Characterization of the HSV-1 strain 17+ neurovirulence gene RL1 and its expression in a bacterial system.

by

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Summary

In HSV-1 strain F, one gene product had previously been localised to the region of the genome between RL2 and the 'a' sequence, and shown to be a determinant of HSV-1 strain F neurovirulence. This protein was designated ICP34.5.

Sequence analysis of the HSV-1 strain 17^+ genome in this region revealed that in HSV-1 strain 17^+, the putative ORF ascribed to ICP34.5 was thoroughly disrupted by a 2bp insert, which rendered 60% of the coding-region out of frame. This did not correlate however, with data obtained by several laboratories which clearly demonstrated that in HSV-1 strain 17^+ this locus encoded an important neurovirulence determinant.

The initial stage of this project involved reinvestigation of the HSV-1 strain 17^+ sequence at the terminus of the long repeat, where the expected frame-shift occurred. By comparison of two newly sequenced clones with the original clone used to sequence this region of the genome, it was demonstrated that the original sequence obtained for this locus of HSV-1 strain 17^+ was inaccurate and came from an atypical plasmid clone. Correction of the strain 17^+ sequence opens the reading frame proposed by Chou and Roizman to encode a protein of 248 amino acids. This ORF has been designated RL1.

Having confirmed that HSV-1 strain 17^+ encodes an ICP34.5 homologue, we wished to confirm the importance of this gene in neurovirulence following intracerebral inoculation of HSV-1 strain 17^+. Verification that lack of synthesis of ICP34.5 alone, was responsible for a non-neurovirulent phenotype was achieved through the construction and characterization of a HSV-1 strain 17^+ RL1 variant, 1771, with a stop-codon in only the ICP34.5 reading frame, 9 base pairs downstream from the initiating ATG. 1771, did not produce ICP34.5 as demonstrated by Western blotting using an ICP34.5 polyclonal antiserum which was generated during the course of this study. It was not impaired in growth in tissue culture, and following intracerebral inoculation of BALB/c mice it was totally non-neurovirulent, with a LD_{50} of $>10^6$ p.f.u./mouse, compared to 7 p.f.u./mouse for the wild-type virus HSV-1 strain 17^+. This lack of neurovirulence was shown to

correlate with an inability to replicate in mouse brain, and clearly demonstrated the crucial role played by ICP34.5 in HSV-1 strain 17^+, following intracerebral inoculation of mice.

Previously, peptide antisera had been raised against 7 different regions of the predicted HSV-1 strain 17^+ RL1 ORF, but none of these, until recently, had successfully identified ICP34.5 in HSV-1 strain 17^+ infected cells. To demonstrate that the HSV-1 strain 17^+ RL1 ORF was capable of expressing a protein, it was placed under the control of a strong T7 promoter and over-expressed in *E.coli* BL21(DE3) cells. A two-step process was developed for partial purification of the *E.coli* -expressed ICP34.5 which involved initial ammonium sulphate precipitation followed by anion-exchange chromatography. The partially purified protein was used as an immunogen for the production of a rabbit polyclonal anti-ICP34.5 serum. This antiserum successfully recognised ICP34.5 in HSV-1 strain 17^+ and HSV-1 F infected cell extracts and revealed that previous results which had indicated that ICP34.5 was underproduced in HSV-1 strain 17^+ were inaccurate, and the protein was produced in similar quantities by both strains. Using this polyclonal antiserum, and 1771 as a negative control, ICP34.5 was specifically localised to the cytoplasm of HSV-1 strain 17^+ infected cell extracts.

An antiserum, raised against a peptide corresponding to 10 copies of a Proline-Alanine-Threonine amino acid repeat predicted by the HSV-1 strain F RL1 ORF, had been previously shown to recognise a polypeptide of M_r 39K in HSV-1 strain F infected cell extracts, but only recently, following extensive optimization of our assay conditions has it specifically recognised ICP34.5 in HSV-1 strain 17^+ infected cells. A strain F mutant, F11, with a deletion in RL1 was constructed to confirm the specifity of this antiserum, to use as a negative control in ICP34.5 localization studies, and to show that loss of ICP34.5 expression specifically correlated with a loss of neurovirulence.

F11 was shown to be totally non-neurovirulent following intracerebral inoculation of mice with a LD_{50} of $>10^7$ p.f.u./mouse, compared to $<10^2$ p.f.u./mouse for the parental wild-type HSV-1 strain F. Unlike the strain 17^+ mutant 1716 (which has an identical deletion in its genome) F11 was not significantly impaired , compared to the wild-type virus, in reactivating from latency. Using Western blot analysis we were able to demonstrate that

lack of synthesis of ICP34.5 correlated with a loss of neurovirulence. Using F11 as a negative control, ICP34.5 was localised to the cytoplasm of HSV-1 strain F infected cells by immunofluorescence and cell fractionation studies.

During the analysis of single plaques from a transfection, a variant designated 1772, was isolated which had a large deletion in U_S. It was observed that this variant had a small plaque morphology on BHK21/C13 cells which was indicative of a defect in cell-to-cell spread. This property had not previously been ascribed to any gene in U_S, and for this reason initially, it was decided to further localize the deletion in 1772. Initial characterization demonstrated that 1772 had an extended lag phase following one-step but more noticably following multi-step growth analysis. Sequencing revealed that 1772 had a 630bp deletion which removed the initiating methionine of US7, which encodes gI, and extended to the 3' end of the gene. This was subsequently confirmed by immunoprecipitation, using a gI specific monoclonal antibody. 1772 was not impaired in adsorption or penetration onto BHK21/C13 cells, compared to the wild-type virus HSV-1 strain 17^+. However it was intermediate in neurovirulence following intracerebral inoculation of mice. Due to time constraints, further characterization of the defect in 1772 was not possible.

Abbreviations

A	adenine
APS	ammonium persulphate
ATP	adenosine triphosphate
BHI	brain heart infusion
BHK21/C13	baby hamster kidney cells batch 21 clone 13
BHV	bovine herpesvirus
bp	base pairs
BSA	bovine serum albumin
C	cytosine
^{o}C	degrees centigrade
CAT	chloramphenicol acetlytransferase
CCV	channel catfish virus
CHO	Chinese hamster ovary
Ci	Curie
c.p.e.	cytopathic effect
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxy cytidine-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
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DRG	dorsal root ganglia
DTT	dithiothreitol
EBV	Epstein-Barr virus
E.coli	*Eschericia coli*
EDTA	ethylenediaminetetra acetic acid
EHV	equine herpesvirus
G	guanine
HBLV	human B-cell lymphotropic virus
HCMV	human cytomegalovirus
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid
HHV	human herpesvirus

HSV-1	herpes simplex virus 1
HSV-2	herpes simplex virus 2
HVA	herpesvirus ateles
HVS	herpesvirus saimiri
ICP	infected cell polypeptide
IE	immediate early
Ig	immunoglobulin
IPTG	isopropyl-β-D-thiogalactoside
IR_L	internal long repeat
IR_S	internal short repeat
k	kilo (ie. 10^3)
kbp	kilobase pairs
L	long segment
LAT	latency associated transcript
MDV	Marek's disease virus
mg	milligrams
ml	millilitres
mM	millimolar
m.o.i.	multiplicity of infection
M_r	molecular weight
m.u.	map units
ng	nanogram
np	nucleotide position
NP40	Nonidet P40
OD	optical density
ORF(s)	open reading frame(s)
oriL	origin of replication in U_L
oriS	origin of replication in U_S
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
p.f.u.	plaque forming units
p.i.	post-infection
PRV	pseudorabies virus
R1	large subunit of ribonucleotide reductase
R2	small subunit of ribonucleotide reductase
RE	restriction enzyme
RNA	ribonucleic acid

r.p.m.	revolutions per minute
RR	ribonucleotide reductase
RT	room temperature
S	short segment
SDS	sodium dodecyl sulphate
T	thymidine
TEMED	N, N, N', N', -tetramethlyethylene diamine
Tris	tris (hydroxymethyl) aminomethane
TK	thymidine kinase
TR_L	long terminal repeat
TR_S	short terminal repeat
ts	temperature sensitive
uCi	microcurie
U_L	long unique
ul	microlitre
uM	micromolar
U_S	short unique
U.V.	ultraviolet
V	volts
Vmw	molecular weight in kilodaltons of HSV induced polypeptides
VP	virion protein
v/v	volume/volume (ratio)
VZV	varicella zoster virus
w/v	weight/volume (ratio)
w/w	weight/weight (ratio)

ONE AND THREE LETTER ABBREVIATIONS FOR AMINO ACIDS

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

Chapter 1- INTRODUCTION

1.1. The herpesviruses

1.1.1. General properties of the herpesviruses

There are now more than 80 members of the herpesvirus group (Nahmias *et al.*, 1972) which all conform to a set of basic morphological characteristics (Fenner, 1976). The virion (120-200nm in diameter) consists of 4 structural components; (a) a core made up of a linear double-stranded DNA molecule of 120-240kbp is wrapped; (b) an icosahedral capsid 100-110nm in diameter composed of 162 capsomers; (c) an amorphous lipid-containing layer termed the tegument which surrounds the capsid- this is often distributed asymmetrically and may be variable in amount; (d) the envelope, a bilayered membrane which surrounds the tegument and has surface projections (Wildy *et al.*, 1960; Roizman and Furlong, 1974).

All members of the family possess a linear double-stranded genome with a molecular weight of approximately $80\text{-}150 \times 10^6$ (Roizman, 1982) and are readily separable using criteria such as base composition, genome structure, immunological cross-reactivity, biological properties and size. Classification of the *Herpesviridiae* in terms of biological properties (Roizman *et al.*, 1978; Mathews, 1982; Roizman, 1982) and genome structure (Honess and Watson, 1977; Roizman, 1982; Honess, 1984) is described below.

1.1.2. Classification according to biological characteristics

Members of the family *Herpesviridiae* are classified, primarily on the basis of biological behaviour, into 3 sub-groups, the *Alpha-*, *Beta-* and *Gammaherpesviriniae* (Roizman, 1982). The differentiation between the *Alpha-* and *Gamma-* subfamilies is based on host-range and *in vitro* characteristics, while the differentiation between the *Beta-* subfamily and the others is based primarily on the length of the reproductive cycle and slow development of cytopathology in cell cultures.

The *Alphaherpesviriniae* are neurotropic and frequently, but not exclusively, become latent in neurons of the dorsal root ganglia of the peripheral nervous system (PNS). They are

characterised by their short reproductive cycle and rapid spread of infection in cell culture resulting in mass destruction of susceptible cells. This subfamily can be further sub-divided into 2 genera; the *Simplexvirus* -the type virus of which is HSV-1- and the *Varicellovirus* whose members include VZV, EHV-1 and PRV.

In contrast the *Betaherpesviriniae* which comprise the Cytomegaloviruses have a relatively long reproductive cycle. In tissue culture they produce slowly progressing lytic foci and infected cells frequently become enlarged (cytomegalia) both *in vitro* and *in vivo*. Members of this group can establish latent infections in secretory glands, lymphoreticular tissue, kidneys and other tissues. They include HCMV and EHV-2.

The third family in this grouping are the *Gammaherpesviriniae,* which are lymphotropic and specifically infect B- and T- lymphocytes. In the lymphocyte, infection is frequently arrested either at a pre-lytic stage with persistence and minimal expression of the viral genome, or at a lytic stage, causing death without production of complete virions. EBV and HVA are members of this subfamily.

1.1.3. Classification on the basis of genome structure

Base composition, size of the genome and arrangement of reiterated sequences within the genome are 3 factors which can be considered when classifying the *Herpesviridiae* in terms of genome structure. As herpesviruses differ significantly in their overall base composition (32-75% G+C) and the size of their genome, the most useful characteristics for the classification of herpesviruses is the arrangement of reiterated sequences. On that basis the viral genomes fall into 5 classes (A through E). These are illustrated diagramatically in figure 1.1..

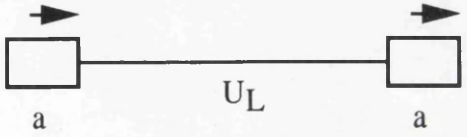
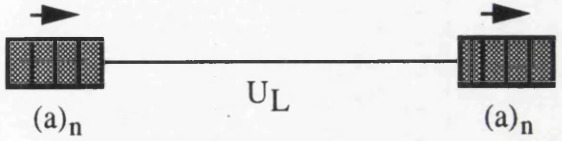
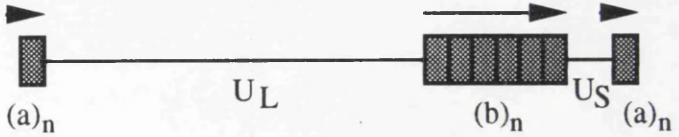
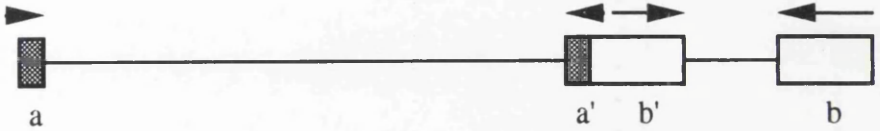
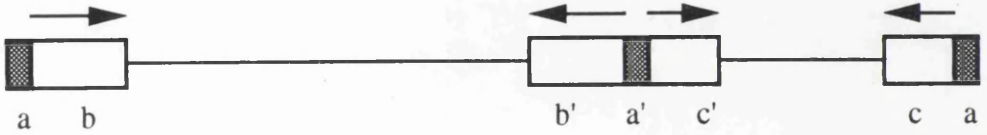
Group A: The genomes of members of this group have a single direct repeat at the termini which is in the same orientation at either end. The DNA exists in only one isomeric arrangement. CCV is a member of this group (Chousterman *et al.*, 1979).

Group B: The DNA is present in only 1 isomeric arrangement. HVS is a member of this group. The genome is characterized by containing multiple copies of a sequence present as a

Figure 1.1. Genome structures of the herpesviruses.

Schematic diagram of the herpesvirus genomes. Solid lines represent unique sequences (U_S and U_L). Repeat sequences larger than 1kb are shown in open boxes and those of small reiterated sequences are shown in shaded boxes. Letters $(a)_n$ and $(b)_n$ signify multiple tandem repeat sequences. Arrowheads above boxes indicate the orientation of the repeat sequences. An example of each group A-E2, is illustrated and the number of isomers indicated. In VZV, U_S is in either orientation 50% of the time, while U_L is in 1 orientation 95% of the time.

a, b and c indicate repeat sequences with a', b' and c' their complement respectively.

Genome structure	group	no. of isomers	example
	A.	1	CCV
	B.	1	HVS
	C.	1	EBV
	D.	2	PRV
	E1.	2 major 2 minor	VZV
	E2.	4	HSV-1

direct repeat at each termini (Bornkamm *et al.*, 1976; Albrecht *et al.*, 1992).

Group C: EBV is a member of this group, the DNA of which is present again in only 1 isomer (Raab-Traub *et al.*, 1980). The termini of the DNA molecules are formed by several copies of a directly repeated sequence and internally, the genome carries another set of directly repeated elements. Both the terminal repeats and the major internal repeat families vary in copy number.

Group D: The viral DNA of this group is characterized by having two unique regions (U_L and U_S) with U_S being flanked by inverted repeats (IR_S and TR_S) which allow inversion of U_S, giving rise to 2 isomeric forms. PRV (Stevely, 1977) and EHV-1 (Chowdhury *et al.*, 1990; Yamanchili and O'Callaghan, 1990) are members of this group.

