virus genome: Domain and structural features. *Proc. Natl. Acad. Sci. USA* **78**, 7047-7051.

Mocarski, E.S. and Roizman, B. (1982). Structure and role of the herpes simplex virus DNA termini on inversion, circularization and generation of virion DNA. *Cell* **31**,89-97.

Moore, P., Gao, S.J., Dominguez, G., Cesarman, E., Lungo, O., Knowles, D.M., Garber, R., Pellett, P.E., McGeoch, D.J. and Chang, Y. (1996). Primary characterisation of a herpesvirus agent associated with Kaposis-sarcoma. *J. Virol.* **70**, 549-558.

Morgan, C., Rose, H.M., Holden, M. and Jopes, E.D. (1959). Electron microscopic observations on the development of herpes simplex virus. *J. Exp. Med.* **110**, 643-656.

Moyal, M., Berkowitz, C., Rosen-Wolff, A., Darai, G. and Becker, Y. (1992). Mutation in the UL53 gene of HSV-1 abolish virus neurovirulence to mice by the intracerebral route of infection. *Virus Res.* **26**, 99-112.

Muggeridge, M.I., Cohen, G.H. and Eisenberg, R.J. (1992). Herpes simplex virus infection can occur without involvement of the fibroblast growth factor receptor. *J. Virol.* **66**, 824-830.

Muggeridge, M.I., Wilcox, W.C., Cohen, G.H. and Eisenberg, R.J. (1990). Identification of a site on herpes simplex virus type 1 glycporotein D that is essential for infectivity. *J. Virol.* **64**, 3617-3626.

Mullaney, J., Moss, H.W.McL. and McGeoch. D.J. (1989). Gene UL2 of herpes simplex virus type 1 encodes a uracil-DNA glycosylase. *J. Gen. Virol.* **70**, 449-454.

Muller, M.T., Bolles, C.S. and Parris, D.S. (1985). Association of I DNA topoisomerase with herpes simplex virus. *J. Gen. Virol.* **66**, 1565-1574.

Munoz, A. and Carrasco, L. (1984). The formation of non-infectious herpesvirus particles in cultured cells treated with human interferon. *J. Gen. Virol.* **65**, 1089-1078.

Nahmias, A.J., Keyserling, H. and Lee, F.K. (1991). Herpes simplex viruses 1 and 2: Epidemiology and control. In: *Viral infections of humans*, Chapter 14, 3rd edition, pp 393-418. Ed A. Evans. Plenum press, New York and London.

Nash, A.A., Leung, K.N. and Wildy, P. (1985). The T-cell mediated immune response of mice to herpes simplex virus. In: *The Herpesviruses.* B. Roizman and C. Lopez (ed's). Plenum Press, New York.

Nash, T.C. and Spivack, J.G. (1994). The UL55 and UL56 genes of herpes simplex virus type 1 are not required for viral replication, intraperitoneal virulence or establishment of latency. *Virology* **204**, 794-798.

Neidhardt, H., Schrode,r C.H. and Kaerner, H.C. (1987). Herpes simplex virus type 1 glycoprotein E is not indespensible for viral infectivity. *J. Virol.* **61**, 600-603.

Newcomb, W.M. and Brown, J.C. (1991). Structures of the herpesvirus capsid: Effect of extraction with guanidine hydrochloride and partial reconstruction of extracted capsids. *J. Virol.* **65**, 613-620.

Newcomb, W.W., Trus, B.L., Booy, F.P., Steven, A.C., Wall, J.S. and Brown, J.C. (1993). Structure of the herpes simplex virus capsid. Molecular composition of the pentons and the triplexes. *J. Mol. Biol.* **232**, 499-511.

Nicholas, J. (1996). Determination and analysis of the complete nucleotide sequence of Human herpesvirus 7. *J. Virol.* **70**, 5975-5989.

Nilheden, E., Jeansson, S.I. and Vahlne, A. (1985 a). Herpes simplex virus latency in hyperresistant clone of mouse neuroblastoma (C1300) cells. *Arch. Virol.* **83**, 319-325.

Nilheden, E., Jeansson, S.I. and Vahlne, A. (1985 b). Amplification of herpes simplex virus resistance in mouse neuroblastoma (C1300) cells. *Arch. Virol.* **83**, 269-283.

Nishiyama, Y. Yamada, Y., Kurachi, R. and Daikoko, T. (1992). Construction of a US3 *lacZ* insertion mutant of HSV-2 and characterisation of its phenotype *in vitro* and *in vivo*. *Virology* **190**, 256-268.

Notarianni, E.L. and Preston, C.M. (1982). Activation of cellular stress protein genes by herpes simplex virus temperature sensitive mutants which overproduce immediate-early polypeptides. *Virology* **123**, 113-122.

Oakes, J.E., Gray, W.L. and Lausch, R.N. (1986). Herpes simplex virus type 1 DNA sequences which direct spread of virus from cornea to central nervous system. *Virology* **150**, 513-517.

Oberman, F. and Panet, A. (1988). Inhibitors of herpes simplex virus immediate early genes in interferon-treated human cells. *J. Gen. Virol.* **69**, 1167-1177.

Oberman, F. and Panet, A. (1989). Characterisation of the early steps of herpes virus replication in interferon-treated human cells. *J. Interferon Res.* **9**, 563-571.

O'Hare, P. (1993). The virion transactivator of herpes simplex virus. *Seminars in Virology* **4**, 145-155.

O'Hare, P. and Goding, C.R. (1988). Herpes simplex virus regulatory elements and the immunoglobulin octamer domain bind a common factor and are both targets for virion transactivation. *Cell* **52**, 435-445.

O'Hare, P., Goding, C.R. and Haigh, A. (1988). Direct combinatorial interaction between a herpes simplex virus regulatory protein and a cellular octamer-binding factor mediates specific induction of virus immediate-early gene expression. *EMBO J.* **7**, 4231-4238.

O'Hare, P. and Hayward, G.S. (1985a). Evidence for a direct role for both the 175,000 and 110,000-molecular weight immediate early proteins of herpes simplex in the transactivation of delayed early promotors. *J. Virol.* **53**, 751-760.

O'Hare, P. and Hayward, G.S. (1985b). Three trans-acting regulatory proteins of herpes simplex virus modulate immediate early gene expressionin a pathway involving negative feedback regulation. *J. Virol.* **56**, 723-733.

Olivio, P.D., Nelson, N.J. and Challberg, M.D. (1988). Herpes simplex virus DNA replication: the UL9 gene encodes an origin-binding protein. *Proc. Natl. Acad. Sci. USA* **83**, 6322-6326.

Ostrander, M. and Cheng, Y.C. (1980). Properties of herpes simplex virus type 1 and type 2 DNA polymerase. *Biochem. Biophys. Acta.* **609**, 232.

Overton, H., McMillan, D., Hope, L. and Wong-Kainin, P. (1994). Production of host shutoff-defective mutants of herpes simplex virus type 1 by inactivation of the UL13 gene. *Virology* **202**, 97-106.

Pappin, D.J.C., Hojrup, P. and Bleasby, A.J. (1993). Rapid identification of proteins by peptide-mass fingerprinting. *Curr. Biol.* **3**, 327-332.

Pappin, D.J.C., Rahman, D., Hansen, H.F., Jeffery, W. and Sutton, C.W. (1994). Peptide-mass fingerprinting as a tool for the rapid identification and mapping of cellular proteins. In: *Methods in protein structural analysis*. Edited by M. Atassi and E. Appella. In press, Plenum, New York.