Group E: This group has been further divided into 2 subgroups E1 and E2.

Subgroup E1 consists of viruses eg.VZV whose unique regions (U_S and U_L) are flanked by inverted repeats (TR_L/IR_L, TR_S/IR_S). U_L is flanked by small inverted repeats and inverts inefficiently, while U_S is flanked by longer repeats and inverts to give equimolar amounts of both orientations of U_S. In the case of VZV two of the isomers, representing one orientation of U_L are about 20-fold more abundant than the other two (Davison, 1984).

HSV-1 and HSV-2 belong to the subgroup E2. They have 2 unique segments (U_L and U_S) both of which are flanked by inverted repeats (TR_L/IR_L, TR_S/IR_S) which share a short region of DNA directly repeated at the termini and indirectly repeated at the junction between the internal inverted repeats. Preparations of DNA contain equimolar amounts of 4 sequence-orientation isomers.

The complete DNA sequences have been published of EBV (Baer *et al.*, 1984), VZV(Davison and Scott, 1986a), HSV-1 (McGeoch *et al.*, 1985,1986,1988a; Perry and McGeoch, 1988), HCMV (Chee *et al.*, 1990), EHV-1 (Telford *et al.*, 1992) HVS (Albrecht *et al.*, 1992) CCV (Davison, 1992) and partial sequence data are available for many others. This information has enabled the re-evaluation of the original classification and has shown that although a virus may have the biological characteristics of one sub-family, it may be genetically related to a different family.

In general, assessment of genetic relationships between individual viruses are based primarily on comparisons of predicted amino acid sequences and are in agreement with the biological classification, but there are exceptions. On the basis of morphology and biology CCV was classified as a member of the *Alphaherpesviriniae* (Roizman, 1982). Sequence analysis of this virus (Davison, 1992) has shown that genetically, CCV should not be included in any of the recognised herpesvirus subfamilies, but should be classified as the sole member of a new subfamily, or possibly as a new virus family altogether.

HHV-6, being a lymphotrophic virus, would appear to belong in the *Gammaherpesviriniae*, but in fact its sequence is closely related to HCMV, the prototype member of the *Betaherpesviriniae*. Similarly, two avian herpesviruses, MDV and the closely related herpesvirus of turkeys (HVT) had originally been assigned to the *Gammaherpesviriniae* because of their tropism for lymphocytes (Roizman, 1982), but comparison with other herpesviruses has indicated that they share greater homology to VZV (a member of the *Alphaherpesviriniae*) than to EBV (Buckmaster *et al.*, 1988). Furthermore, it has been suggested that PRV and BHV-1, which have been classified with VZV in the *Varicellovirus* genus, should perhaps be considered along with HSV-1 as members of the *Simplexvirus* genus, on the basis of their similar mechanism of entry into cells (Spear, 1993).

1.1.4. The Human Herpesviruses

There are 7 herpesviruses known to infect man. These are HSV-1, HSV-2, VZV, EBV, HCMV, HHV-6 and HHV-7. A brief outline of the pathogenesis of each is given below.

HSV-1 and **HSV-2** (reviewed by Whitley, 1990) usually cause cold sores and genital lesions respectively. At the time of primary infection, the virus replicates lytically in the peripheral tissues around the site of infection and enters sensory nerves at the termini of axons of sensory neurons. A lifelong latent infection is established in the neurons of the sensory ganglia and periodically, various stimuli including stress, exposure to ultraviolet light, hormonal changes and immune suppression induce reactivation and production of infectious virus. The reactivated virus then travels back to the tissues around the site of

primary infection, where it may again replicate lytically, destroying cells and causing the formation of recurrent lesions.

VZV (reviewed by Gelb, 1990) produces two distinct clinical syndromes- chickenpox and shingles. Chickenpox (varicella) is a ubiquitous, highly contagious, generalized exanthem that spreads rapidly in a susceptible population. Herpes zoster, however appears to be a secondary reactivation of VZV that has remained latent since an earlier attack of varicella. It usually occurs in older and immunocompromised individuals.

EBV (reviewed by Miller, 1990) was first discovered in the lymphoblastoid cells of the tumour known as Burkitt's lymphoma and was subsequently found to be the causative agent of infectious mononucleosis or ' glandular fever'; it has also been associated with the human malignancy nasopharyngeal carcinoma. Although EBV exists worldwide, nasopharyngeal carcinoma is endemic only in Southern China, and Burkitt's lymphoma only in tropical Africa, suggesting the involvement of genetic or environmental factors in progression towards these diseases.

HCMV (reviewed by Alford and Britt, 1990). In immunocompetent individuals, HCMV infections are generally asymptomatic, however it is one of the most common opportunistic infections associated with people with AIDS. CMV recurrence is the major viral complication in bone marrow transplantation (Meyers *et al.*, 1986) as well as in organ transplantation, including liver, kidney, heart and lung (Ho, 1991). It is established that recurrence from the transplant itself as well as within the transplant recipient both contribute to the overall risk of HCMV disease.

HCMV has been isolated from biopsies of cervical carcinoma, adenocarcinoma and Kaposi's sarcoma, indicating that it may be associated with these cancers. However the ubiquitous distribution of the virus has made an aetiological association between HCMV infection and human cancer difficult to establish.

HHV-6 was originally designated human B lymphotrophic virus, because it was first isolated in 1986 from peripheral blood leukocytes of AIDS patients and patients with lymphoproliferative disorders (Salahuddin *et al.,* 1986). It is now thought that this virus can grow in B- and T- cell lineages (Downing *et al.,* 1987; Lopez *et al.,* 1988) and it has been renamed HHV-6. HHV-6 is the causative agent of exanthem subitum (roseola infantum), a common childhood disease characterized by high fever and skin rash. Using PCR, HHV-6 DNA sequences have been detected in peripheral blood and saliva samples from the majority of healthy individuals (Gopal *et al.,* 1990; Jarret *et al.,* 1990), and it is likely that saliva is an important vehicle for transmission of HHV-6.

HHV-7 was first isolated in 1990 from $CD4^+$ T-cells purified from peripheral blood mononuclear cells of a healthy individual (Frenkel *et al.,* 1990) but little is known about this virus. Berneman *et al.* (1992) independently isolated HHV-7 from peripheral blood mononuclear cells of a patient with chronic fatigue syndrome. Using the serum of a patient infected with HHV-7 they demonstrated by immunofluoresence that HHV-7 has a tropism for primary human T-lymphocytes and for at least one immature T-cell line. Although HHV-6 and HHV-7 were found to be molecularly and immunologically related, the molecular divergence between HHV-6 and HHV-7 is enough for HHV-7 to be considered another herpesvirus rather than a 'sub-type' of HHV-6.

1.1.5. Morphological structure of the herpesviruses

Herpesviruses consist of 4 morphologically distinct structures: core, capsid, tegument and envelope. Previously, the core was thought to have the form of a cylindrical protein plug around which the DNA was wound (Nazerian, 1974; Irmiere and Gibson, 1983) but this has recently been shown to be incorrect (F. Rixon, personal communication).

In the case of HSV, the cylindrical protein plug appears to be largely composed of the single protein VP21 (Gibson and Roizman, 1972). The cylinder provides a mechanical support for the orderly winding of the virus DNA, which in HSV is spooled around the cylinder- with a spacing of 4-5nm generating a toroid arrangement of DNA in the core (Furlong *et al.,*

1972).

The herpesvirus capsid is approximately 100nm in diameter and surrounds the core. It exhibits 2-, 3- and 5- fold symmetry and is composed of 162 capsomeres, of which 150 are hexameric and 12 are pentameric (Wildy *et al.*, 1960) arranged in the form of an icosahedron. It is composed of 7 proteins: VP5 (UL19), VP19C (UL38), VP22a (UL26.5), VP21 (UL26 C-terminal), VP23 (UL18), VP24 (UL26 N-terminal) and VP26 (UL35) (Gibson and Roizman,1972; Cohen *et al.*, 1980).

VP5, encoded by the UL19 gene (Costa *et al.*, 1984; McGeoch *et al.*, 1988a) is the major capsid protein. It has a M_r of 155,000 and forms the main component of the hexameric and probably the pentameric capsomeres (Newcomb and Brown, 1989,1991). VP19C and VP23 are located near the capsid surface (Newcomb and Brown, 1989) and may be components of the trimeric structures linking adjacent capsomeres. VP22a is a highly processed protein, comprising many forms with apparent M_rs in the region of 40,000, several of which are phosphorylated (Gibson and Roizman, 1974; Preston *et al.*, 1983). VP26 is a component of the capsid interior, whereas VP21 and VP24 are core proteins (Newcomb and Brown, 1989).

An amorphous layer known as the tegument surrounds the capsid (Roizman and Furlong, 1974). VP1-3 (Gibson and Roizman, 1972) the products of the UL36 gene (McGeoch *et al*, 1988a), and the 65K virion trans-activating factor are amongst several proteins believed to reside within the tegument and intercalate with the capsid and envelope (Batterson and Roizman, 1983; Campbell *et al.*, 1984). Although the size of the capsid is well conserved between herpesviruses, the dimensions of the tegument vary and appear to be determined at least in part by the virus (Nazerian and Witter, 1970; McCombs *et al.*, 1971).

The tegument is completely enclosed in a trilaminar membrane described as the envelope (Wildy *et al.*, 1960; Darlington and Moss, 1968). The envelope has a mean diameter of 180nm and exhibits spikes 8-10nm long spaced 5nm apart over the surface (Wildy *et al.*, 1960). The use of monoclonal antibodies coupled to immunogold labelling identified some of these spikes as being HSV encoded glycoproteins (Stannard *et al.*, 1987). There are at least 8 distinct HSV-1 glycoproteins in the virion envelope: gB(UL27), gC (UL44), gD

(US6), (Spear, 1976), gE (US8), (Baucke and Spear, 1979), gG (US4), (McGeoch *et al.*, 1985; Frame *et al.*, 1986), gH (UL22), (Buckmaster *et al.*, 1984), gI (US7), (Sullivan and Smith, 1988) and gM (UL10), (Baines and Roizman, 1993). No direct evidence is yet available for gK (UL53), (Hutchinson *et al.*, 1992b; Ramasway and Holland, 1992) or gL (UL1), (Hutchinson *et al.*, 1992a). In addition to HSV encoded glycoproteins, the envelope is believed to contain cell lipids (Asher *et al.*, 1969) deduced from the sensitivity of virions to lipid solvents and detergents, and spermidine (Gibson and Roizman, 1971).

1.1.6. Light-particle production

Ficoll gradient purification of HSV-1 strain 17^+ virus particles has demonstrated the existence of a non-infectious particle termed 'light-particle'. As well as normal virions, light particles are generated during HSV-1 infection (Szilagyi and Cunningham, 1991). These particles lack capsids and viral DNA and consist predominantly of tegument and envelope proteins. They contain at least 5 phosphoproteins not normally observed in virion profiles. The UL26 mutant *ts* 1201 (Preston *et al.*, 1983) which does not package DNA at the non-permissive temperature, has been used to demonstrate that L-particle formation is independent of virion formation (Rixon *et al.*, 1992). Other HSV-1 strains (eg. HSV-1 strain F) and other *Alphaherpesviruses* including HSV-2, PRV, BHV and EHV-1 also produce 'light-particles' (McLaughlan and Rixon, 1992; Dr.D. Dargan, personal communication). The role of these light particles in the HSV life-cycle is currently being determined (Dr.D. Dargan, personal communication).

It is possible that 'light-particles' could be used for the production of a non-infectious sub-unit vaccine. Initial experiments have shown that vaccination with HSV-1 strain 17^+ light particles protects against subsequent challenge both with wild-type HSV-1 strain 17^+ in a mouse model and with wild-type HSV-2 in a guinea-pig model; while in a therapeutic model, innoculation with HSV-1 strain 17^+ light particles reduces the severity of recrudescent disease symptoms from a previously established latent HSV-2 infection in a guinea pig model (Dr.A.R. MacLean, personal communication).

Table 1.1. The HSV-1 genes identified to date.

The status of genes known to be essential for virus growth in culture cells is marked 'e', and non-essential 'ne'. e? indicates that data regarding status is not conclusive. e/ne, necessity depends on culture conditions or temperature. Immediate early proteins are termed 'IE'. The absence of information regarding status is indicated by a dash. References are omitted because of space constraints; recent referenced lists of HSV-1 functions are published by McGeoch *et al.*, 1988a and McGeoch and Schaffer, 1993. Adapted with permission from Dr. D.J. McGeoch from McGeoch *et al.*, 1993.

HSV-1 gene	Protein / function	Status
RL1	Neurovirulence factor (ICP34.5)	ne
RL2	IE protein; transcriptional regulator (Vmw110)	ne
UL1	Glycoprotein L	e?
UL2	Uracil-DNA glycosylase	ne
UL3	Function unknown	ne
UL4	Function unknown	ne
UL5	Component of DNA helicase-primase	e
UL6	Role in virion morphogenesis	e
UL7	Function unknown	-
UL8	Component of DNA helicase primase	e
UL9	Ori-binding protein essential for DNA replication	e
UL10	Function unknown; probable integral membrane protein	ne
UL11	Myristylated tegument protein; role in envelopment and transport of nascent virions	ne
UL12	Deoxyribonuclease; role in maturation/ packaging of nascent DNA	e
UL13	Tegument protein; probable protein kinase	ne
UL14	Function unknown	-
UL15	Function unknown; possible NTP-binding motifs	e?
UL16	Function unknown	ne
UL17	Function unknown	e?
UL18	Capsid protein (VP23)	-
UL19	Major capsid protein (VP5)	e
UL20	Integral membrane protein; role in egress of nascent virions	e/ne
UL21	Function unknown	-
UL22	Virion surface glycoprotein H; role in virus entry	e
UL23	Thymidine kinase	ne
UL24	Function unknown	ne
UL25	Virion protein	e
UL26	Proteinase, acts in virion maturation; N-terminal portion is capsid protein VP24; C-terminal is VP21	e
UL26.5	Internal protein of immature capsids (VP22a); processed by UL26 proteinase	e?
UL27	Virion surface glycoprotein B; role in cell entry	e
UL28	Role in capsid maturation/ DNA packaging	e
UL29	ssDNA binding protein; essential for DNA replication	e
UL30	Catalytic subunit of replicative DNA polymerase	e
UL31	Function unknown	-

Table 1.1 continued.

HSV-1 gene	Protein / function	Status
UL32	Function unknown	-
UL33	Role in capsid maturation/ DNA packaging	e
UL34	Membrane associated phosphoprotein	-
UL35	Capsid protein (VP26)	e
UL36	Very large tegument protein (VP1-3)	e
UL37	Function unknown; may have DNA-binding role	-
UL38	Capsid protein (VP19C)	e
UL39	Ribonucleotide reductase large subunit (R1)	e/ne
UL40	Ribonucleotide reductase small subunit (R2)	e/ne
UL41	Virion protein; host shutoff factor	ne
UL42	Subunit of replicative DNA polymerase; increases processivity	e
UL43	Function unknown; probable integral membrane protein	ne
UL44	Virion surface glycoprotein C; role in cell entry	ne
UL45	Virion protein	ne
UL46	Modulates IE gene transactivation by UL48 gene	ne
UL47	Tegument protein; modulates IE gene transactivation by UL48 protein	ne
UL48	Major tegument protein; transactivates IE genes (VP16, Vmw65, αTIF)	e
UL49	Tegument protein (VP22)	-
UL49A	Possible membrane protein	e?
UL50	Deoxyuridine triphosphatase	ne
UL51	Function unknown	-
UL52	Component of DNA helicase-primase	e
UL53	Glycoprotein K	e?
UL54	IE protein; post-translational regulator of gene expression (ICP27, Vmw63)	e
UL55	Function unknown	ne
UL56	Function unknown	ne
LAT	Family of transcripts expressed during latency; function unknown protein-coding capacity uncertain	ne
RS1	IE protein; transcriptional regulator (ICP4, Vmw175)	e
US1	IE protein; function unknown (ICP22, Vmw68)	e/ne
US2	Function unknown	ne
US3	Protein kinase	ne
US4	Virion surface glycoprotein G	ne
US5	Virion surface glycoprotein J ?	ne
US6	Virion surface glycoprotein D; role in entry	e

Table 1.1 continued.

HSV-1 gene	Protein / function	Status
US7	Virion surface glycoprotein I	ne
US8	Virion surface glycoprotein E	ne
US8.5	Function unknown	ne
US9	Virion protein	ne
US10	Virion protein	ne
US11	Virion protein; ribosome associated in infected cells	ne
US12	IE protein; function unknown (ICP47, Vmw12)	ne

1.2. The HSV-1 genome

The sequence of the HSV-1 strain 17^+ genome published in 1988, contains 152,260 residues in each strand (McGeoch *et al.*, 1985,1986,1988a; Perry and McGeoch, 1988). As with all herpesvirus genomes studied in detail, the existence of variation means that this number is not fixed.

The complete sequence of HSV-1 strain 17^+ was predicted to contain 70 distinct genes (McGeoch *et al.*, 1988a). Determination of the coding potential was based on critical sequence interpretation with gene designations requiring evidence beyond the presence of an open-reading frame (ORF) starting with a methionine, of which many existed other than those considered genuinely functional. Specifically, the protein-coding potential of ORFs was evaluated on the basis of codon-usage triplet periodicity in base frequencies compatible with the high G+C content of the genome and size of ORF (>M_r 9K). The number of ORFs was a conservative estimate and at the time, it was expected that extra genes (especially small genes) would be discovered. In HSV-1 strain F, the region upstream of IE1 has been shown to encode the gene RL1 (Chou and Roizman, 1986; Ackermann *et al.*, 1986). A homologous gene was subsequently identified in HSV-1 strain 17^+ (Dolan *et al.*, 1992) and HSV-2 strain HG52 (McGeoch *et al.*, 1991). Three additional genes have since been identified, namely UL26.5 (Liu and Roizman, 1991a) UL49A (Barker and Roizman, 1992; Barnett *et al.*, 1992) and US8.5 (Georgopoulou *et al.*, 1993; Dr. B. Barnett, personal communication). The HSV-1 genes identified to date are listed in Table 1.1..

1.2.1. Comparison of the HSV-1 genome with other alphaherpesvirus genomes.

Comparisons of genome organization between five alphaherpesviruses, HSV-1, HSV-2, VZV, PRV and EHV-1 has shown extensive genome colinearity between the viruses (Davison and Wilkie, 1983b). In contrast, when the genome organizations of VZV and the gammaherpesvirus EBV are compared, three regions containing conserved genes are observed. Although the genes are generally colinear within these regions, the three are arranged differently in the two genomes (Davison and Taylor, 1987).

HSV-1 and VZV differ substantially in terms of genome size, and organization of repeat elements, but they have largely corresponding sets and layout of genes. Like HSV-1, VZV has elements equivalent to U_L, R_S and U_S, however in VZV R_L is only 88bp (Davison, 1984). VZV has an overall base composition of 46% G+C, compared with 68.3%G+C of HSV-1. Although the sequence of VZV U_L is grossly colinear with that of HSV-1 the S region varies to a much greater extent, with VZV lacking counterparts of 6 genes found in HSV-1 (Davison and McGeoch, 1986); namely US2, US4 (gG), US5, US6 (gD), US11 (virion protein) and US12 (Vmw12).

With more sequencing data becoming available, it has now become possible not only to determine the genome organization of different herpesvirus but to compare the amino acid sequences of individual genes. It has been found that in general, the S component is the most variable region in alphaherpesviruses, ranging in size from 19,871bp in VZV to 37,289bp in EHV-1. Genes corresponding to HSV-1 RS1 (IE175), US1 (Vmw68), US3 (protein kinase), US7 (gI) and US9 (tegument protein) are conserved in VZV, HSV-1, PRV and EHV-1 (Telford *et al.*, 1992), although the relative positions of some genes differ. Davison and McGeoch (1986) concluded that differences in gene layout between the S components of HSV-1 and VZV have resulted from expansion and contraction of IR_S/TR_S during evolution, and this is perhaps true for all members of the *alphaherpesviriniae.*

EHV-1 and HSV-1 share a common gene arrangement as demonstrated by the mapping of several EHV-1 genes including the glycoproteins gC (Allen and Coogle, 1988), gB (Whalley *et al.*, 1989), gD (Audonnet *et al.*, 1990; Whalley *et al.*, 1991), gI and gE (Audonnet *et al.*, 1990), the transcriptional regulator IE110 (Grundy *et al.*, 1989) and thymidine kinase (Robertson and Whalley, 1988). The complete DNA sequence of the Ab4 strain of EHV-1 has recently been published (Telford *et al.*, 1992). The EHV-1 genome contains 76 distinct genes and the transcript pattern of the genome, as predicted from the position of potential polyadenylation sites is largely similar to those proposed for VZV (Davison and Scott, 1986a) and HSV-1 (McGeoch *et al.*, 1988a). Only 3 EHV-1 genes were found to lack positional and sequence counterparts in VZV and HSV-1, while 4 EHV-

1 genes lack amino acid homologues in VZV and HSV-1 but have positional counterparts in VZV or HSV-1 to which they are probably related evolutionarily.

HSV-1 and HSV-2 have a very similar G+C content (68.3% and 69% respectively) and share extensive homology in 50% of their sequences (Kieff *et al.*, 1972). Coding sequences of corresponding genes in general show 70-80% identities, with gG being the most highly divergent gene identified so far (McGeoch *et al.*, 1987) while non-coding regions, in particular the major repeats typically show greater differences and in some cases have become highly diverged (Davison and Wilkie, 1981; Whitton and Clements, 1984).

The entire HSV-2 strain HG52 genome is currently being sequenced (Dr. B. Barnett, personal communication). Comparison of the genetic content of R_L and the adjacent parts of U_L with the equivalent region in HSV-1 has revealed that their genetic content is very similar (Perry and McGeoch, 1988; McGeoch *et al.* , 1991). At the left-hand end of U_L, HSV-2 has coding regions of genes with counterparts to HSV-1 genes UL1 to UL4 and part of UL5. At the right-hand end, counterparts to HSV-1 genes UL54 to UL56 and part of UL53 are found. In HSV-2 R_L sequences were identified corresponding to HSV-1 genes encoding ICP34.5 and IE110 (RL1 and RL2 respectively). In HSV-2 RL1 contains an intron which is not present in the HSV-1 RL1 ORF. The most highly conserved region in the 2 proteins is near the C-terminus in which 63 amino acids in each are aligned without introduction of gaps, and show 83% homology. Homologues of this protein are not found in other herpesviruses (Dr. B. Barnett, personal communication). In HSV-2 the R_L sequence encoding the LATs was found to be dissimilar to that of HSV-1, however the proposed promoter regions of both were found to be similar. The two parts of HSV-2 U_L sequenced are closely similar to, and colinear with the corresponding parts of HSV-1 U_L, whereas the R_L sequences are generally more divergent.

Certain herpesvirus genes show conservation in their encoded amino acid sequence to nonherpesvirus genes; these include the genes for ribonucleotide reductase (Gibson *et al.*, 1984), DNA polymerase (Wong *et al.*, 1988), and helicase (Hodgman, 1988). It is possible that these genes have been acquired either directly or indirectly from a cellular genome

during evolution.

1.2.2. Organization of the HSV-1 genome

Herpes simplex virus DNA consists of 2 unique segments, the long unique (U_L) and short unique (U_S), each bounded by a set of internal (I_R) and terminal (T_R) inverted repeats, IR_L/TR_L and IR_S/TR_S respectively (Sheldrick and Berthelot, 1974; Delius and Clements, 1976). A short sequence, known as the 'a' sequence is present as a direct repeat at the termini and in an inverted orientation at the L-S junction (Sheldrick and Berthelot, 1974; Wadsworth *et al.*, 1976; Davison and Wilkie, 1981). U_S and U_L can invert relative to one another resulting in 4 possible isomeric arrangements (Clements *et al.*, 1976; Wilkie and Cortini, 1976; Roizman, 1979). These isomers occur in equimolar amounts and are termed P (prototype), I_L (inversion of the long segment), I_S (inversion of the short segment) and I_{SL} (inversion of both the long and short segments).The gross organization of the HSV-1 genome is illustrated in fig.1.2..

Because the HSV-1 DNA molecule exists in 4 equimolar isomers, restriction enzyme analysis of the DNA yields 3 classes of fragment (Skare and Summers, 1977; Clements *et al.*, 1976) which occur at different frequencies. Fragments derived entirely from the unique regions of the molecule (U_S and U_L) appear in 1M quantities relative to the molarity of intact viral DNA (Skare and Summers, 1977; Wilkie, 1976; Wilkie and Cortini, 1976). Since each terminus is present in only 2 of the 4 isomers, terminal sequence fragments are present in 0.5M quantities whereas fragments which consist of the L-S joint sequences are present in only 1 of the 4 isomers so they are found in 0.25M quantities.

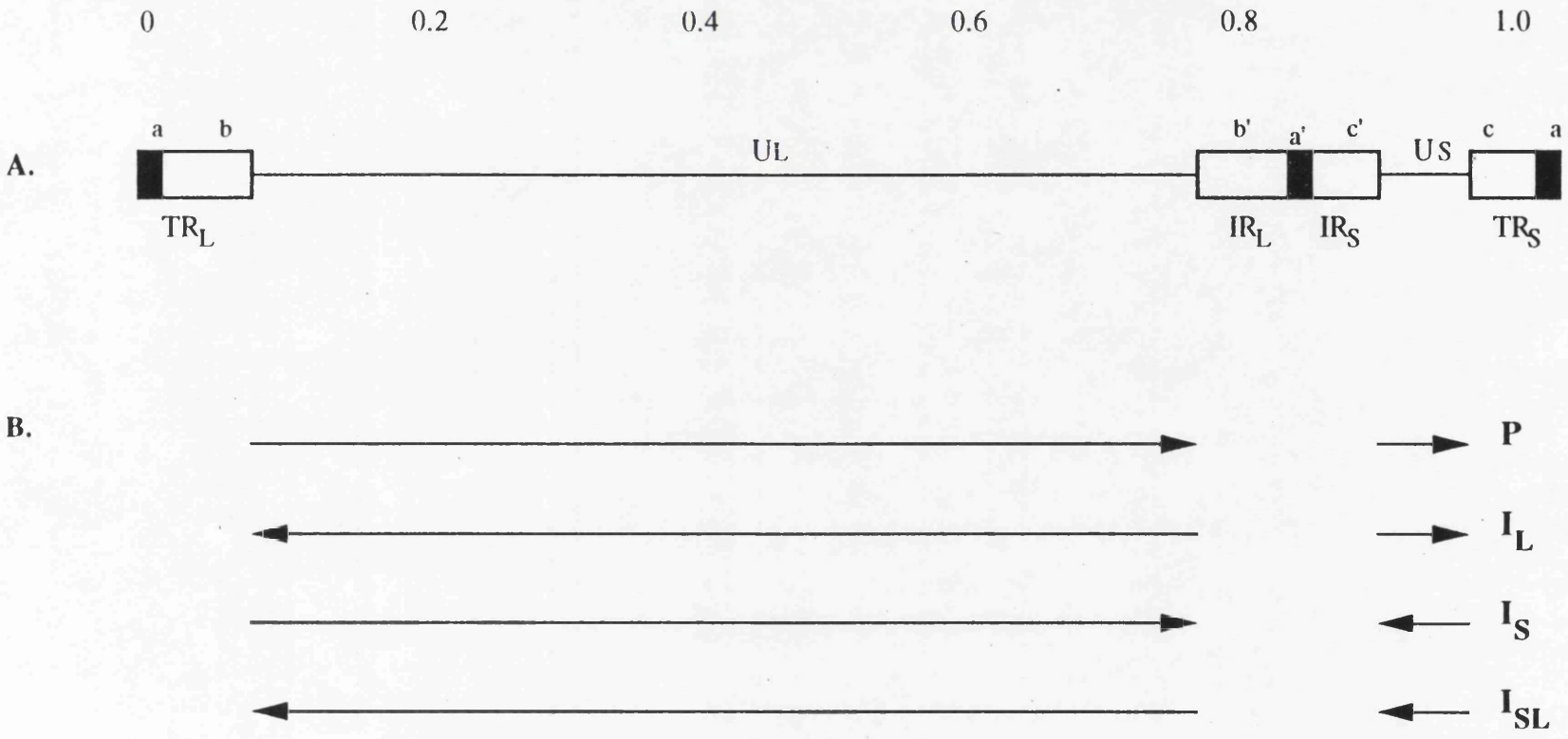
1.2.3. The 'a' sequence

The 'a' sequence is a direct repeat found at the termini and the L-S junction of the HSV-1 genome, and varies in size from 250-550bp. This variation is seen both within (Wagner and Summers, 1978) and between strains (Locker and Frenkel, 1979).

Tandem reiterations of the 'a' sequence may be found at the L terminus and joint region of the genome, but only a single copy is found at the S terminus (Wilkie, 1976; Wagner and

Figure 1.2. Structure of the HSV-1 genome

The HSV genome is shown, with unique sequences as solid lines (U_S and U_L). The repeats TR_L, IR_L, IR_S and TR_S are illustrated as boxes with their component parts a, b, c and a', b', c' designated. Below the genome representation, the isomerisation of the HSV-1 genome is illustrated. The 4 isomers are: P (prototype), I_L (L inverted with respect to P), I_S (S inverted with respect to P) and I_{SL} (S and L inverted with respect to P).


0
0.2
0.4
0.6
0.8
1.0
A.
a
b
UL
b'
a'
c'
US
c
a
TRL
IRL
IRS
TRS
B.
P
IL
IS
ISL

Summers,1978).

Several important functions are mediated by the 'a' sequence, including circularization of the genome following infection, site specific recombination and cleavage/packaging of HSV DNA (Davison and Wilkie, 1983a; Poffenberger and Roizman, 1985). It can be divided into several structural regions (Mocarski and Roizman,1981) which consist of both unique (U) and directly repeated regions (DR); the difference in size between different 'a' sequences being due to variation in the copy number of these direct repeats. In HSV-1 strain F (Mocarski and Roizman, 1981), the L-S junction 'a' sequence can be represented as DR_1-U_b-$(DR_2)_{19}$-$(DR_4)_3$-U_c-DR_1 (see fig.1.3.), where DR_1 is a 20bp sequence present at both ends, U_b and U_c are unique sequences of 58bp and 65bp respectively and are named by virtue of their proximity to the b' and c' repeated regions respectively; DR_2 is a 12bp sequence present in 19-22 tandem copies and DR_4 is a 37bp sequence present in 3 tandem copies.