Parker, R.C. (1980). Conversion of circular DNA to linear strands for mapping. *Methods Enzymol.* **65**, 415-426.

Parker, B.D., Bankier, A., Satchwell, S., Barrell, B and Farrell, P.J. (1990). Sequence and transcription of Raji Epstein-Barr virus DNA spanning the B95-8 deletion region. *Virology* **179**, 339-346.

Patel, A.H. and MacLean, J.B. (1995). The product of the UL6 gene of herpes simplex virus type 1 is associated with virus capsids. *Virology* **206**, 465-478.

Patel, A.H., Rixon, F.J., Cunningham, C. and Davison, A.J. (1996). Isolation and characterisation of herpes simplex virus type 1 mutants defective in the UL6 gene. *Virology* **217**, 111-123.

Patterson, T. and Everett, R.D. (1988a). Mutational dissection of the HSV-1 immediate early protein Vmw175 involved in transcriptional transactivation and repression. *Virology* **166**, 186-196.

Patterson, T. and Everett, R.D. (1988b). The regions of the herpes simplex virus type 1 immediate early protein Vmw175 required for site specific DNA binding closely correspond th those involved in transcriptional regulation. *Nucl. Acids Res.* **16**, 11005-11025.

Patterson, T., Preston, V.G. and Everett, R.D. (1990). A mutant herpes simplex virus type 1 immediate early polypeptide Vmw175 binds to the cap site of it's own promoter *in vitro* but fails to autoregulate *in vivo*. *J. Gen. Virol.* **71**, 851-861.

Pederson, M., Tally-Brown, S. and Millet, R.L. (1981). Gene expression of herpes simplex virus. III. Effect of arabinosyladenine on viral polypeptide synthesis. *J. Virol.* **38**, 712-719.

Pelham, H.R.B. (1986). Speculations on the functions of the major heat shock and glucose-related proteins. *Cell* **46**, 944-959.

Pereira, L., Wolff, H.M., Fenwick, M. and Roizman, B. (1977). Regulation of herpesvirus macromolecular synthesis. V. Properties of polypeptides made in HSV-1 and HSV-2 infected cells. *Virology* **77**, 733-749.

Perng, G.C., Ghiasi, H., Slanina, Nesburn, A.B. and Wechsler, S.L. (1996). High dose ocular infection with a herpes simplex virus type 1 ICP34.5 deletion mutant produces no corneal disease or neurovirulence yet results in *wt* levels of spontaneous reactivation. *J. Virol.* **70**, 2883-2893.

Perng, G.C., Thompson, R.L., Sawtell, N.M., Taylor, W.E., Slanina, S.M., Ghiasi, H., Kaiwar, R., Nesburn, A.B. and Wechsler, S.L. (1995). An avirulent ICP34.5 deletion mutant of herpes-simplex virus type-1 is capable of *in vivo* spontaneous reactivation. *J. Virol.* **69**, 3033-3041.

Perry, L.J., Rixon, F.J., Everett,R.D., Frame, M.C. and McGeoch, D.J. (1986). Characterisation of the IE110 gene of herpes simplex virus type 1. *J. Gen. Virol.* **67**, 2365-2380.

Pertuiset, B., Boccara, M., Cebrian, J., Berthelot, N., Chousterman, S., Puvion-Dutilleui, F., Sisman, J. and Sheldrick, P. (1989). Physical mapping and nucleotide sequence of a herpes simplex virus type 1 gene required for capsid assembly. *J. Virol.* **63**, 2169-2179.

Phillips, B., Abraavaya, K. and Morimoto, R.I. (1991). Analysis of the specificity and mechanism of transcriptional activation of the human hsp 70 gene during infection by DNA viruses. *J. Virol.* **65**, 5680-5692.

Phelan, A., Carmo-Fonesca, M., McLaughlan, J., Lamond, A.I. and Clements, J.B. (1993). A herpes simplex virus type 1 IE gene product, IE63, regulates small nuclear ribonucleoprotein distribution. *Proc. Natl. Acad. Sci. USA* **90**, 9056-9060.

Poffenberger, K.L. and Roizman, B. (1985). A non-inverting genome of a viable herpes simplex virus 1: presence of head-to-tail linkages in packaged genomes and requirements for circularization after infection. *J. Virol.* **53**, 582-595.

Poffenberger, K.L., Idow, A.D., Fraser-Smith, E.B., Raichlen, P.E. and Herman, R.C. (1994). A herpes simplex virus type 1 ICP22 deletion mutant is altered for virulence and latency *in vivo*. *Arch. Virol.* **139**, 111-119.

Poffenberger, K.L.Raichlen, P.E. and Herman, R.C. (1993). *In vitro* characterisation of a herpes simplex virus type 1 ICP22 deletion mutant. *Virus Genes* **7**, 171-186.

Poffenberger, K.L., Tabares, E. and Roizman, B. (1983). Characterisation of a viable, non-inverting herpes simplex virus 1 genome derived by insertion of sequences at the L-S component junction. *Proc. Natl. Acad. Sci. USA* **80**, 2690-2694.

Polvino-Bodnar, M., Orberg, P.K., and Schaffer, P.A. (1987). Herpes simplex virus type 1 ori_L is not required for virus replication or for the establishment and reactivation of latent infection in mice. *J. Virol.* **61**, 3528-3535.

Poon, A.P.W. and Roizman, B. (1993). Characterization of a temperature-sensitive mutant of the U_L15 open reading frame of herpes simplex virus 1. *J. Virol.* **67**, 4497-4503.

Portolani, M., Cermelli, C., Mirandola, P. and Luca, D.D. (1995). Isolation of human herpes virus 7 from an infant with febrile syndrome. *J. Med. Virol.* **45**, 282-283.

Post, L.E., Mackem, S. and Roizman, B. (1981). Regulation of α genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with α gene promoters. *Cell* **24**, 555-565.

Post, L.E. and Roizman, B. (1981). A generalized technique for deletion of specific genes in large genomes: α gene 22 of herpes simplex virus 1 is not essential for growth. *Cell* **25**, 227-232.

Powell, D., Franklin, J., Arisaka, F. and Mosig, G. (1990). Bacteriophage T4 DNA packaging genes 16 and 17. *Nucl. Acids Res.* **18**, 4005.

Powell, K.L., Littler, E. and Purifoy, D.J.M. (1981). Nonstructural proteins of HSV-1. II. Major virus-specific DNA binding protein. *J. Virol.* **39**, 894-902.

Preston, C.M. (1979). Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or temperature-sensitive mutant *ts*K. *J. Virol.* **29**, 275-284.

Preston, C.M. and Russell, J. (1991). Retention of non-linear viral DNA during herpes simplex virus latency *in vitro*. *Intervirology* **32**, 69-75.

Preston, V.G., Al-Kobaisi, M.F., McDougall, I.M. and Rixon, F.J. (1994). The herpes simplex virus gene UL26 proteinase in the presence of the UL26.5 gene product promotes the formation of scaffold-like structures. *J. Gen. Virol.* **75**, 2355-2366.

Preston, V.G., Coates, J.A.V. and Rixon, F.J. (1983). Identification and characterisation of a herpes simplex virus gene product required for encapsidation of virus DNA. *J. Virol.* **45**, 1056-1064.

Preston, V.G., Darling, A.J. and McDougall, I.M. (1988). The herpes simplex virus type 1 temperature sensitive mutant ts1222 has a single base pair deletion in the small subunit of ribonucleotide reductase. *Virology* **169**, 458-467.

Preston, V.G., Davison, A.J., Marsden, H.S., Timbury, M.C., Subak-Sharpe, J.H. and Wilkie, N.M. (1978). Recombinants between herpes simplex virus type 1 and type 2: analysis of genome structures and expression of immediate early polypeptides. *J. Virol.* **28**, 499-517.

Preston, V.G., Rixon, F.J., McDougal, I.M., McGregor, M. And Al-Kobaisi, M.F. (1992). Processing of the herpes simplex virus assembly protein-ICP35 near its carboxy terminal end requires the product of the whole of the UL26 reading frame. *Virology* **186**, 87-98.

Price, R.W., Katz, B.J. and Notkins, A.L. (1975). Latent infection of the peripheral ANS with herpes simplex virus. *Nature* **257**, 686-688.

Purifoy, D.M.J. and Powell, K.L. (1977). Herpes simplex virus DNA polymerase as the site of phosphonoacetate sensitivity: temperature sensitive mutants. *J. Virol.* **24**, 470-477.

Purves, F.C., Spector, D. and Roizman, B. (1991). The herpes simplex virus 1 protein encoded by the US3 gene mediates post translational modification of the phosphoprotein encoded by the UL34 gene. *J. Virol.* **65**, 5757-5764.

Pyles, R.B., Sawtell, N.M. and Thompson, R.L. (1992). Herpes simplex virus type 1 dUTPase mutants are attenuated for neurovirulence, neuroinvasiveness and reactivation from latency. *J. Virol.* **66**, 6706-6713.

Pyles, R.B. and Thompson, R.L. (1994). Evidence that the herpes simplex virus type 1 Uracil DNA glycosylase is required for efficient viral replication and latency in the murine nervous system. *J. Virol.* **68**, 4963-4972.

Quinlan, M.P., Chen, L.B. and Knipe, D.M. (1984). The internuclear localisation of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. *Cell* **36**, 857-868.

Ramakrishnan, R., Levine, M. And Fink, D.J. (1994). PCR-based analysis of herpes simplex virus type 1 latency in the rat trigeminal ganglion established with a ribonucleotide reductase-deficient mutant. *J. Virol.* **68**, 7083-7091.

Ramaswamy, R. and Holland, T.C. (1992). *In vitro* characterization of the HSV-1 UL53 gene product. *Virology* **186**, 579-587.

Rao Basaveswara, V. and Black, L.W. (1988). Cloning, overexpression and purification of the terminase proteins gp16 and gp17 of bacteriophage T4. *J. Mol. Biol.* **200**, 475-488.

Read, G.S. and Frenkel, N. (1983). Herpes simplex virus mutants defective in the virion-associated host shutoff of host polypeptide synthesis and exhibiting abnormal synthesis of the α (immediate-early) viral polypeptides. *J. Virol.* **46**, 498-512.

Read, G.S., Karr, B.M. and Knight, K. (1993). Isolation of a herpes simplex virus type 1 mutant with a deletion in the virion host shutoff gene and identification of multiple forms of the vhs (UL41) polypeptide. *J. Virol.* **67**, 7149-7160.

Reed, L.J. and Meunch, H. (1938). A simple method of estimating fifty percent endpoints. *Am. J. Hygiene* **27**, 493-497.

Resnick, J., Boyd, B.A. and Haffey, M.L. (1989). DNA binding by the herpes simplex virus type 1 ICP4 promotor is necessary for efficient down regulation of the ICP0 promotor. *J. Virol.* **63**, 2497-2503.

Rice, S.A. and Knipe, D.M. (1988). Gene-specific transactivation by herpes simplex virus type 1 alpha protein ICP27. *J. Virol.* **62**, 3814-3823.

Rice, S.A. and Knipe, D.M. (1990). Genetic evidence for two distinct transactivation functions of the herpes simplex virus α protein ICP27. *J. Virol.* **64**, 1704-1715.

Rice, S.A., Lam, V. and Knipe, D.M. (1993). The acidic amino-terminal region of herpes simplex virus type 1 alpha protein ICP27 is required for an essential lytic function. *J. Virol.* **67**, 1778-1787.

Rixon, F.J. (1993). Structure and assembly of herpesviruses. *Seminars in Virology* **4**, 135-144.

Rixon, F.J., Addison, C. and McLauchlan, J. (1992). Assembly of enveloped tegument structures (L-particles) can occur independently of virion maturation in herpes simplex virus type 1-infected cells. *J. Gen. Virol.* **73**, 277-284.

Roberts, M.S., Boundy, A., O'Hare, P., Pizzorno, M.C., Ciufo, D.M. and Hayward, G.S. (1988). Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175 (α4) promoter and a specific binding site for the IE175 (ICP4) protein. *J. Virol.* **62**, 4307-4320.

Robertson, L.M., MacLean, A.R. and Brown, S.M. (1992). Peripheral replication and latency reactivation kinetics of the non-neurovirulent herpes simplex virus type 1 varient 1716. *J. Gen. Virol.* **73**, 967-970.

Rock, D.L. and Fraser, N.W. (1983). Detection of HSV-1 genome in the central nervous system of latently infected mice. *Nature* **302**, 523-525.

Rock, D.L. and Fraser, N.W. (1985). Latent herpes simplex virus type 1 DNA contains two copies of the virion DNA joint region. *J. Virol.* **55**, 849-852.

Rock, D.L., Nesburn, A.B., Ghiasi, H., Ong, J., Lewis, T.L., Lokensgard, J.R. and Wechsler, S.L. (1987). Detection of latency-associated viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. *J. Virol.* **61**, 3820-3826.

Roizman, B. (1979). The structure and isomerisation of herpes simplex virus genomes. *Cell* **16**, 481-494.

Roizman, B. and Batterson, W. (1985). Herpesviruses and their replication. In: *Virology* pp 497-526. B.N. Fields (ed). Raven press, New York.

Roizman, B., Carmichael, L.E., Deinhardt, F., de-The, G., Nahmias, A.J., Plowright, W., Rapp, F., Sheldrick, P., Takahashi, M. and Wolf, K. (1981). Herpesviridae. Definition, provisional nomenclature, and taxonomy. *Intervirology* **16**, 201-217.

Roizman, B., Desrosiers, R.C., Fleckenstein, B., Minson, A.C. and Studdert, M.J. (1992). The family herpesviridae: an update. *Arch. Virol.* **123**, 425-449.

Roizman, B. and Jenkins, F.J. (1985). Genetic engineering of novel genomes of large DNA viruses. *Science* **229**, 1208-1214.

Roizman, D. and Roane, P.R. (1964). The multiplication of herpes simplex virus. II. The relation between protein synthesis and the duplication of viral DNA in infected Hep-2 cells. *Virology* **22**, 262-269.

Roizman, B. and Sears, A.E. (1987). An enquiry into the mechanisms of herpes simplex virus latency. *Ann. Rev. Microbiol.* **41**, 543-571.

Roizman, B. and Sears, A.E. (1990). Herpes simplex viruses and their replication. In: *Virology* 2nd Ed. 65, pp 1795-1841. B.N. Fields, D.M. Knipe et al., (ed's). Raven, New York.

Roizman, B. And Sears, A.E. (1993). Herpes simplex viruses and their replication. In: *The human herpesviruses*, pp11-68. Edited by Roizman *et al.* Raven press, NY.

Roller, R.J. and Roizman, B. (1992). Herpes simplex virus 1 RNA-binding protein-US11 negatively regulates the accumulation of a truncated viral messenger-RNA. *J. Virol.* **65**, 5873-5879.