Chou and Roizman (1985) demonstrated that deletion of the U_b or U_c domains does not affect the ability of the 'a' sequence to mediate inversion, whereas deletion of DR_4 drastically reduces inversion and deletion of both DR_2 and DR_4 completely abolishes inversion.

1.2.4. Cleavage /packaging of DNA

Following replication of the HSV-1 genome, the large concatemeric DNA molecules (section 1.5.) are processed into unit length genomic molecules and packaged into viral capsids. Cleavage of these concatemers occurs within terminally reiterated 'a' sequences situated at the junctions between adjacent viral genomes (Vlazny and Frenkel, 1981; Mocarski and Roizman, 1982; Stow *et al.*, 1983). The distal $DR1_b$ and $DR1_c$ elements in the ba and ac termini respectively, together constitute a complete DR1 sequence. Thus in HSV-1 strain F (Mocarski and Roizman, 1982), the ba terminus was reported to contain 18.5bp (ie.18bp plus one 3' overhang) of the DR1 sequence, whereas the ac terminus contained the remaining 1.5bp. Mocarski and Roizman (1982) proposed a model whereby a baac junction is cleaved within the shared DR1 element to generate the ba and ac termini.

Figure 1.3. Structure of the HSV-1 'a' sequence

A. The HSV-1 genome.

B. An expansion of the IR_L/IR_S junction showing the structure of the 'a' sequence. The 'a' sequence consists of unique and directly repeated elements (the information given concerns the HSV-1 strain F 'a' sequence [Mocarski and Roizman,1981,1982]).

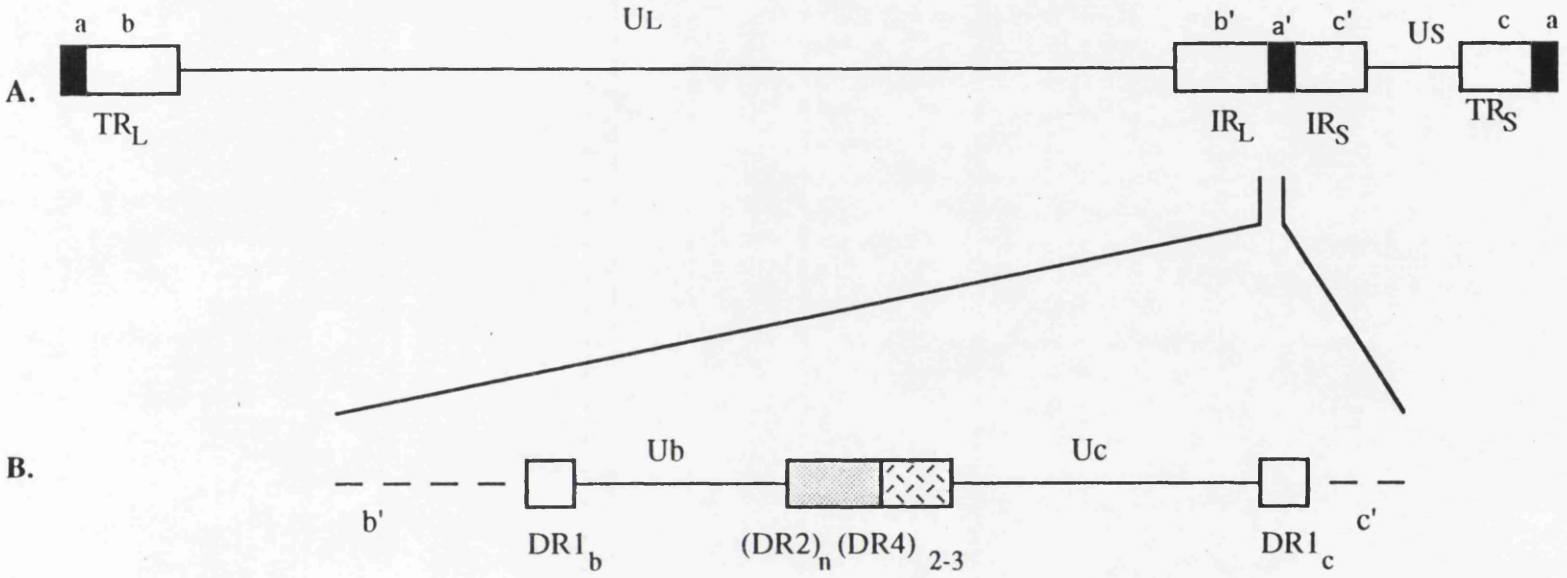
Ub- a unique region located towards the b' region of the genome.

Uc- a unique region located towards the c' region of the genome.

$DR1_b$ and $DR1_c$ - 20bp elements present as direct repeats at the edge of the 'a' sequence

DR2- a directly repeated 12bp element present in 19-22 copies

DR4- a directly repeated 37bp sequence present in 2-3 copies.


A.
a
b
UL
b'
a'
c'
US
c
a
TR_L
IR_L
IR_S
TR_S
B.
Ub
Uc
b'
DR1_b
(DR2)_n (DR4)_2-3
DR1_c
c'

Although the nucleotide sequence of the 'a' sequence varies between different virus strains, two highly conserved regions termed pac-1 and pac-2 are present in U_b and U_c respectively and appear to be essential for the cleavage/packaging process (Varmuza and Smiley, 1985; Deiss *et al.*, 1986).

1.2.5. Promoter activity of the 'a' sequence

The feature of the 'a' sequence most relevant to this study is its promoter activity. Chou and Roizman (1986) reported that in HSV-1 strain F, the 'a' sequence contains the promoter-regulatory domain and the transcription initiation sites of a gene located in the 'b' region of the genome. By fusing the 'a' sequence to a promoterless thymidine kinase gene, they found that when the 'a' sequence was orientated with Ub adjacent to the thymidine kinase gene, expression of thymidine kinase occurred, whereas when the 'a' sequence was in the opposite orientation (with Uc adjacent to the thymidine kinase gene) no expression occurred. Sequence analysis of the region between IE110 and the 'a' sequence predicted a protein of 358 amino acids in size which was designated ICP34.5. A peptide antiserum raised against a Proline-Alanine-Threonine repeat sequence found 10 times in the strain F sequence detected a protein of 43,500K in HSV-1 strain F infected cell extracts (Ackermann *et al.*, 1986). This protein will be dealt with in greater detail in a later chapter.

1.3. HSV latent infection

The basis of most clinical disease arising from HSV infection is the ability of the virus to preferentially and specifically interact with components of the peripheral nervous system (PNS). HSV has evolved a complex interaction with the neurons of the PNS whereby it can survive throughout the life of the infected individual in a latent state and thus evade the hosts immune response. Following primary infection and active replication at peripheral sites, virus attaches to the sensory nerve terminals (Vahlne *et al.*, 1978), enters them and travels centripetally via axons to neuronal cell bodies within the sensory ganglia (Cook and Stevens, 1973; McLennan and Darby, 1980). In the case of HSV-1 these are mainly

trigeminal ganglia (Bastian *et al.*, 1972; Baringer and Swoveland, 1973; Efstathiou *et al.*, 1986) and in the case of HSV-2 dorsal root ganglia (DRG) including sacral, lumbral and thoracic (Baringer, 1974; Galloway *et al.*, 1979, 1982). Latent virus has also been recovered from cells of peripheral tissues eg. corneas of humans (Schimeld *et al.*, 1982; Cook, 1988), and experimentally infected rabbits (Cook *et al.*, 1987), and mice (Openshaw, 1983), and footpads of experimentally infected mice (Al-Saadi *et al.*, 1983). Latency in peripheral tissues is thought to be a secondary sequelae to primary HSV infection (Hill, 1985).

As a working hypothesis in the laboratory, latency is defined as the ability to isolate infectious virus from explant cultures (of sensory ganglia) after a period of time in culture, where there has been failure to isolate virus from the explant tissue homogenate immediately post explantation. The phases of a latent infection are broadly divided into 3 main components- establishment, maintenance and reactivation. The phenomenon of latency has been the subject of extensive research over many decades and an outline of this is presented below.

1.3.1. Animal models of latency

A number of animal models have been developed in which HSV latency has been studied. By far, the most common is the mouse (Stevens and Cook, 1971; Walz *et al.*,1974; Al-Saadi *et al.*,1983; Clements and Subak-Sharpe, 1983, 1988). Additional experimental systems have been provided by rabbits (Nesburn, 1967, 1977), guinea pigs (Scriba, 1975, 1976) and monkeys (Reeves *et al.*, 1976, 1981). Peripheral inoculation sites of footpad (Stevens and Cook, 1971; Scriba, 1975), eye (Nesburn *et al.*, 1967), ear pinnae (Hill *et al.*, 1975, 1978, 1980) , flank (Underwood and Weed, 1974; Whitby *et al.*, 1987) and vagina (Scriba, 1976) have been used. Inevitably none of these models of HSV infection fully mimic the pathogenesis of HSV infection in humans in terms of eg. innoculation site, dose of input virus, reactivation triggers or immune response. They have however provided invaluable information on the three basic components of latency although none provide the ideal system to study establishment, maintenance and reactivation in a single host.

The first demonstration that HSV could establish a latent infection in sensory ganglia was provided following footpad inoculation of mice and the subsequent explantation of DRG which released infectious virus after a period of time in culture (Stevens and Cook, 1971). This fundamental study provided the basic model of *in vitro* reactivation as a measure of latent infection. It was consequently demonstrated by explantation following post mortem dissection that HSV resides in a latent state in human sensory ganglia (Warren *et al.*, 1978). The footpad model has been used extensively (Stevens and Cook, 1971; Walz *et al.*, 1974; Al-Saadi *et al.*, 1983; Clements and Subak-Sharpe, 1983, 1988) and has allowed the demonstration that HSV can also establish a latent infection within cell populations within the footpad skin (Al-Saadi *et al.*, 1983, 1988). Infection by the footpad route does not provide a system to study reactivation *in vivo* as recurrences neither occur spontaneously, nor can they be induced (Hill, 1985).

Another model which is widely used, is that of peripheral inoculation of the eye of both mouse (Knotts *et al.*, 1974; Walz *et al.*, 1974) and rabbit (Nesburn *et al.*, 1967; Stevens *et al.*, 1972). In the mouse spontaneous or induced reactivation is not reproducible (Harbour *et al.*, 1983; Shimeld *et al.*, 1990) and hence only factors affecting establishment and maintenance *in vivo* can be addressed. The rabbit on the other hand, provides a system where reactivation can be induced "on demand" by iontophoresis of adrenalin into the eye (Nesburn *et al.*,1977). Frequent spontaneous recurrent shedding also takes place (Nesburn *et al.*, 1977) and this makes interpretation of factors influencing reactivation difficult.

Both ear pinnae and flank inoculation of mice have facilitated the study of reactivation as virus recrudesence can be induced by mild trauma such as cellophane stripping (Hill et. al, 1978), U.V. irradiation (Blyth *et al.*, 1976) or treatment with chemicals, (Harbour *et al.*, 1983) at the site of inoculation.

A novel model of HSV latency has recently been described (Sawtell and Thompson, 1992a) which is based on the strong correlation between fever and HSV reactivation in humans (Boak *et al.*, 1934; Roizman and Sears, 1990). Latently infected mice are briefly immersed in hot water to induce mild hyperthermia, with the rate of rise in core temperature being controlled by the depth of immersion of the animal. Using this method virus could be

isolated from the trigeminal ganglia of mice within 14hrs post-induction; the approximate time required for 1 round of viral replication in culture. It is not clear if virus is shed at the appropriate peripheral site.

HSV-2 latency has historically been studied following intravaginal inoculation of guinea pigs which results in vaginal recurrences (Scriba, 1976). This system has been hampered by the high incidence of viral persistence in the cells of the vagina (Stanberry *et al.*, 1985).

The use of mutants in early stages of gene transcription has suggested replication is not required for the establishment of a latent infection (Clements and Stow, 1989; Coen *et al.*, 1989; Efstathiou *et al.*, 1989; Leib *et al.*, 1989a; Steiner *et al.*, 1990). Null mutants with lesions in Vmw63 and Vmw175 do not replicate in the eye or ganglia and fail to establish reactivable latent infection (Leib *et al.*, 1989a); since both Vmw63 and Vmw175 are essential for virus replication, it is possible that input virus leads to latent viral DNA below the level of detection.

Thymidine kinase mutant viruses can establish latent infection in neurons of mouse trigeminal ganglia but do not replicate productively in these cells (Coen *et al.*, 1989; Efstathiou *et al.*, 1989). During the early stages of establishment of latency by these mutants, expression of viral lytic genes is drastically reduced or undetectable as assayed by *in situ* hybridization (Kosz-Vnenchak *et al.*, 1990), suggesting that the block to productive replication during establishment of latent infection by HSV occurs before or early during the expression of α genes.

It has been shown that the vast majority of latent HSV genomes are in a linear non-integrated and possibly episomal state *in vivo* (Rock and Fraser, 1983; Efstathiou *et al.*, 1986), and a similar situation has been reported *in vitro* (Section 1.3.2.).

1.3.2. *In vitro* models of HSV latency.

Animal models by their very nature have intrinsic problems which make them inefficient as a system to study latency, especially at the molecular level. Tissue culture systems have therefore been developed in which latency can be mimicked and manipulated. As with

animal models, any tissue culture system can never be fully satisfactory but they can provide information which may facilitate the design of more meaningful *in vivo* experiments.

Various tissue culture systems have been studied as models of " *in vitro* latency". Cells of both neuronal and non-neuronal origin have been used eg. rat and human sensory neurons (Wigdahl *et al.*, 1983, 1984a,b; Wilcox and Johnson, 1987, 1988, 1990), corneal cells (Cook and Brown, 1987) and HFL cells (Russell and Preston, 1986; Harris and Preston, 1991). The systems have for the most part relied on the inhibition of genome replication by the use of inhibitors eg. AraC, acycloguanosine and/or the use of elevated temperatures generally >40^{o}C. The cultures have been assessed as containing HSV genomes in a "latent" state by the criteria of ability to reactivate infectious virus following manipulations such as temperature reduction (Russell and Preston, 1986), superinfection with HSV or other herpesviruses (Colberg-Poley *et al.*, 1979; Russell and Preston, 1986; Russell *et al.*, 1987) and BVDU/ IFN α treatment (Wigdahl *et al.*, 1983).

The question arises whether repressed equals latent ? In this Institute a system has been developed which does not rely on the use of inhibitors. Use of HSV-2 at very low m.o.i., elevated temperature of 42^{o}C and HFL cells has produced a working system which is reproducible and demonstrates some of the features of *in vivo* latency (Russell and Preston, 1986). It has led to an understanding of the role of the HSV gene IE1 in reactivation from latency (Russell *et al.*, 1987; Harris *et al.*, 1989; Zhu *et al.*, 1990) and Vmw65 αTIF (Harris and Preston, 1991) in the establishment of latency. Modification using the HSV-1 Vmw65 αTIF mutant (*in* 1814) has allowed higher levels of latency to be established. This has provided information on the episomal nature of the latent genome (Preston and Russell, 1991) and its relationship with chromatin (Dr D.R.S. Jamieson, personal communication). Unfortunately the system falls down in that there is no evidence of latency associated transcript expression (Section 1.3.3.) during the "latent" state.

1.3.3. Transcription during latency- latency associated transcripts (LATs).

Until recently there had been no convincing evidence of transcription from the latent HSV genome. In 1987, both Stevens *et al.*, and Spivak and Fraser discovered that a region of the

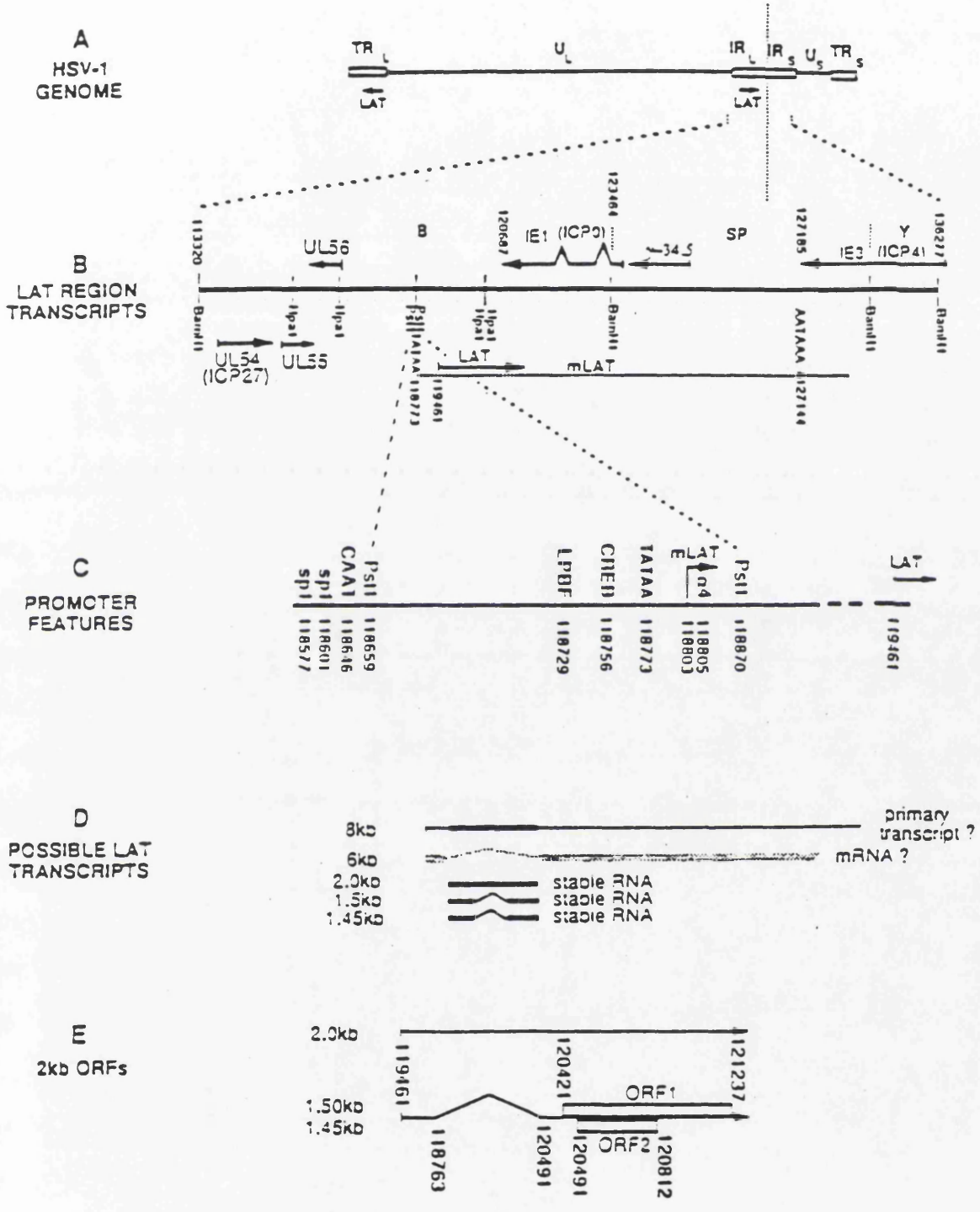
HSV-1 genome is transcribed during latency to preduce LATs. These have subsequently been demonstrated in human sensory ganglia (Krause *et al.*, 1988). Their discovery has led to inumerable investigations into their organization, relevance and role in latency. They are transcribed in an antisense direction from a genomic region adjacent to and partially overlapping the IE1 gene (Rock *et al.*, 1987; Spivak and Fraser, 1987; Stevens *et al.*, 1987; Krause *et al.*, 1988). Transcripts of size ~2, and 1.2-1.5 kb have been identified in various systems (Rock *et al.*, 1987; Spivak and Fraser, 1987; Krause *et al.*, 1988) and are thought to be the stable intron products of an 8kb primary transcript (Zwaagstra *et al.*, 1990). The location and organization of the LATs is shown diagramatically in Fig 1.4..

Although no protein products have been found or reported, with the exception of one unrepeated *in vitro* experiment (Doerig et.al., 1991) there are ORFs within the LATs. Many hypothesis regarding their role, especially in suppressing IE1 transcription, have been put forward (Stevens *et al.*, 1987). Various mutants in the LATs and LAT promoter regions have been constructed (Ho and Mocarski, 1989; Leib *et al.*, 1989b; Steiner *et al.*, 1989; Block *et al.*, 1990; Natarajan *et al.*, 1991; Trousdale *et al.*, 1991). The mutants appear to establish latency efficiently but the efficiency of and temporal control of reactivation both *in vitro* and *in vivo* is affected (Leib *et al.*, 1989b; Steiner *et al.*, 1989; Trousdale *et al.*, 1991). However a recent experiment suggests that LAT mutants may be impaired in establishing latency (Sawtell and Thompson, 1992b). The role of LATs is therefore still open to question.

Peripheral nervous system sensory neurons are undoubtly privileged sites for the establishment of HSV latent infections (Baringer and Swoveland, 1973; Warren *et al.*, 1978; Krause *et al.*, 1988). Despite the important discovery of LATs, a comprehensive understanding of the factors controlling this particular virus/host interaction has not been achieved. It may be pertinent in the future to investigate (i) the strategic importance of the juxtaposition of three ORFs whose products have defined roles in HSV/neuronal interactions ie. LATs, Vmw110 (Harris *et al.*, 1989; Zhu *et al.*, 1990) and ICP34.5 (Chou and Roizman, 1991; MacLean, A., *et al.*, 1991a; Dr S.M. Brown, personal

Figure 1.4. The LAT region of HSV-1

(A) The HSV-1 genome is shown. The LATs are encoded by a diploid gene that maps to the repeat regions. (B) The LAT region. LAT is overlapped by transcripts expressed during a lytic infection. (C) LAT promoter features. The putative LAT promoter maps to a TATA element between two PstI sites at nucleotide 118,773 (McGeoch *et al.*, 1988a). Within this region are several transcription factor binding sites, which probably play a role in LAT regulation. (D) Possible LAT transcripts. From the LAT promoter, an approximately 8-kb long primary transcript is proposed to be transcribed during latency. Stable 2-, 1.5- and 1.45-kb RNA species which map towards the 5' end of LAT have been identified in tissue culture and accumulate to high levels in latently infected tissue. The 6.0-kb LAT which would result from splicing, if the 2.0-kb transcript is an intron has not been detected. (E) 2.0-kb LAT open-reading frames. Within the 2-kb RNA, there are two open-reading frames that are conserved within several HSV-1 strains but not in HSV-2 strain HG52 (McGeoch *et al.*, 1991). However, no convincing evidence for expression of any viral polypeptide during *in vivo* latency exists (Adapted from Fraser *et al.*, 1992).


A
HSV-1
GENOME
TR L
U L
IR L
IR S
U S
TR S
LAT
LAT
B
LAT REGION
TRANSCRIPTS
UL56
B
IE1 (ICP0)
SP
IE3 (ICP4)
Y
UL54
(ICP27)
UL55
LAT
mLAT
AATAAA
C
PROMOTER
FEATURES
CAAT
Sp1
Sp1
Sp1
LPDF
CTED
TATAA
mLAT
n4
LAT
D
POSSIBLE LAT
TRANSCRIPTS
8kb
6kb
2.0kb
1.5kb
1.45kb
primary
transcript ?
mRNA ?
stable RNA
stable RNA
stable RNA
E
2kb ORFs
2.0kb
1.50kb
1.45kb
ORF1
ORF2

communication) (ii) whether other cellular factors which may or may not be present in neurons, make a contribution to the latency process such as has been demonstrated for Oct 2 (Lillycrop *et al.,* 1991), and (iii) whether control is exercised in the form of differential transcriptional splicing of defined genes in cells of neuronal and non-neuronal origin.

1.4. The HSV-1 lytic cycle

1.4.1. Adsorption (attachment of HSV to the cell surface).

Entry of HSV-1 into cells requires a cascade of events that occur at the cell surface (reviewed by Spear,1993). Adsorption is mediated, at least in part, by the binding of virions to heparin sulphate moieties. There are several lines of evidence to support this: (i) HSV does not bind to surfaces stripped of heparin sulphate by enzymatic treatment with heparinase or hepartinase, or to mutant cells that fail to synthesize heparin sulphate (WuDunn and Spear, 1989), (ii) HSV virions can bind to immobilized heparin and soluble heparin can block the binding of HSV to cells (WuDunn and Spear, 1989) and (iii) Heparin-binding proteins can inhibit HSV adsorption and plaque formation (WuDunn and Spear, 1989; Kaner *et al.,* 1990), probably by competing with virus for receptor sites associated with cell-surface heparin sulphate.

Shieh *et al.* (1992) used CHO cell mutants, defective in various aspects of heparin sulphate synthesis to provide genetic evidence that heparin sulphate serves as a cell surface receptor for HSV. Binding of virus to heparin sulphate-deficient mutant cells was severely impaired and mutant cells were resistant to HSV infection.

Glycoprotein C (gC) appears to be the glycoprotein principally involved in adsorption of HSV-1 to the cell surface (Kuhn *et al.,* 1990; Herold *et al.,* 1991), but gD and gB also probably play a role in adsorption as monoclonal antibodies against these glycoproteins can inhibit virion binding to cellular surface components (Kuhn *et al.,* 1990). Virions lacking gC bind to significantly fewer cells than wild-type virus under conditions approaching saturation of available sites on the cell surface (Herold *et al.,* 1991). In PRV (Robbins *et al.,* 1986b; Mettenleiter, 1989) and BHV-1 (Liang *et al.,* 1991) homologues of gC have been

reported to play a similar role in mediating stable attachment of virions to the cell surface.

1.4.2. Penetration (entry of bound virus into the cell).

At least 5 viral glycoproteins, gD, gB, gH, gK and gL (Fuller and Spear, 1987; Cai *et al.*, 1988; Ligas and Johnson, 1988; Fuller *et al.*, 1989; Fuller *et al.*, 1989; Hutchinson *et al.*, 1992a, 1992b; Roop *et al.*, 1993) are involved in virus entry into cultured cells. Several results suggest that gD in virions interacts with a cellular receptor after virion attachment to heparin sulphate proteoglycans. Cell lines which express large amounts of gD are resistent to infection (Campadelli-Fiume *et al.*, 1988; Johnson and Spear, 1989), suggesting that intracellular gD sequesters a receptor. Infection can be prevented by prior exposure to U.V.-inactivated wild-type virus but not gD minus virus (Ligas and Johnson, 1988). The interactions of gD with its receptor are required for virus entry into cells but not for adsorption, which occurs whether or not the critical gD receptors are blocked (Johnson and Ligas, 1988; Ligas and Johnson, 1988). In addition, HSV-1 and HSV-2 plaque production can be inhibited by treatment of cells with soluble forms of gD (Johnson *et al.*, 1990). Binding of soluble gD was inhibited by treatment of cells with certain proteases, but not by enzymatic treatment to remove cell surface heparin sulphate glucosaminoglycans or when binding was carried out in the presence of heparin. This suggests that gD binds to proteins, or to structures dependent on proteins on the cell surface following initial adsorption of gC to heparin sulphate moieties.

Kaner *et al.* (1990) suggested that fibroblast growth factor receptor (bFGFR) was a portal of entry for HSV-1 as they found that Chinese Hamster Ovary (CHO) cells expressing a transfected receptor for bFGF bound and internalized greater amounts of radiolabelled HSV than did the bFGF deficient parental cells or control cells. Binding was inhibited by bFGF. The drawback with this study was that only one cell line- bovine arterial smooth muscle- was used and several groups (Shieh and Spear, 1991; Muggeridge *et al.*, 1992) have since shown that inhibition by bFGF is restricted to a minority of cell types. Muggeridge *et al.* (1992) tested several cell lines, but only one showed comparable inhibition by bFGF. Furthermore, they could not find a correlation between the amount of bFGF receptor and the

ability of bFGF to block infection, as cell lines expressing low levels of bFGF receptor were permissive for HSV. FGF receptor- positive and FGF receptor-negative rat myoblasts do not differ in susceptibility to HSV infection (Mirda *et al.*, 1992) and soluble forms of FGF fail to inhibit HSV infection whereas they inhibit basic FGF binding and biological activity. Taken together, these results suggest that different cell types harbour different receptors for HSV.

Productive entry of HSV-1 can also occur through pH-independent direct fusion of the virion envelope with the plasma membrane that leads to disassembly of the virion and initiation of gene expression (Fuller and Spear, 1987). Neither the molecular interactions which mediate this entry nor the specific roles which the essential glycoproteins play in penetration have been defined. Fuller and Lee (1992) proposed a model for pH-independent entry of HSV-1 into cells with separate roles for gD and gH. gD was shown to play a role in the early stage of virus penetration, contributing to a stable virion attachment and close association of the virion envelope and cell membrane, while gH neutralized virus proceeded to events post-attachment but did not completely disassemble or fuse with the cell membrane. It appears that HSV-1 entry into cells is mediated by a cascade of virus-cell interactions in which each glycoprotein plays a specific role in triggering or signalling changes in the virion which result in membrane fusion and nucleocapsid uncoating.

1.4.3. Additional functions of the HSV-1 glycoproteins

Cell fusion

The HSV-1 glycoprotein gK (Hutchinson *et al.*, 1992b) is the product of the UL53 gene (DebRoy *et al.*, 1985; McGeoch *et al.*,1988a). It was detected using a peptide antiserum raised against the hydrophilic region of the predicted protein. gK is thought to play an important role in regulating membrane fusion and the majority of well characterized syncytial mutations map to this gene (Ruyechan *et al.*, 1979; Bond and Person,1984; Pogue-Geile and Spear,1987). The EHV-1 UL4 gene codes for a protein homologous to HSV-1 gK (Telford *et al.*, 1992; Zhao *et al.*, 1992).

Mutations affecting the cytoplasmic C-terminal domain of gB also induce a syncytial

phenotype (DeLuca *et al.*, 1982; Bzik *et al.*, 1984; Cai *et al.*, 1988). gB-negative virus cannot enter cells, but its infectivity is increased by treatment with polyethylene glycol (PEG) to promote cell fusion (Cai *et al.*, 1988). It appears likely that gB is directly involved in cell fusion.