Romanelli, M.G., Mavromaranazos, P., Spector, D. And Roizman, B. (1992). Mutational analysis of the ICP4 binding-sites in the 5' transcribed noncoding domains of the herpes simplex virus- 1 UL49.5 gamm-2 gene. *J. Virol.* **66**, 4855-4863.

Romanowski, E., Aravllocruz, T., Zhang, Y., McKnight, J.L.C. and Gordon, J. (1991). Acute pathogenicity of HSV-1 UL46 and UL47 deletion mutants in Balb/c mice and New Zealand rabbits. *Invest. Opthalmol. Vis. Sci.* **31**, 853.

Roop, C., Hutchinson, L. and Johnson, D.C. (1993). A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells, and its particles lack glycoprotein H. *J. Virol.* **67**, 2285-2297.

Rosen, A., Gelderblom, H. and Darai, G. (1985). Transduction of virulence in herpes simplex virus type 1 from a pathogenic to a non pathogenic strain by a cloned viral DNA fragment. *Med. Microbiol. Immun. 173*, 257-278.

Rosen, A., Ernst, F., Koch, H.G., Gelderblom, H., Darai, G., Hadar, J., Tabor, E., Ben-Hur, T. and Becker, Y. (1986). Replacement of the deletion in the genome (0.762-0.789 mu) of avirulent HSV-1 HFEM using cloned MluI DNA fragment (0.7615-0.796 mu) of virulent HSV-1 F leads to generation of virulent intretypic recombinant. *Virus Research* **5**, 157-175.

Rosen-Wolff, A. and Darai, G. (1991). Identification and mapping of the UL56 gene transcript of herpes simplex virus type 1. *Virus Res.* **19**, 115-126.

Rosen-Wolff, A., Ben-Hur, T., Becker, Y. and Darai, G. (1988). Comparative analysis of the transcripts mapped in the BamHI DNA fragment B of avirulent HSV-1 HFEM, virulent HSV-1 F and their intertypic recombinant. *Virus Res.* **10**, 315-324.

Rosen-Wolff, A., Lamade, W., Berkowitz, C., Becker, Y. And Darai, G. (1991). Elimination of UL56 gene by insertion of LacZ cassette between nucleotide position 116030 to 121753 of the herpes simplex virus type 1 genome abrogates intraperitoneal pathogenicity in tree shrews and mice. *Virus Res.* **20**, 205-221.

Russell, J. and Preston, C.M. (1986). An *in vitro* latency system for herpes simplex virus type 2. *J. Gen. Virol.* **67**, 397-403.

Russell, J., Stow, N.D., Stow, E.C. and Preston, C.M. (1987a). Herpes simplex virus genes involved in latency *in vitro*. *J. Gen. Virol.* **68**, 3009-3018.

Russell, J., Stow, N.D., Stow, E.C. and Preston, C.M. (1987b). Abnormal forms of herpes simplex virus IE polypeptide Vmw175 induce the cellular stress response. *J. Gen. Virol.* **68**, 3009-3018.

Ruvkun, G. and Finney, M. (1991). Regulation of transcription and cell identity by POU domain proteins. *Cell* **64**, 475-476.

Sacks, R.W., Greene, C.C., Aschman, D.P. and Schaffer, P.A. (1985). Herpes simplex virus type 1 ICP27 is an essential regulatory protein. *J. Virol.* **55**, 796-805.

Sadowski, I., Triezenberg, S. and Ptashne, M. (1988). GAL4-VP16 is an unusually potent transcriptional activator. *Nature* **335**, 563-564.

Sakaoka, H., Kurita, K., Gouro, T., Kumamoto, Y., Sawada, S., Ihara, M. and Kawana, T. (1995). Analysis of genomic polymorphism among herpes simplex virus type-2 isolates from 4 areas of Japan and 3 other countries. *J. Med. Virol.* **45**, 259-272.

Sakaoka, H., Kurita, K., Iida, Y., Takada, S., Umene, K., Kim, Y.T., Ren, C.S. and Nahmias, A.J. (1994). Quantitative analysis of genomic polymorphism of herpes simplex virus type-1 strains from 6 countries - studies of molecular evolution and molecular epidemiology of the virus. *J. Gen. Virol.* **75**, 513-527.

Salohuddin, S.Z., Abalashi, D.V., Markham, P.D., Josephs, S.F., Sturzenegger, S., Kaplan, M., Halligan, G., Biberfeld, P., Wong, S.F. and Kramarsky, B. (1986). Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. *Science* **232**, 596-600.

Samuel, C.E. (1991). Antiviral actions of interferon: interferon-regulated proteins and their surprisingly selective antiviral activities. *Virology* **183**, 1-11.

Sandri-Golden, R.M., Hibbard, M.K. and Hardwicke, M.A. (1995). The c-terminal repressor region of herpes simplex virus type 1 ICP27 is required for the redistribution of small nuclear ribonucleoprotein particles and splicing factor SC35; however, these alterations are not sufficient to inhibit host cell splicing. *J. Virol.* **69**, 6063-6076.

Sandri-Goldin, R.M. and Mendoza, G.E. (1992). A herpes virus regulatory protein appears to act post-transcriptionally by affecting mRNA processing. *Genes Dev.* **6**, 848-863.

Sawtell, N.M. and Thompson, L.R. (1992a). Rapid *in vivo* reactivation of herpes simplex virus in latently infected murine ganglionic neurons after transient hyperthermia. *J. Virol.* **66**, 2150-2156.

Sawtell, N.M. and Thompson, L.R. (1992b). Herpes simplex virus type 1 latency-associated transcriptional unit promotes anatomical site-dependent establishment and reactivation from latency. *J. Virol.* **66**, 2157-2169.

Schaffer, P.A., Preston, V.G., Wagner, E.K. and Devi-Rao, G.B. (1987). Herpes simplex virus. In: *Genetic. Maps.* 4, pp 93-98. Cold Spring Harbor Laboratories.

Schaffer, P., Vonka, V., Lewis, R. and Benyesh-Melnick, M. (1970). Temperature-sensitive mutants of herpes simplex virus. *Virology* **42**, 1144-1146.

Schek, N. and Bachenheimer, S.L. (1985). Degradation of cellular messenger-RNAs induced by a virion-associated factor during herpes-simplex virus-infection. *J. Virol.* **55**, 601-610.

Scherer, W.F., Syverton, J.T. and Gey, G.O. (1953). Studies on the propagation *in vitro* of poliomyelitis viruses IV: Viral mulitplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. *J. Exp. Med.* **97**, 695-709.

Schmitz, J.B., Albright, A.G., Kinehington, P.R. and Jenkins, F.J. (1995). The UL37 ptotein of herpes simplex virus type 1 is associated with the tegument of purified virions. *Virology* **206**, 1055-1065.

Schrag, J.D., Venkataram, Prasad, B.V., Rixon, F.J. and Chiu, W. (1989). Three dimentional structure of the herpes simplex virus type 1 nucleocapsid. *Cell* **56**, 651-660.

Schroder, H.C., Falke, D., Weise, K., Bachmann, M., Carmo-Fonseca, M., Zaubitzer, T. And Muller, W./E.G. (1989). Change of processing and nucleocytoplasmic transport of mRNA in HSV-1 infected cells. *Virus Res.* **13**, 61-78.

Schroder, C.H., Stegmann, B., Lauppe, H.F. and Kaerner, H.C. (1976). A universal defective genotype derived from herpes simplex virus strain ANG. *Intervirol.* **6**, 270-284.