The glycoproteins gH and gD also play a role in virus entry into cells, and although mutations in these genes do not give rise to a syncytial phenotype, there is evidence that both may be involved in cell fusion. gH-negative virions bind to cells, but do not enter them unless treated with PEG (Forrester *et al*,.1992). Similarly anti-gD mAbs can neutralize HSV infectivity by blocking penetration of the virus at the cell surface, (Fuller and Spear, 1987) apparently by blocking fusion between the virion envelope and plasma membrane. Neutralized virus binds to cells and accumulates on the surface with reversal of the effect of neutralizing antibody again being achieved by treatment with PEG.

Other syncytial mutations have been mapped to non-glycoprotein encoding regions of the HSV-1 genome including UL24 (Sanders *et al.*, 1982; Jacobson *et al.*, 1989a) and the *Syn* 6 locus (Romanelli *et al.*,1991) which maps between the 5' end of IE110 and the 3' end of RL1. Deletion of the UL20 gene also appears to give rise to a syncytial phenotype (Baines *et al.*, 1991). Previous marker rescue experiments had erroneously suggested that a syn mutation mapped to the UL1 gene (Little and Schaffer,1981; McGeoch *et al.*, 1988a), which was recently shown to encode gL (Hutchinson *et al.*, 1992a), but more recently this mutation was found to map to the UL53 gene (Roop *et al.*, 1993).

Fc receptor

Receptors which have affinity for the Fc domain of immunoglobulin G (IgG) are expressed on the surface of HSV-1 infected cells and on virion envelopes (McTaggart *et al.*, 1978; Westmoreland and Watkins, 1974). In 1979, Bauke and Spear identified a group of electrophoretically similar HSV-1 polypeptides which bound with high affinity to IgG columns. These proteins were all thought to be derived by post-translational modification of gE (Cross *et al.*, 1987). Johnson and Feenestra (1987) subsequently detected a novel HSV-1 induced polypeptide which was structurally distinct from gE and was provisionally named

g70. It could be detected on the surfaces of infected cells and was co-precipitated using rabbit or human IgG or anti-gE antibody.

Johnson *et al.* (1988) used a monoclonal antibody (MAb 3104) to further characterize g70. Using extracts from pulse-labelled cells, a polypeptide was precipitated with MAb 3104, which had an electrophoretic mobility identical to the smaller polypeptide precipitated with rabbit IgG. This polypeptide was the mature form of a protein with an electrophoretic mobility very similar to the immature form of gE. Insertional mutagenesis and antipeptide sera were used to map g70 to the US7 open reading frame and gI (Johnson *et al.*, 1988; McGeoch *et al.*, 1988a). US7 had previously been shown to be non-essential (Longnecker *et al.*, 1987).

Expression of gE alone is sufficient for induction of Fc activity, whereas gI alone gives no detectable Fc binding (Bell *et al.*, 1990; Dublin *et al.*, 1990). Coexpression of gI and gE gives better binding than expression of gE alone, suggesting that HSV-1 has the potential to induce 2 Fc receptors- one a low affinity receptor composed of gE alone and a high affinity receptor composed of gE + gI. gE appears to be the Fc receptor for IgG complex, while gE and gI form the Fc receptor for monomeric IgG (Dublin *et al.*, 1990).

C3b binding

Mammalian cells in culture which are infected with HSV-1 express receptors for the C3b component of complement (Cines *et al.*, 1982). These receptors are not present following HSV-2 infection. Friedman *et al* (1984) demonstrated that gC-1 but not gC-2, when expressed on cells, functions as a C3b receptor. Sequence analysis has shown that gC-1 relative to gC-2 has a 28 amino acid insert (Frink *et al.*, 1983; Swain *et al.* ,1985) which may explain the functional differences between these 2 polypeptides.

Purified gC-1 has been found to demonstrate dose-dependent acceleration of decay in the activity of C3 convertase of the alternative pathway and, by this mechanism, can block the effects of complement (Fries *et al.*, 1986). gC-1 can thus protect against complement mediated cytolysis by inhibition of the alternative pathway (Harris *et al.*, 1990) The presence of gC-1 or gC-2 in virions appears to provide protection against complement mediated

neutralization (McNearney *et al.*, 1987).

1.4.4. Effects on host cell macromolecular synthesis

A characteristic of herpesvirus-infected cells is the rapid shut-off of host macromolecular metabolism early in infection (reviewed by Fenwick, 1984). In the host cell, DNA synthesis is shut-off, protein synthesis declines, RNA synthesis is reduced and glycosylation of host proteins ceases.

HSV host shut off occurs in two stages, one (early shut-off, or *vhs*) is due to the action of a constituent of the infecting virus particle and does not require *de novo* protein synthesis (Fenwick and Walker, 1978; Kwong and Frenkel, 1987; Kwong *et al.*, 1988), whereas the other (delayed shut-off) requires virus gene expression (Read and Frenkel, 1983; Fenwick and Clark, 1982).

The viral component responsible for *vhs* activity has been mapped to the UL41 open reading frame of the HSV-1 genome (Kwong *et al.*, 1988). It is predicted to encode a 489 amino acid protein of 58K (Frink *et al.*, 1981; McGeoch *et al.*, 1988a). which is a virion component (Fenwick and Everett, 1990a) that is phosphorylated in infected cells (Smibert *et al.*, 1992). *vhs* activity is not essential for virus replication. The level of *vhs* activity varies markedly between different HSV strains and is particularly weak in HSV-1 strain 17^+ (Fenwick and Everett, 1990b). Several conserved regions have been identified by protein sequence alignment with various alphaherpesviruses (HSV-1, HSV-2, VZV and EHV-1) counterparts (Berthomme *et al.*, 1993).

1.4.5. Virion assembly

Very little is known about how the virus particle is formed but the available evidence supports a model in which the viral genome is inserted into a pre-formed capsid (Preston *et al.*, 1983). One protein involved in virion assembly is VP22a (ICP35) which is present in large amounts in intermediate capsids which lack virion DNA but is only found in trace amounts in empty capsids devoid of any internal structure, in DNA-containing capsids and in mature virions (Rixon *et al.*, 1988; Sherman and Bachenheimer, 1988). The precise

function of VP22a is unclear, but its ability to self-assemble into defined structures (toroids) *in vitro* (Newcomb and Brown, 1991), in the absence of preformed capsids raises the possibility that its *in vivo* function may be to form a scaffold or jig around which other proteins (VP5, VP19 and VP23) could condense to form the capsid shell.

VP22a was initially identified as the product of HSV-1 gene UL26 from the genome location of the lesion in the mutant *ts* 1201 which has a defect in the N-terminal part of UL26 and is defective in processing VP22a (Preston *et al.*, 1983). This region has since been shown to encompass 2 genes designated UL26 and UL26.5 (Liu and Roizman, 1991a) which encode 2 related proteins specified by overlapping transcripts with a unique 5' end but common 3' ends (see fig 1.5). The inability of *ts* 1201 to process VP22a at non-permissive temperatures was explained by the recent discovery that UL26 has proteolytic activity which is responsible for cleaving UL26.5 to produce VP22a (Liu and Roizman, 1991b, 1992; Preston *et al.*, 1992). The protease undergoes self cleavage at 2 sites; one near the C terminus and the other at a second position upstream from the UL26.5 sequence to release 2 large fragments VP21 and VP24. These have recently been identified as capsid components (Davison *et al.*, 1992).

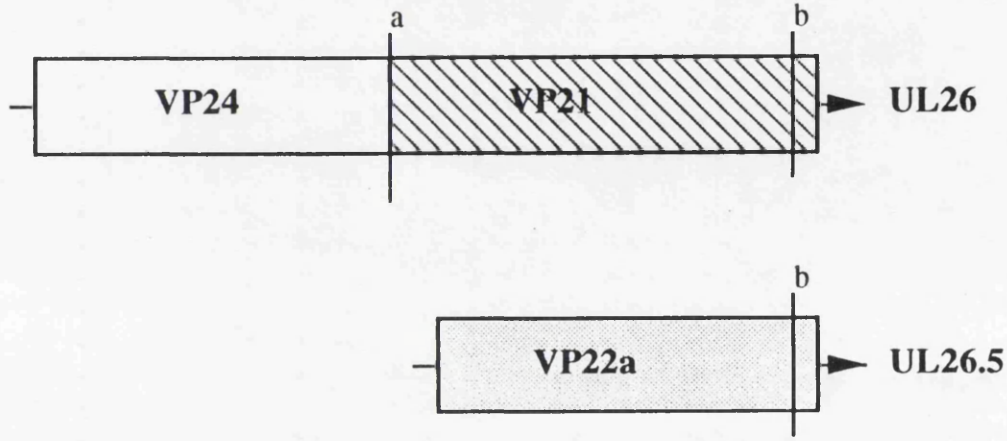
Recently infection of cells with baculovirus expressing all the capsid genes has allowed reconstitution of virus capsids (J. Tatman, personal communication).

1.4.6. Envelopment and Egress

HSV capsids acquire a tegument and envelope *en route* from the nucleus to the oustide of the cell. The precise details of events are still unclear, but it is apparent that capsids leave the nucleus by budding through the inner membrane into the space between the inner and outer membrane (Darlington and Moss, 1968; Nii *et al.*, 1968). Johnson and Spear (1982) proposed a model for HSV-1 envelopment and viral egress by which virions were first enveloped at the inner membrane where they acquired viral glycoproteins lacking golgi modifications. Subsequently these "immature" virions are transported to the Golgi apparatus, where precursor glycoproteins are modified as the enveloped particles move through the Golgi stacks. It was concluded that complete processing of viral glycoproteins is

Figure 1.5. Organization of the HSV-1 genes UL26 and UL26.5

The two 3' co-terminal mRNAs are shown as rightward orientated arrows. These are overlaid by boxes indicating the positions of the open reading frames specifying the protease (UL26) and VP22a (UL26.5). Proteolytic cleavage sites (a and b) are indicated by vertical lines. Cleavage of the protease at site *a* releases the capsid proteins VP21 and VP24 (adapted from Rixon,1993).


a
b
VP24
VP21
UL26
b
VP22a
UL26.5

not essential for virion morphogenesis or infectivity but is associated with egress of virions The first evidence that a viral gene product was involved in egress of the virus was provided by Baines *et al.* (1991). They demonstrated that deletion of the UL20 gene (McGeoch *et al.*, 1988a) resulted in accumulation of enveloped and unenveloped capsids in the cytoplasm. The product of the UL20 gene was required in some cell lines although not in Vero cells, possibly indicating the presence of a cellular gene product which can complement the UL20 deletion.

It is suspected that the product of the UL11 gene- a myristylated virion protein- also plays a role in virus assembly, envelopment or release (MacLean *et al.*, 1989, 1992)

1.5. HSV-1 DNA replication

HSV-1 DNA replication is first detected 3 hours post-infection at 37°C, peaks by about 9-11 hours and is virtually complete by 16 hours post-infection (Wilkie, 1973). The majority of the progeny viral DNA are large concatemeric molecules (Jacob *et al.*, 1979) and hence, it has been hypothesized that viral DNA replication occurs by a rolling-circle mechanism (Roizman, 1979).

Initially, synthesis is localised within the nucleus to discrete sites , which grow and coalesce as infection proceeds, until the entire nucleus is filled with replicating viral DNA (Rixon *et al.*, 1983). The initial sites of viral DNA replication appear to be virus specific structures induced by infection, and have been called "replication compartments" (Quinlan *et al.*, 1984).

1.5.1. HSV-1 origins of replication

Herpes simplex virus has been shown to contain 3 origins of DNA replication. These sequences were originally identified as those required for replication of defective genomic DNA in infected cells (Frenkel *et al.*, 1976; Schroder *et al.*, 1975). The first HSV origin of replication, oriS was identified using a plasmid based system (Stow, 1982). Fragments of DNA from the S region of the wild-type HSV-1 genome were cloned into plasmids and

analysed for their ability to enable amplification of the linked plasmid vector sequences, when helper functions were provided by superinfecting virus. An origin was mapped within a 955bp fragment contained entirely within the short repeat genome segment, and was thus present in 2 copies. Deletion analysis of this oriS fragment defined a 90bp region containing all the necessary *cis* -acting signals required for the initiation of HSV DNA replication (Stow, 1982; Stow and McMonagle, 1983).

The other HSV-1 origin of DNA replication, oriL, is located at map units 0.4 in the unique sequences of the L component of the HSV-1 genome (Gray and Kaerner, 1984; Weller *et al.*, 1985). The sequences of oriS and oriL are closely related (Weller *et al.*, 1985; Murchie and McGeoch, 1982). Both contain an extensive inverted repeat sequence, the central 18bp of which are exclusively AT base pairs. The inverted repeat region of oriL is considerably longer than that of oriS. For this reason plasmids containing oriL, but not oriS are highly unstable in *E.coli* and analysis of this region has proved difficult.

Weller *et al.* (1985) successfully cloned the sequences between 0.398 and 0.413 map units in the HSV-1 genome, which contain oriL, into a yeast cloning vector in an undeleted form. Sequence analysis of a 425bp region revealed a 144bp perfect palindrome with striking homology to oriS. The minimum sequences required for the function of oriS correspond well to the region of highest similarity with oriL (Lockshon and Galloway, 1988) indicating that they are functionally equivalent.

1.5.2.Gene products required for HSV-1 replication

The complete set of viral genes that are required for HSV-1 DNA replication were identified by means of a transient complementation assay in which cloned segments of HSV-1 DNA were tested for the ability to support the replication of co-transfected plasmids containing oriL or oriS. Seven genes (UL5, UL8, UL9, UL29, UL30, UL42 and UL52) were found to be both necessary and sufficient for origin dependent DNA replication (McGeoch *et al.*, 1988b; Wu *et al.*, 1988). Similar results were obtained in insect cells transfected with an oriS-containing plasmid and superinfected with a mixture of 7 baculovirus recombinants expressing the HSV-1 DNA replication proteins (Stow, 1992). These genes are all essential

for virus replication and DNA synthesis in tissue culture (Weller, 1991). A brief outline of each is given below and their positions on the HSV-1 genome are illustrated in figure 1.6. (for review, see Challberg, 1991).

UL9

The initial event in the activation of a HSV-1 origin of replication is likely to be the sequence specific binding of an origin binding protein (OBP) to oriL or oriS.

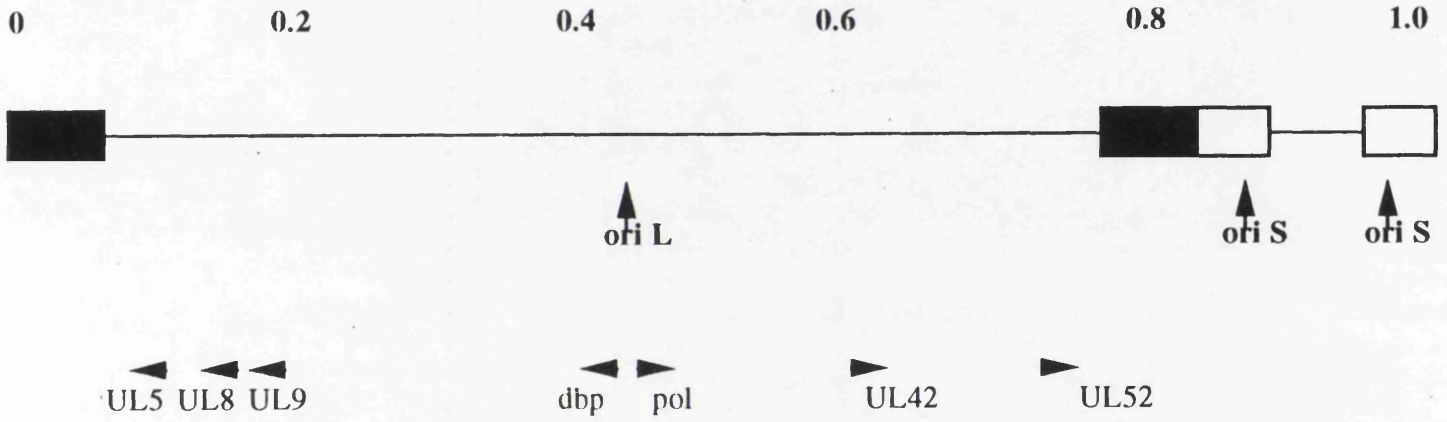
Olivio *et al.* (1988) expressed the UL9 gene using a baculovirus expression system and using an immunoassay for protein-DNA interaction, they demonstrated that the UL9 protein binds specifically to the HSV origins of replication. Two pieces of evidence suggested that origin-binding is an intrinsic property of UL9; protein DNA complexes containing oriS and an origin specific binding activity from HSV-1 infected cells were immunoprecipitated with UL9 antiserum, and extracts from insect cells infected with recombinants expressing UL9 also contain an HSV-1 origin binding activity. Again, this binding activity can be precipitated with UL9 antiserum. DNase I footprinting analysis showed that UL9 interacts with 2 related high affinity sites (site I and site II) on oriS. As had previously been shown (Stow *et al.*, 1983), site I occurs within a region of hyphenated dyad symmetry, whereas site II is located in a region homologous to site I on the other side of the dyad axis.

It has subsequently been demonstrated that oriS contains a third UL9 binding site (site III) which is of much lower affinity (Elias *et al.*, 1990; Dabrowski and Schaffer, 1991). Analysis of sites I and II has identified an 11bp sequence-CGTTCGCACTT- which is specifically recognised by UL9 (Koff and Teghnmeyer, 1988; Elias *et al.*, 1990). Mutations in site I or site II which abolish UL9 binding eliminate or greatly reduce DNA replication, whereas mutations in site III reduce replication efficiency by a factor of around 5 (Weir and Stow, 1990; Hernandez *et al.*, 1991).

The sequence of events which follow UL9 binding are unclear. The two parental strands must be unwound before replication is initiated. This is consistent with the finding that UL9 has intrinsic helicase activity (Bruckner *et al.*, 1991). When cells expressing truncated forms of OBP are infected with HSV-1 they do not replicate the DNA efficiently (Stow, 1992;

Figure 1.6. Locations of the genes determining DNA replication functions on the herpes simplex virus genome.

The HSV-1 genome is shown schematically in the prototype orientation with the unique regions drawn as thin lines and the inverted repeats flanking the long and short unique regions as closed and open boxes respectively. The positions and orientations of the ORF's encoding the seven proteins essential for viral DNA replication are shown by arrows. The positions of the origins of replication, oriL and oriS are also indicated (adapted from Arbuckle, 1993).


0
0.2
0.4
0.6
0.8
1.0
ori L
ori S
ori S
UL5 UL8 UL9
dbp
pol
UL42
UL52

Perry *et al.*, 1993; Stow *et al.*, 1993). The transdominant phenotype of truncated forms of OBP is probably not due to a loss of helicase activity, but retention of binding capacity; hence the truncated forms bind to one or both high-affinity sites in a replication origin, thereby blocking that origin's participation in a productive replication complex. This shows that DNA binding alone is insufficient for UL9 function and that helicase activity is also required (Stow,1992).

UL42 / Pol(UL30)

The HSV-1 DNA polymerase (UL30) co-purifies from infected cells in a heterodimeric form with its accessory protein UL42 (Gallo *et al.*, 1988; Crute *et al.*, 1988). One known function of UL42 is to increase the ability of Pol to synthesize longer DNA products (Gottlieb *et al.*, 1990; Hernandez and Lehman, 1990) through an increase in processivity. The DNA polymerase has intrinsic 3'-5' exonuclease activity which probably serves a proof-reading function to increase the fidelity of DNA replication (O'Donnell *et al.*, 1987; Purifoy and Powell, 1977) and a 5'-3' exonuclease/RNase activity similar to *E.coli* Pol I (Crute and Leihman, 1989). This activity may play a role in the removal of the RNA primers present on the 5' end of Okazaki fragments which are made on the lagging strand during semi-discontinuous synthesis.

As UL42 and Pol are essential for virus growth (Aron *et al.*, 1975; Marchetti *et al.*, 1988; Marcy *et al.*, 1990; Johnson *et al.*, 1991) this would indicate that the interaction between the 2 polypeptides is also essential (Gottlieb *et al.*, 1990; Digard and Coen, 1990) making it a valid target for antiviral agents.

Owsianka *et al.* (1993) attempted to identify the regions of UL42 which affect DNA polymerase activity.

They synthesized overlapping decapeptides spanning the entire 488 amino acids of the UL42 protein and tested their ability to inhibit polymerase activity on a defined template primer in the presence or absence of UL42. Peptides from 5 separate regions were found to interfere with HSV polymerase activity, but there was no evidence that these peptides acted by disruption of the Pol/UL42 interaction. Two other groups (Digard *et al.*, 1993; Tenney *et*

al., 1993) used combinations of deletion and insertion mutagenesis to demonstrate that sequences mapping in the carboxyterminal 40 amino-acids of DNA polymerase are crucial for the stability of UL42 binding. In addition, a region which is highly conserved in several other Alphaherpesviruses (HSV-2, EHV-1 and VZV), between amino acids 1176 and 1195 of UL30, was found to be crucial for a productive interaction with UL42 (Tenney *et al.*, 1993).

UL5/UL8/UL52 (helicase-primase)

Crute *et al.* (1988), identified a DNA-dependent ATPase, present specifically in HSV-1 infected cells. Partial purification of this activity and SDS-polyacrylamide gel electrophoresis of the fractions obtained by Superose 12 gel-filtration showed that 3 major polypeptides with masses of 130, 97 and 70 kDa coincided with the peak of DNA dependent ATPase activity.

These proteins were purified to homogeneity (Crute *et al.*, 1989) and immunochemical analysis revealed that they were the products of the UL52, UL5 and UL8 open-reading frames (Wu *et al.*, 1988; McGeoch *et al.*, 1988b). In addition, analysis of DEAE-sephadex fractions for DNA primase activity showed that the primase co-eluted perfectly with the herpes-induced DNA-dependent ATPase. The association of these 2 activities enables close coordination of the priming of the lagging strand DNA with unwinding of the replication fork.

The precise role which each subunit plays in these activities is unclear, but a complex consisting of only the UL5 and UL52 subunits *in vitro* exibits all the known activities of the 3 component complex (Calder and Stow, 1990; Dodson and Lehman, 1991). The predicted UL5 amino acid sequence contains 6 sequence motifs which are characteristic of helicases (Hodgman,1988; McGeoch *et al.*, 1988b). However purified UL5 has not been demonstrated to act as a helicase in the absence of UL52 (Calder and Stow, 1990; Dodson and Lehman, 1991).

Calder *et al.* (1992) used immunofluoresence to demonstrate that the UL8 protein is important for efficient nuclear uptake of the helicase-primase complex. In addition, UL8 acts to increase the efficiency of primer utilization by stabilizing the association between nascent

oligoribonucleotide primers and template DNA (Sherman *et al.*, 1992). UL8 has recently been expressed in insect cells (Parry *et al.*, 1993), but no binding of the purified protein to ssDNA, dsDNA or to a DNA/RNA hybrid was observed, suggesting that UL5 and UL52 are required for binding of UL8 to nucleic acid.

UL29

The major ssDNA-binding protein (mDBP) was first recognised as an abundant HSV induced protein of about 130kDa (Honess and Roizman, 1973; Powell and Courtney, 1975) which binds more tightly to ssDNA columns than to dsDNA columns (Bayliss *et al.*, 1975; Purifoy and Powell,1976) based on DNA cellulose chromatography of infected cell extracts. Analysis of *ts* mutants (Conley *et al.*, 1981; Powell *et al.*, 1981; Weller *et al.*, 1983; Holland *et al.*,1984) later demonstrated that the mDBP (also known as ICP8) is the product of the UL29 gene.

UL29 has many characteristics of a helix destabilizing protein. It not only binds preferentially and co-operatively to ssDNA templates with no detectable sequence specificity (Ruyechan, 1983; Ruyechan and Weir, 1984) but it can also enhance the denaturation of a polydeoxyadenylic acid-polydeoxythymidylic acid duplex (Powell *et al.*, 1981). It is likely that the HSV-1 mDBP has a function analogous to that of the *E.coli* ssDNA binding protein, which binds to and stabilizes regions of ssDNA at the replication fork (Powell *et al.*, 1981; Ruyechan, 1983; Ruyechan and Weir, 1984).

UL29 is known to form intracellular complexes with viral DNA (Lee and Knipe, 1983). It accumulates at specific regions within the cell nucleus by 4-5hrs post-infection (Quinlan *et al.*, 1984) and under certain conditions where DNA synthesis is permitted UL29 is found at discrete pre-replicative sites throughout the nucleus where it plays a role in organizing DNA replication proteins (deBruyn Kops and Knipe, 1988; Bush *et al.*, 1991).

There is evidence to suggest that UL29 may interact specifically with other replication proteins. Bush *et al.* (1991) used immunofluoresence to examine the intracellular localization of HSV-1 Pol in infected cells. Pol was found to localize to the nucleus in the absence of any other replication proteins, but functional mDBP was required for localization of Pol to

the pre-replicative sites. In cells infected with mutant viruses encoding defective Pol molecules, mDBP localizes to pre-replicative sites, indicating that Pol is not required for formation of pre-replicative sites or the localization of mDBP to these structures. Although several studies have supported a possible direct interaction between Pol and mDBP (Hernandez and Lehman, 1990; Ruyechan and Weir,1 984; Chiou *et al.,* 1985; O'Donnell *et al.,* 1987) no direct biochemical evidence for an interaction has been presented.

Several laboratories (Weller *et al.,* 1983; Leinbach *et al,* 1984; Leinbach and Heath, 1988; Gao and Knipe, 1989) have attempted to identify functional domains of UL29. It appears that the carboxyterminal region of the gene contains the ssDNA binding site (Leinbach and Heath, 1988; Gao and Knipe, 1989) while the amino-terminus contributes to the ssDNA binding activity of the intact protein by maintaining the carboxyterminus in an active configuration (Leinbach and Heath, 1988).

1.5.3. Enzymes required for DNA replication

HSV-1 specifies a large array of enzymes which are involved in nucleic acid metabolism. With the exception of the alkaline DNase (Weller *et al.,* 1990) these are generally dispensible for growth in tissue culture.

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

A HSV encoded dUTPase has been identified (Caradonna and Cheung, 1981) which maps to the UL50 open reading frame (Preston and Fischer, 1984; McGeoch *et al.,* 1988a). dUTPases act to hydrolyse dUTP to dUMP, providing both a mechanism to prevent incorporation of dUTP into DNA and a pool of dUMP for conversion to dTMP by thymidylate synthetase.

The HSV ribonucleotide reductase (RR) functions to reduce ribonucleotides to

deoxyribonucleotides creating a pool of substrates for DNA synthesis (Bacchetti *et al.*, 1986, 1986; Ingemarson and Lankinen, 1987). In HSV-1 the enzyme consists of a large subunit R1, and a small sub-unit R2, which have M_r values of 136K and 38K respectively (Frame *et al.*, 1985; Bacchetti *et al.*,1986; Darling *et al.*, 1988). The subunits consist of a tight complex in an $\alpha 2\beta 2$ structure similar to the RR enzymes of *E.coli* and mammalian cells (Ingemarson and Lankinen, 1987). The large sub-unit, designated ICP6 (Honess and Roizman ,1973, 1974), is encoded by the UL39 gene; the small subunit, designated ICP38, is encoded by the UL40 gene (McGeoch *et al.*, 1988a). These genes map between 0.562 and 0.597 m.u. on the viral genome and are translated from 3'-coterminal RNAs of 5.0 and 1.3kb respectively (Anderson *et al.*, 1981; Swain and Galloway, 1986; McLauchlan and Clements, 1983). A synthetic nonapeptide (YAGAVVNDL), corresponding to the carboxy-terminal 9 amino acids of the small subunit specifically inhibits the HSV-1 enzyme (Dutia *et al.*, 1986; Cohen *et al.*, 1986) by dissociation of the 2 subunits (McClements *et al.*, 1988).

HSV-1 thymidine kinase phosphorylates thymidine (Kit and Dubbs, 1963; Dubbs and Kit, 1964), deoxycytidine (Jamieson and Subak-Sharpe, 1974) and thymidylate (Chen and Prusoff, 1978).

A virus specific topoisomerase has also been reported (Muller *et al.*, 1985) but has not been shown to be virally encoded (Bapat *et al.*, 1987).

These enzymes will be discussed more fully in a later section in relation to their role in HSV pathogenicity.

1.6. Regulation of transcription of viral genes by HSV-1

The expression of viral genes during infection of tissue culture cells by HSV-1 can be divided into 3 broad classes termed α, β and γ (Honess and Roizman, 1974, 1975) or immediate-early (IE), early (E) and late (L), (Clements *et al.*, 1977) based on their kinetics of appearance and requirements for either *de novo* protein or DNA synthesis. This "cascade" pattern is however, more complex than a simple 3-fold one (Wagner, 1985). The late genes can be subclassed into leaky-late ($\beta\gamma$, $\gamma 1$) which require 3 to 5hrs of protein synthesis before

expression, or true-late (γ2) which require in addition the onset of viral DNA replication (Weinheimer and McKnight, 1987; Jones and Roizman, 1979; Harris-Hamilton and Bachenheimer, 1985; Clements *et al.*, 1977). In general, the IE genes code for transcriptional regulatory proteins, E genes are involved in DNA replication and L genes are structural and assembly proteins.

In the presence of inhibitors of protein synthesis, mRNAs from the 5 IE genes accumulate in relatively large quantities. The IE genes are defined as those genes which can be transcribed in the absence of *de novo* protein synthesis. Following separation by gel electrophoresis, IE-mRNAs labelled with ^{32}P were used to locate the IE genes on the viral genome and *in vitro* translation was used to define their products (Watson *et al.*, 1979).

1.6.1. Transcriptional control of IE genes by Vmw65

The regulatory regions of the IE gene promoters of HSV-1 contain 1 or more copies of a conserved DNA sequence element which has a minimum consensus TAATGARAT (where R=purine) (Mackem and Roizman, 1982; Whitton and Clements, 1984) ,which is required for response of the promoter to transactivation by a major component of the virus particle Vmw65 (Batterson and Roizman., 1983; Campbell *et al.*, 1984; Marsden *et al.*, 1987) the product of the gene UL48 (Dalrymple *et al.*, 1985; Parris *et al.*, 1988). Fragments or short oligonucleotides containing this consensus sequence can confer Vmw65 inducibility upon otherwise non-responsive promoters (Cordingley *et al.*, 1983; Preston and Tannahill, 1985; Preston *et al.*, 1984). Vmw65 does not bind DNA by itself (Marsden *et al.*, 1987) but its effect is to induce transcription of the IE genes by cellular RNA polymerase II (Post *et al.*, 1981; Batterson and Roizman, 1983, Campbell *et al.*, 1984).