Sears, A.E., Halliburten, I.W., Meignier, B., Silver, S. and Roizman, B. (1985). Herpes simplex virus type 1 mutant deleted in the α 22 gene: Growth and gene expression in permissive and restricted cells and establishment of latency. *J. Virol.* **55**, 338-346.

Sen, G.C. and Lengyel, P. (1992). The interferon system: a birds eye view of its biochemistry. *J. Biol. Chem.* **267**, 5017-5020.

Serafini-Cessi, F., Dall'Olio, F., Scannavini, M. and Campadelli-Fiume, G. (1983). Processing of herpes simplex virus 1 in cells defective in glycosyl transferases of the Golgi system: relationship to cell fusion and virion egress. *Virology* **131**, 59-70.

Shao, L., Rapp, L.M. and Weller, S.K. (1993). Herpes simplex virus 1 alkaline nuclease is required for the efficient egress of capsids from the nuclei. *Virology* **196**, 146-162.

Sheih, M-T. and Spear, P.G. (1991). Fibroblast growth factor: does it have a role in the binding of herpes simplex virus ? *Science* **253**, 208-209.

Sheih, M-T., WuDunn, D., Montgomery, R.I., EsKo, J.D. and Spear, P.G. (1992). Cell surface receptors for herpes simplex virus are heparan sulphate proteoglycans. *J. Cell Biol.* **116**, 1273-1281.

Sheldrick, P. and Berthelot, N. (1974). Inverted repetitions in the chromosome of herpes simplex virus. Cold Spring Harbor Symposia on Quantitative Biology, 39, part 2.

Shelton, L.S.G., Pensiero, M.N. and Jenkins, F.J. (1990). Identification and characterisation of the herpes simplex virus type 1 protein encoded by the UL37 open reading frame. *J. Virol.* **64**, 6101-6109.

Shepard, A.A., Imbalanzo, A.N. and DeLuca, N.A. (1989). Separation of the primary structural components conferring autoregulation transactivation and DNA binding properties of the herpes simplex virus transcriptional regulatory protein ICP4. *J. Virol.* **63**, 3714-3728.

Sherman, G., Gottlieb, J. and Challberg, M.D. (1992). The UL8 subunit of the herpes simplex virus helicase-primase complex is required for efficient primer utilisation. *J. Virol.* **66**, 4884-4892.

Shimeld, C., Hill, T., Blyth, B. and Easty, D. (1989). An improved model of recurrent herpetic eye disease in mice. *Curr. Eye Res.* **8**, 1193-1205.

Sinclair, M.C. (1992). Antisence RNA transcription regulates IE2 expression in the HSV-1 deletion varient 1703. Ph.D. Thesis, Glasgow University.

Smibert, C.A., Johnson, D.C. and Smiley, J.R. (1992). Identification and characterisation of the virion-induced host shutoff product of herpes simplex virus gene UL41. *J. Gen. Virol.* **73**, 467-470.

Smibert, C.A., Popova, B., Xiao, P., Capone, J.P. and Smiley, J.R. (1994). HSV VP16 forms a complex with the virion host shutoff protein *vhs*. *J. Virol.* **68**, 2339-2346.

Smibert, C.A. and Smiley, R.J. (1990). Differential regulation of endogenous and transduced β globin genes during infection of erythroid cells with a herpes simplex virus type 1 recombinant. *J. Virol.* **64**, 3882-3894.

Smiley, J.R., Johnson, D.C., Pizer, I.L. and Everett, R.D. (1992). The ICP4 binding site in the herpes simplex virus type 1 glycoprotein D (gD) promotor are not essential for efficient gD transcription during virus infection. *J. Virol.* **66**, 623-631.

Smith, C.A., Bates, P., Rivergonzalez, R., Gu, B.H. and DeLuca, N.A. (1993). ICP4, the major transcriptional regulatory protein of herpes simplex virus type 1, forms a tripartite complex with TATA-binding protein and TFIIB. *J. Virol.* **67**, 4676-4687.

Smith, I.L., Hardwicke, M.A. and Sandri-Goldin, R.M. (1992). Evidence that the herpes simplex virus immediate early protein ICP27 acts post-transcriptionally during infection to regulate gene expression. *Virology* **186**, 74-86.

Smith, C.A., Marchetti, M.E., Edmonsin, P. and Schaffer, P.A. (1989). Herpes simplex virus type 2 mutants with deletions in the intergenic region between ICP4 and ICP22/47: identification of nonessential *cis*-acting elements in the context of the viral genome. *J. Virol.* **63**, 2036-2047.

Smith, R.F. and Smith, T.F. (1989). Identification of new protein kinase-related genes in three herpesviruses, herpes simplex virus, varicella-zoster virus, and Epstein-Barr virus. *J. Virol.* **63**, 450-455.

Spadari, S. (1976). Properties of DNA ligase from uninfected and virus infected HeLa cells. *Nucl. Acids Res.* **3**, 2155-2167.

Spear, P.G. (1985). Glycoproteins specified by herpes simplex viruses. In: *The Herpesviruses* 3, pp 315-356. Roizman, B., ed. Plenum press, New York and London,.

Spear, P.G. and Roizman, B. (1972). Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpes virion. *J. Virol.* **9**, 431-439.

Spivack, J.G., Fareed, M.U., Valgi-Nagy, T., Nash, A.C., O'Keefe, J.S., Gesser, R.M., McKie, E.A., MacLean, A.R., Fraser, N.W. and Brown, S.M. (1995). Replication, establishment of latent infection, expression of the latency-associated transcripts and explant reactivation of herpes simplex virus type 1 α 34.5 mutants in a mouse eye model. *J. Gen. Virol.* **76**, 321-332.

Spivack, J.G. and Fraser, N.W. (1987). Detection of herpes simplex virus type 1 transcripts during latent infection in mice. *J. Virol.* **61**, 3841-3847.

Spivack, J.G., Woods, G.M. and Fraser, N.W. (1991). Identification of a novel latency-specific splice donor signal within the herpes simplex virus type 1 2.0-kilobase latency-associated transcript (LAT); translation inhibition of LAT open reading frames by the intron within the 2.0-kilobase LAT. *J. Virol.* **65**, 6800-6810.

Sprecher, E. and Becker, Y. (1986). Skin langerhans play an essential role in the defence against herpes simplex virus type 1 infection. *Arch. Virol.* **91**, 341- 349.

Stanberry, L.R., Bourne, N., Bravo, F.J. and Bernstein, D.I. (1992). Capsaicin-sensitive peptidergic neurons are involved in the zosteriform spread of herpes simplex virus infection. *J. Med. Virol.* **38**, 142-146.

Stanberry, L.R., Kern, E.R., Richards, J.T. and Overall Jr, J.C. (1985b). Recurrent genital herpes simplex virus infection in guinea pigs. *Intervirology* **24**, 226-231.

Stanberry, L.R., Kit, S. and Myers, M.G. (1985a). Thymidine kinase deficient herpes simplex virus type 2 infection in guinea pigs. *J. Virol.* **55**, 322-328.

Steiner, I. and Kennedy, P.G.E. (1991). Herpes simplex virus latency in the nervous system - a new model. *Neuropathol. Appl. Neurobiol.* **17**, 433-440.

Steiner, I. And Kennedy, P.G.E. (1993). Molecular biology of herpes simplex virus type 1 latency in the nervous system. *Mol. Neurobiol.* **17**, 137-159.

Steiner, I., Spivack, J.G., Deshmane, S.L., Ace, C.I., Preston, C.M. and Fraser, N.W. (1990). A herpes simplex virus type 1 mutant containing a non-transinducing Vmw65 protein establishes latent infection *in vivo* in the absence of viral replication and reactivates efficiently from explanted trigeminal ganglia. *J. Virol.* **64**, 1630-1638.

Steiner, I., Spivack, J.G., Lirette, R.P., Brown, S.M., MacLean, A.R., Subak-Sharpe, J. and Fraser, N.W. (1989). Herpes simplex virus type 1 latency-associated transcripts are evidently not essential for latent infection. *EMBO J.* **8**, 505-511.

Stern, S. and Herr, W. (1991). The herpes simplex virus *trans*-activator VP16 recognises the Oct-1 homeo domain: evidence for a homeo domain recognition subdomain. *Genes Dev.* **5**, 2555-2566.

Stern, S., Tanaka, M. and Herr, W. (1989). The Oct-1 homeo-domain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16. *Nature* **341**, 624-630.

Stevens, J.G. (1978). Latent characteristics of selected herpesviruses. *Adv. Cancer Res.* **26**, 227-256.

Stevens, J.G. and Cook, M.L. (1971). Latent herpes simplex virus in spinal ganglia of mice. *Science* **173**, 843-845.

Stevens, J.G., Haarr, L., Porter, D.D., Cook, M.L. and Wagner, E.K. (1988). Prominence of the herpes simplex virus latency-associated transcript in trigeminal ganglia from seropositive humans. *J. Infect. Dis.* **158**, 117-122.

Stevens, J.G., Wagner, E.K., Devi-Rao, G.B., Cook, M.L. and Feldman, L.T. (1987). RNA complementary to a herpesvirus alpha gene mRNA is promenent in latently infected neurons. *Science* **235**, 1056-1059.

Stow, N.D. (1992). Herpes simplex virus origin-dependant DNA replication in insect cells using recombinant baculoviruses. *J. Gen. Virol.* **73**, 313-321.

Stow, N.D. (1993). Sequences at the C-terminus of the herpes simplex virus type 1 UL30 protein are dispensable for DNA-polymerase-activity but not for viral origin-dependent DNA-replication. *Nucl. Acids Res.* **21**, 87-92.

Stow, N.M. and McMonagle, E.C. (1983). Characterisation of the TR_S/IR_S origin of replication of HSV-1. *Virology* **130**, 427-438.

Stow, N.D. and Stow, E.C. (1986). Isolation and characterisation of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110. *J. Gen. Virol.* **67**, 2571-2585.

Strelow, L.I. and Leib, D.A. (1995). Role of virion host shutoff (*vhs*) of herpes simplex virus type 1 in latency and pathogenesis. *J. Virol.* **69**, 6779-6786.

Strelow, L.I. and Leib, D.A. (1996). Analysis of conserved domains of UL41 of herpes simplex virus type 1 in virion host shutoff and pathogenesis. *J. Virol.* **70**, 5665-5667.

Strobel-Fidler, M. and Francke, B. (1980). Alkaline deoxyribonuclease induced by herpes simplex virus type 1: composition and properties of the purified enzyme. *Virology* **103**, 493-501.

Su, L. and Knipe, D.M. (1989). Herpes simplex virus α protein ICP27 can inhibit or augment viral gene transactivation. *Virology* **170**, 496-504.

Su, Y.H., Oakes, J.E. and Lausch, R.N. (1993). Mapping of the genetic region coding for herpes simplex virus-resistance to mouse interferon-alpha/beta. *J. Gen. Virol.* **74**, 2315-2332.

Subak-Sharp H. (1969). Proceedings of the first international congress for virology, Helsinki, 1968. p 252.

Sunstrum, J.C., Chrisp, C.E., Levine, M. and Glorioso, J.C. (1988). Pathogenicity of glycoprotein C negative mutants of herpes simplex virus type 1 for the mouse central nervous system. *Virus Res.* **11**, 17-32.

Sussman, M.D., Kutish, G. and Roberts, P. (1992). Identification of an African swine fever virus gene with similarity to a myeloid differentiation primary response gene and a neurovirulence-associated gene of herpes-simplex virus. *J. Virol.* **66**, 5586-5589.

Svennerholm, B., Jeansson, S., Vahlne, A. and Lycke, E. (1991). Involvement of Glycoprotein C (gC) in adsorption of herpes simplex virus type 1 (HSV-1) to the cell. *Arch. Virol.* **120**, 273-279.

Szilagyi,, J.F. and Cunningham, C. (1991). Identification and characterisation of a novel non-infectious herpes simplex virus-related particle. *J. Gen. Virol.* **72**, 661-668.

Talsinger, R., Eisenberg, R.J., Valyinagy, T., Fraser, N.W. and Cohen, G.H. (1994). N-linked oligosaccharides on herpes-simplex virus glycoprotein gD are not essential for establishment of viral latency or reactivation in the mouse eye model. *Virology* **202**, 1050-1053.

Tatman,J.D., Preston, V.G., Nicholson, P., Elliott, R.M. and Rixon, F.J. (1994). Assembly of herpes simplex virus type 1 capsids using a panel of recombinant baculoviruses. *J. Gen. Virol.* **75**, 1101-1113.

Tedder, D.G., Everett, R.D., Wilcox, K.W., Beard, P. and Pizer, L.I. (1989). ICP4 binding sites in the promoter and coding regions of the herpes simplex virus gD gene contribute to activation of *in vitro* transcription by ICP4. *J. Virol.* **63**, 2510-2520.

Telford, E.A.R., Studdert, M.J., Agius, C.T., Watson, M.S., Aird, H.C. and Davison, A.J. (1993). Equine herpesviruses-2 and herpesviruses-5 are gamma-herpesviruses. *Virology* **195**, 491-499.

Telford, E.A.R., Watson, M.S., McBride, K. and Davison, A.J. (1992). The DNA sequence of equine herpes virus-1. *Virology* **189**, 304-316.

Telford, E.A.R., Watson, M.S., Aird, H.C., Perry, J. and Davison, A.J. (1995). The DNA sequence of equine herpes virus-2. *J. Mol. Biol.* **249**, 520-528.

Tengelsen, L.A., Pederson, N.E., Shaver, P.R., Wathen, M.W. and Homa, F.L. (1993). Herpes simplex virus type 1 DNA cleavage and encapsidation require the product of the UL28 gene: isolation and characterisation of two UL28 deletion mutants. *J. Virol.* **67**, 3470-3480.

Tenser, R.B. (1991). Role of herpes simplex virus thynidine kinase expression in viral pathogenesis and latency. *Intervirol.* **32**, 76-92.

Tenser, R.B., Ressel, S. and Dunstan, M.E. (1981). Herpes simplex virus thymidine kinase expression in trigeminal ganglion infection: correlation of enzyme activity with ganglion virus titre and evidence of *in vivo* complementation. *Virology* **112**, 328-241.

Thomas, M.S., Gao, M., Knipe, D.M. and Powell, K.L. (1992). Association between the herpes simplex major DNA-binding protein and alkaline nuclease. *J. Virol.* **66**, 1152-1161.

Thompson, R.L., Cook, M.L., Devi-Rao, G.B., Wagner, E.K. and Stevens, J.G. (1986). Functional and molecular analyses of the avirulent wild-type herpes simplex virus type 1 strain KOS. *J. Virol.* **58**, 203-211.

Thompson, R.L., Rogers, S.K. and Zerhusen, M.A. (1989). Herpes simplex virus neurovirulence and productive infection of neuronal cells is associated with a function

which maps between 0.82 and 0.832 map units on the HSV genome. *Virology* **172**, 435-450.

Thompson, R.L. and Stevens, J.G. (1983). Biological characterisation of a herpes simplex virus intertypic recombinant which is completely and specifically non-neurovirulent. *Virology* **131**, 171-179.

Thompson, R.L. and Wagner, E.K. (1988). Partial rescued herpes simplex virus neurovirulence with a 3.2 kb cloned fragment. *Virus Genes* **1**, 261-273.

Thompson, R.L., Wagner, E.K. and Stevens, J.G. (1983). Physical location of a herpes simplex virus type 1 function (s) specifically involved in a 10 million fold increase in HSV neurovirulence. *Virology* **131**, 180-192.

Thomsen, D.R., Roof, L.L. and Homa, F.L. (1994). Assembly of herpes simplex virus (HSV) intermediate capsids in insect cells infected with recombinant baculoviruses expressing HSV capsid proteins. *J. Virol.* **68**, 2442-2457.

Timbury, M.C. (1991). Herpes simplex virus infections. In: *Notes on medical virology* pp 84-98. Churchhill Livingstone.

Timbury, M.C., Theriault, A. and Elton, R.A. (1974). A stable syncitial mutant of herpes simplex type 2 virus. *J. Gen. Virol.* **23**, 219-224.

Tomazin, R., Hill, A.B., Jugovic, P., York, I., van Endert, P., Ploegh, H.L., Andrews, D.W. and Johnson, D.C. (1996). Stable binding of the herpes simplex virus ICP47 protein to the peptide binding site of TAP. *EMBO. J.* **15**, 3256-3266.

Torrisi, M.R., DiLazzaro, C., Pavan, A., Pereira, L. and Campadelli-Fume, G. (1992). Herpes simplex virus envelopment and maturation studied by fracture label. *J. Virol.* **66**, 554-561.

Turk, S.R., Kik, N.A., Birch, G.M., Chiego Jr, D.J. and Shipman Jr, C. (1989). Herpes simplex virus type 1 ribonucleotide reductase null mutants induce lesions in guinea pigs. *Virology* **173**, 733-735.

Umene, K. (1986). Conversion of a fraction of the unique sequence to part of the inverted repeats on the S component of the herpes simplex virus type 1 genome. *J. Gen. Virol.* **67**, 1035-1048.

Valyi-Nagy, T., Deshmane, S.L., Spivack, J.G., Steiner, I., Ace, C.I., Preston, C.M. and Fraser, N.W. (1991a). Investigation of herpes virus type 1 (HSV-1) gene expression and DNA synthesis during the establishment of latent infection by an HSV-1 mutant, *in*1814, that does not replicate in mouse trigeminal ganglia. *J. Gen. Virol.* **72**, 641-649.

Valyi-Nagy, T., Deshmane, S., Dillner., A. and Fraser, N.W. (1991b). Induction of cellular transcription factors in trigeminal ganglia of mice by corneal sacrification, herpes simplex virus type 1 infection, and explantation of trigeminal ganglia. *J. Virol.* **63,** 4142-4152.

Van Zijl, M., Quint, W., Briaire, J., De Rover, T., Gielkens, A. and Berns, A. (1988). Regeneration of herpesviruses from molecularly cloned subgemomic fragments. *J. Virol.* **62,** 2191-2195.

Varmnza, S.L. and Smiley, R.J. (1985). Signals for site-specific cleavage of HSV DNA: maturation involves two separate cleavage events at sites distal to the recognition sequences. *Cell* **41,** 793-802.

Vaughn, P.J., Thibault, K.T., Hardwicke, M.A. and Sandri-Goldin, R.M. (1992). The herpes simplex virus immediate early protein ICP27 encodes a potential metal binding domain and binds zinc *in vitro*. *Virology* **189,** 377-384.

Visalli, R.J. and Brandt, C.R. (1991). The HSV-1 UL45 gene-product is not required for growth in Vero cells. *Virology* **185,** 419-423.

Visalli, R.J. and Brandt, C.R. (1993). The HSV-1 UL45 18 kDa gene-product is a true late protein and a component of the virion. *Virus Res.* **29,** 167-178.

Vlazny, D.A. and Frenkel, N. (1981). Replication of herpes simplex virus DNA: location of replication recognition signals within defective virus genomes. *Proc. Natl. Acad. Sci. USA* **78,** 742-746.

Vlcek, C., Benes, V., Lu, Z., Kutish, G.F., Paces, V., Rock, D., Letchworth, G.J. and Schwyzer, M. (1995). Nucleotide sequence analysis of a 30-kb region of the bovine herpesvirus 1 genome which exhibits a colinear gene arrangement with the UL21 to UL4 genes of herpes simplex virus. *Virology* **210,** 100-108.

Wagner, E.K. (1985). Individual HSV transcripts. In: *The Herpesviruses* vol 3 pp 45-104. B. Roizman (ed). Plenun publishing corp. New York.

Wagner, E.K. (1994). Molecular biology. In: *Encyclopedia of virology* vol 2 pp 593-603. Edited by R.G. Webster and A. Granoff. Academic press.

Wagner, E.K., Devi-Rao, G., Feldman, L.T., Dobson, A.T., Zhang, Y.F., Flanagan, W.M. and Stevens, J.G. (1988a). Physical characterisation of the herpes simplex virus latency-associated transcripts in neurons. *J. Virol.* **62,** 1194-1202.

Wagner, E.K., Flanagan, W.M., Devi-Rao, G., Zhang, Y.F., Hill, J.M., Anderson, K.P. and Stevens, J.G. (1988b). The herpes simplex virus latency-associated transcript is spliced during the latent phase of infection. *J. Virol.* **62,** 4577-4585.

Wagner, M.J. and Summers, W.C. (1978). Structure of the joint region and the termini of the DNA of herpes simplex virus type 1. *J. Virol.* **27**, 374-387.

Ward, P.L., Barker, P.E. and Roizman, B. (1996). A novel herpes simplex virus-1 gene, U(L) 43.5, maps antisence to the U(L) 43 gene and encodes a protein which colocalises in nuclear-structures with capsid proteins. *J. Virol.* **70**, 2624-2690.

Weber, P.C., Levine, M., Glorioso, J.C. (1987). Rapid identification of nonessential genes of herpes simplex virus type 1 by Tn5 mutagenesis. *Science* **236**, 576-579.

Weber, P.C. and Wigdahl, B. (1992). Identification of dominant-negative mutants of herpes simplex virus type 1 immediate-early protein ICP0. *J. Virol.* **66**, 2261-2267.

Weiheimer, S.D., Boyd, B.A., Durham, S.K., Resnick, J.L. and O'Boyle, D.R. (1992). Deletion of the VP16 open reading frame of herpes simplex virus type 1. *J. Virol.* **66**, 258-269.

Weller, S.K., Aschman, D.P., Sacks, W.R., Coen, D.M. and Schaffer, P.A. (1983). Genetic analysis of temperature sensitive mutants of HSV-1: the combined use of complementation and physical mapping for cistron assignment. *Virology* **130**, 290-305.

Weller, S.K., Carmichael, E.D., Aschman, D.P., Goldstein, D.J. and Schaffer, P.A. (1987). Genetic and phenotypic characterisation of mutnts in 4 essential genes that map to the left half if the HSV-1 UL DNA. *Virology* **161**, 1198-1210.

Weller, S.K., Seghatoleslami, M.R.. Shae, L., Rowse, D. and Carmichael, E.P. (1990). The herpes simplex virus type 1 alkaline nuclease is not essential for viral DNA synthesis: isolation and characterisation of a *lacZ* insertion mutant. *J. Gen. Virol.* **71**, 2941-2952.

Weller, S.K., Sparado, A., Schaffer, J.E., Murray, A.W., Maxam, A. and Schaffer, P.A. (1985). Cloning, sequencing and functional analysis of ori_L, a herpes simplex virus type 1 origin of DNA synthesis. *Mol. Cell Biol.* **5**, 930-942.

Werstuck, G. and Caopne, J.P. (1989). Mutational analysis of the herpes simplex virus *trans*-inducing factor Vmw65. *Gene* **75**, 213-224.

White, D.O. and Fenner, F.J. (1986). Herpesviruses. In: *Medical virology*, third edn. Ch 16, pp 401-431. Academic press.

Whitley, R.J. (1985). Epidemiology of herpes simplex viruses. In: *The Herpesviruses* vol 3. B. Roizman (ed). Plenum Press, New York and London.

Whitley, R.J. (1990). Herpes simplex viruses. In: *Virology* (2nd edn), pp 1844-1877. B.N. Fields, D.M. Knipe, R.M. Chanode, M.S. Hirsch, J.L. Melnick, T.P. Monath and B. Roizman (eds), Raven press New York.

Wigdahl, B.L., Scheck, A.C., Declercq, E. and Rapp, F. (1982). High efficiency latency and reactivation of herpes simplex virus in human cells. *Science* **217**, 1145-1146.

Wigdahl, B.L., Schech, A.C., Ziegler, R.J., Declercq, E. and Rapp, F. (1984). Analysis of the herpes simplex virus genome during latency in human diploid fibroblasts and rat sensory neurons. *J. Virol.* **49**, 205-213.

Wigdahl, B.L., Ziegler, R.J., Sneve, M. and Rapp, F. (1983). Herpes simplex virus latency and reactivation in isolated rat sensory neurons. *Virology* **127**, 159-167.

Wilcox, C.L. and Johnson, E.M. (1987). Nerve growth factor deprivation results in reactivation of latent herpes simplex virus *in vitro*. *J. Virol.* **61**, 2311-2315.

Wilcox, C.L. and Johnson, E.M. (1988). Characterisation of nerve growth factor-dependent herpes simplex virus latenct in neurons *in vitro*. *J. Virol.* **62**, 393-399.

Wilcox, C.L., Smith, R.L., Freed, C.R. and Johnson, E.M. (1990). Nerve growth factor-dependence of herpes simplex virus latency in peripheral symathetic and sensory neurons *in vitro*. *J. Neurosci.* **10**, 1268-1275.

Wilkie,N.M. (1973). The synthesis and substructure of herpesvirus DNA: the distribution of alkali-labile single strand interruptions in HSV-1 DNA. *J. Gen. Virol.* **21**, 453-467.

Wilkie, N.M. (1976). Physical maps for herpes simplex virus type 1 DNA for restriction endonucleases *Hind* III, *Hpa* I and *Xba* I. *J. Gen. Virol.* **20**, 222-233.

Wilson, D.W., Davispoynter, N. And Minson, A.C. (1994). Mutations in the cytoplasmic tail of herpes simplex virus glycoprotein-H suppress cell-fusion by a syncitial strain. *J. Virol.* **68**, 6985-6993.

Wing, B.A., Lee, G.C.Y. and Huang, E.S. (1996). The human cytomegalovirus UL94 open reading frame encodes a conserved herpes virus capsid/tegument-associated virion protein that is expressed with true late kinetics. *J. Virol.* **70**, 3339-3345.

Wu, C.A., Nelson, N.J., McGeoch, D.J. and Challberg, M.D. (1988). Identification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis. *J. Virol.* **62**, 435-443.

WuDunn, D. and Spear, P.G. (1989). Initial interaction of herpes simplex virus with cells is binding to heparan sulphate. *J. Virol.* **63**, 52-58.

Yahasz, S.A. and Stevens, J.G. (1993). Glycoprotein B is a specific determinant of herpes simplex virus type 1 neurovirulence. *J. Virol.* **67**, 5948-5954.

Yamada, M., Uno, F. and Nii, S. (1986). *In vitro* cytopathology and pathogenicity to inbread mice shown by five varients of a laboratory strain of type 1 herpes simplex virus. *Arch. Virol.* **90**, 183-196.

Yamanishi, K., Okuno, T., Shiraki, K., Takahashi, M., Kondo, T., Asano, Y. and Kurata, T. (1988). Identification of human herpesvirus-6 as a causitive agent for exanthem-subitum. *Lancet* **1**, 1065-1067.

Yang, Y.T. and Courtney, R.J. (1995). Influence of the host-cell on the association of ICP4 and ICP0 with herpes-simplex virus type-1. *Virology* **211**, 209-217.

Yoa, F. and Courtney, R.J. (1989). A major transcriptional regulatory protein (ICP4) of herpes simplex virus type 1 is associated with purified virions. J. Virol. 63, 3338-3344.

Yoa, F. and Courtney, R.J. (1992). Association of ICP0 but not ICP27 with purified virions of of herpes simplex virus type 1. *J. Virol.* **66**, 2709-2716.

Yoa, F. And Schaffer, P.A. (1994). Physical interaction between herpes simplex virus type 1 inmmediate early regulatory proteins ICP6 and ICP4. *J. Virol.* **68**, 8158-8168.

York, I.A., Roop, C., Andrews, D.W., Riddell, S.R., Graham, F.L. and Johnson, D.C. (1994). A cytosolic herpes-simplex virus protein inhibits antigen presentation to CD8(+) T lymphocytes. *Cell* 77, 525-535.

Youssoufian, H., Hammer, S.M., Hirsch, M.S. and Mulder, C. (1982). Methylation of the viral genome in an *in vitro* model of herpes simplex virus latency. *Proc. Natl. Acad. Sci. USA* **79**, 2207-2210.

Zhang, Y. And McKnight, J.L.C. (1993). Herpes simplex virus type 1 UL46 and UL47 deletion mutants lack VP11 and VP12 or VP13 and VP14, respectively, and exhibit altered viral thymidine kinase expression. *J. Virol.* **67**, 1482-1492.

Zhang, Y., Sirko, D.A. and McKnight, J.L.C. (1991). Role of herpes simplex virus type 1 UL46 and UL47 in alpha-tif-mediated transcriptional induction - characterisation of three viral deletion mutants. *J. Virol.* **65**, 829-841.

Zhu, L. And Weller, S.K. (1992a). The six conserved helicases of the UL5 gene-product, a component of the herpes-simplex virus type-1 helicase-primase, are essential for its function. *J. Virol.* **66**, 469-479.

Zhu, L. And Weller, S.K. (1992b). The UL5 gene of herpes-simplex virus type-1 - isolation of a *LacZ* insertion mutant and association of the UL5 gene-product with other members of the helicase-promase complex. *J. Virol.* **66**, 458-468.

Zwaagstra, J.C., Ghaisi, H., Slanina, S.M., Nesburn, A.B., Wheatley, S.C., Lillycrop, K., Wood, J., Latchman, D.S., Patel, K. and Wechsler, S.L. (1990). Activity of herpes simplex virus type 1 latency-associated transcript (LAT) promoter in neuron-derived cells: evidence for neuron specificity and for a large LAT transcript. *J. Virol.* **64**, 5019-5028.