Kristie and Roizman (1987), first demonstrated the binding of a host protein to each of the IE gene upstream regulatory regions and this was later confirmed by several other laboratories (O'Hare and Goding, 1988; Preston *et al.*, 1988). This factor was subsequently identified as Oct-1 (Stern *et al.*, 1989) a transcriptional factor already implicated in the regulation of a number of cellular genes. Subsequent results obtained on the binding of TAATGARAT by Oct-1 (O'Hare and Goding, 1988) demonstrated that Vmw65 is recruited

onto the TAATGARAT element in a manner that is dependent on Oct-1 binding. Mutational analysis of Vmw65 (Triezenberg *et al.*, 1988; Greaves and O'Hare, 1989; Haigh *et al.*, 1990) showed that it contains 2 separable domains. One of these domains, which is located at the amino terminus interacts with Oct-1 to form the IE complex which binds to TAATGARAT.

A second cellular factor also appears to be required for the regulation of HSV-1 IE genes by Vmw65 (Gerster and Roeder,1988; Kristie *et al.*, 1989).This factor interacts directly with Vmw65 in the absence of other components of the transactivation complex.(Kristie and Sharp , 1990; Katan *et al.*, 1990). Therefore it is likely that it interacts with Vmw65 to form a stable protein complex that subsequently recognises the DNA-bound Oct-1. This factor has been purified from HeLa cell nuclear extracts (Kristie and Sharp, 1993; Wilson *et al.*, 1993), and is composed of several polypeptides which appear to be post-translational products of a larger 300kDa protein (Wilson *et al.*, 1993).

IE gene transinduction by Vmw65 is not essential for virus growth at high m.o.i. but plays a critical role in determining plaquing efficiency and thus whether infection is lytic or non-productive (Ace *et al.*, 1988,1989). Vmw65 is also important for the virulence of HSV-1 in mice (Ace *et al.*, 1989).

VZV ORF10 encodes a protein homologous to HSV-1 Vmw65 (Davison and Scott, 1986b), but it is 80 amino acids shorter and lacks sequences similar to that of the Vmw65 acidic carboxy-terminal tail (Cohen *et al.*, 1993). Furthermore, VZV ORF10 does not form IE complexes with the TAATGARAT sequence element and cellular proteins (McKee *et al.*, 1990) so it was not thought to have gene regulatory activity. Mouriuchi *et al.* (1993) have shown however, that VZV ORF10 can transactivate both the VZV IE gene ORF62 and HSV-1 Vmw110 and Vmw175 promoters. This indicates that ORF10 is a transactivating protein despite the absence of the acidic carboxyterminus, which activates transcription in Vmw65. At present it is not known if VZV ORF10 transregulatory activity is mediated through binding to cellular factors.

1.6.2. The HSV-1 IE gene products

Several nomenclatures exist for the IE genes and their products. The most commonly used are listed below.

The IE genes of HSV-1 and their products.

Glasgow nomenclature			Chicago nomenclature	
Gene*	Gene$^{\theta}$	Product	Gene	Product
IE-1	RL2	Vmw110	α0	ICP0
IE-2	UL54	Vmw63	α27	ICP27
IE-3	RS1	Vmw175	α4	ICP4
IE-4	US1	Vmw68	α22	ICP22
IE-5	US12	Vmw12	α47	ICP47

* Original designation (Clements *et al.*, 1979)

θ Later designation in complete HSV-1 sequence (McGeoch *et al.*, 1988a; McGeoch *et al.*, 1991).

Vmw110

Vmw110 has a size of M_r 110K by SDS-PAGE and is encoded by the diploid gene RL2, contained within R_L. Vmw110 encodes one of the few spliced transcripts in the HSV-1 genome; its protein coding regions are contained within 3 exons (Perry *et al.*, 1986). Vmw110 is not absolutely essential for virus growth in tissue culture (Stow and Stow, 1986; Sacks and Schaffer, 1987) but at low m.o.i. viruses with a deletion in Vwm110 show a defect in growth which is overcome at high m.o.i.. Viruses which lack Vmw110 are unable to stimulate latent viral genomes to reactivate in an *in vitro* latency system (Russell *et al.*, 1987), while expression of Vmw110 also causes reactivation in this system (Harris *et al.*, 1989). However, *in vivo*, the role of Vmw110 in reactivation is less clear as virus lacking Vmw110 can reactivate from explanted latently infected mice ganglia, albeit more slowly than the wild-type virus.

Vmw110 can activate the expression of a large number of HSV-1 and non-HSV-1 promoters both by itself and in a synergistic manner with Vmw175 (O'Hare and Hayward, 1985; Quinlan and Knipe, 1985; Everett, 1986). The production of Vmw110 itself is regulated by Vmw175 (Resnick *et al.*, 1989). Although the efficiency of promoter activation by Vmw110 alone and its degree of synergy with Vmw175 vary depending on the experimental conditions (Everett, 1988b), some of the regions that are involved in its intrinsic and synergistic activation events have been defined (Everett, 1987; 1988a).

The major determinant of transactivation maps to a cysteine rich region in the second exon (Everett *et al.*, 1987, 1988a; Chen *et al.*, 1991). This region is highly conserved in the Vmw110 homologues of other herpesviruses (Perry *et al.*, 1986; Cheung, 1991; van Santen, 1991). Other regions of the protein contribute to its function, but this is dependent on the promoter examined and the cell type in which the assays are performed (Chen *et al.*, 1991). Vmw110 is thought to mediate transactivation by interaction with an unknown cellular factor. Recent results (Weber and Wigdahl, 1992) indicate that the domain of Vmw110 which interacts with the putative cellular factor is encoded by the first two exons.

Vmw63

Vmw63 is encoded by the gene UL54 and migrates as a M_r 63K polypeptide on SDS-PAGE. Evidence from transfection studies has shown that in the presence of Vmw110 and Vmw175, Vmw63 can repress expression of some target plasmids containing HSV-1 early promoters and enhance expression of targets containing late promoters (Everett, 1986; Rice and Knipe, 1988; Su and Knipe, 1989; McMahan and Schaffer, 1990).

Studies with Vmw63 *ts* and deletion mutants have demonstrated that the gene plays an essential role in viral growth, is involved in the negative regulation of IE and some E genes, does not appear to be absolutely essential for DNA synthesis and is required for the expression of later classes of genes (Sacks *et al.*, 1985; McCarthy *et al.* ,1989; Rice and Knipe, 1990).

Data presented by McCarthy *et al.* (1989) suggested that Vmw63 mediates its effect on late gene expression transcriptionally. In contrast however Smith *et al.* (1992) found that while

*

(Watson and Clements, 1978)

the synthesis of new transcripts continued when Vmw63 was defective, the accumulation of late mRNAs and their translation into protein was reduced. The converse was found for IE products suggesting that Vmw63 was acting partly post transcriptionally to regulate the expression of IE and L gene products.

Vmw175

RS1, the gene encoding Vmw175 (McGeoch *et al.*, 1988a) is located in the short repeat genome segment and is therefore present twice. The encoded polypeptide migrates as a M_r 175K species on SDS-PAGE gels (Courtney and Benyesh-Melnick, 1974). However, the size of the primary translation product as predicted by the DNA sequence is only 132,835 daltons. Vmw 175 is a phosphoprotein which appears as several closely migrating species following SDS-PAGE (Wilcox *et al.*, 1980). One of the phosphorylated species is stable throughout the course of infection, whereas phosphate cycles on and off at least two other species. The Vmw175 sequence can be divided into 5 regions on the basis of similarity to the corresponding proteins produced by other alphaherpesviruses (McGeoch *et al.*, 1986; Vleek *et al.*, 1989; Grundy *et al.*, 1989).

Vmw175 is an essential polypeptide (Preston, 1979; Sacks *et al.*, 1985) and a critical transactivator of most HSV genes. Of the 5 IE gene products, Vmw175 is perhaps the most important, since its inactivation results in failure to transcribe early or late genes and an apparent over-expression of the IE genes (DeLuca *et al.*, 1985). Thus, Vmw175 is required directly or indirectly for the normal regulation of HSV-1 genes.

Vmw175 is a phosphorylated nuclear protein which binds to DNA in crude cell extracts and is associated with chromatin (Hay and Hay, 1980; Wilcox *et al.*, 1980). It is known to bind specifically to DNA sequences (Tedder *et al.*,1989; Imbalzano *et al.*, 1990; Pizer *et al.*, 1991) many (but not all) of which contain the motif ATCGTC.

The DNA binding domain of Vmw175 is well defined (Everett *et al.*, 1990,1991; Wu and Wilcox, 1990; Pizer *et al.*, 1991) and consists of the entire highly conserved region 2 of the polypeptide and also the distal part of the less well conserved region 1. The precise relationship between DNA binding and Vmw175 function is unclear, as a virus with

mutations in the Vmw175 binding domain which reduces the ability of the protein to bind DNA still grows normally in tissue culture (Sheperd and deLuca, 1991a,b).

Vmw68

Vmw68 is encoded by the gene US1 and migrates as a M_r 68K species on SDS-PAGE. Vmw68 and Vmw12 are derived from identical promoters in the repeat sequences which bound the short unique region of the genome. The splice donor site used in IE-4 and IE-5 RNAs is also in R_S while their entire coding regions differ, being located at either end of U_S (Watson *et al.*, 1981, Rixon and Clements, 1982).

Post and Roizman (1981) constructed a virus lacking the carboxyterminal third of Vmw68. This virus grew normally in Vero cells, but later analysis using different cell lines showed that in some cases the virus grew poorly (Sears *et al.*, 1985) and the activity of at least 1 true late promoter was substantially reduced. This virus was non-neurovirulent in mice but could establish a latent infection. Since the majority of the ORF in the mutant used was left intact, these studies did not give a clear answer to the role of Vmw68 but it appears to play a role in determining host-range.

Vmw12

Vmw12 is encoded by the gene US12 and has a size of M_r 12K on SDS-PAGE. Its function is unknown, but Marsden *et al.* (1982), suggested that its cytoplasmic location and the observation that it is the only nonphosphorylated IE polypeptide may point to a different role from other IE polypeptides. Viable deletion mutants have been isolated which lack the whole of this gene, indicating that it does not play an obvious or essential role during infection of tissue culture (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987).

1.6.3. Expression of HSV-1 early and late genes.

The mechanisms of activation of HSV-1 E and L promoters remain unclear. E and L promoters do not contain obviously conserved class-specific sequences analogous to TAATGARAT. The E promoters that have been examined consist of a near upstream region

composed of binding sites for cellular transcription factors linked to a TATA box/ cap site (McKnight and Kingsbury, 1982; Everett, 1984; McKnight and Tjian, 1986).

HSV-1 true late promoters appear to have a simpler structure than E promoters. A TATA element is the only consensus element identified in the upstream regulatory region of viral late promoters (Wagner, 1985) and deletion studies have indicated that little sequence information upstream from this element is required for efficient expression (Homa *et al.*, 1986; Homa *et al.*, 1988). However, Kibler *et al.* (1991) demonstrated that sequences downstream of the US11 TATA box play an active role in specifying true L temporal expression.

Steffy and Weir (1991) constructed linker-scanning mutations in the gC and gH promoters to help define the promoter elements common amongst HSV-1 late promoters. Their analysis identified 3 sequence elements in each promoter: a TATA element, an element that overlapped the start of transcription and an element downstream from the start of transcription. The TATA element or initiation sequence appear to be important for normal transcription initiation.

Analysis of expression from hybrid gE/gC ($\gamma 1/\gamma 2$) promoters (Sethna and Weir, 1993) revealed that the initiation elements of these 2 promoters were interchangable. The gE promoter is active to a degree before the onset of viral DNA replication, and this appears to be controlled by a separate regulatory element upstream from the TATA element.

1.7. Pathogenicity of HSV

HSV-1 and HSV-2 are important human pathogens that cause a variety of diseases ranging from benign superficial cutaneous lesions to life-threatening encephalitis (Whitley, 1985). Identification of the genes that control pathogenic properties and elucidation of their function are of fundamental importance if we are to understand the basic mechanisms by which disease is initiated.

Various animal models such as mice, guinea pigs, rabbits, rats and monkeys have been used to study HSV pathogenicity. Several host factors controlling pathogenicity have been

identified; namely, humoral immunity (McKendall *et al.*, 1979), cytotoxic immunity (Nash *et al.*, 1985), interferons (Lopez, 1985), state of skin and mucous membrane, age of host and route of inoculation (Sprecher and Becker, 1986, 1987). Within the same host the degree of pathogencity is affected by virus strain, serial passage of the virus *in vivo* (Kaerner *et al.*, 1983), *in vitro* (Goodman and Stevens, 1986) and body temperature of the host (Thompson and Stevens, 1983a). As HSV is a natural pathogen of man, the question arises whether a direct extrapolation can be made between animals and man in defining genetic loci responsible for differences in pathogenicity.

As HSV-1 glycoproteins play an important role at the early stages of the virus life-cycle (ie. adsorption and penetration) it might be expected that they would also be relevant *in vivo*. Very little has been published on the pathogenicity of HSV-1 glycoprotein mutants. gC is not a virulence determinant in the mouse model as gC^- mutants of both HSV-1 and HSV-2 remain highly virulent following intravaginal (Johnson *et al.*, 1986), intracerebral and footpad inoculation (Dix *et al.*, 1983; Sunstrum *et al.*, 1988). Similarly monoclonal antibody resistent (*mar* $^-$) gD or gB mutants have no significant affect on pathogenicity (Kumel *et al.*, 1985). Using engineered U_S mutants, Meigner *et al.* (1988) demonstrated that a gE^- mutant was not significantly impaired in neurovirulence following intracerebral inoculation of mice, with a LD_{50} less than 100-fold higher than that of the parental virus.

Both HSV-1 Ang and HSV-1 KOS are completely non-neurovirulent following inoculation on the mouse footpad (Kumel *et al.*, 1983; Kaerner *et al.*, 1983; Thompson *et al.*, 1986) although both are neurovirulent following intracranial inoculation. Following passage in the mouse brain, variants of HSV-1 Ang arise (Ang path) which are capable of causing lethal neurologic disease following footpad inoculation. This increase in neuroinvasiveness (as defined by the ability of the virus to spread to the CNS following peripheral inoculation) is associated with a single base change in the gD ORF (Izumi and Stevens, 1990). In HSV-1 KOS, 2 loci are required for complete restoration of a neuroinvasive phenotype (Yuhaz and Stevens, 1993); one within a region of the genome encoding the genes UL10, UL11, UL12, UL13 and parts of UL9 and UL15 has not yet been fully characterized, whereas the other is localized to gB; as with HSV-1 Ang, a single base change in a glycoprotein greatly affects

neuroinvasiveness. As gB and gD play a role in virus adsorption and penetration respectively, the decrease in neuroinvasiveness associated with these amino acid substitutions could be due to a decreased affinity of the mutant glycoproteins for their cellular receptors, leading to less efficient spread throughout the nervous system. Two amino acid substitutions in the C-terminal portion of gB have been shown to result in a virus which is still virulent following footpad inoculation but produces unusual clinical symptoms (Goodman and Engel, 1991; Engel *et al.*, 1993). Unlike inoculation with the wild-type virus HSV-1 strain 17^+, an acute inflammatory response occurs in inoculated footpads with subsequent death of the inoculated animal without hind limb paralysis. It is possible that in this case the alterations in gB may alter antigen presentation, thus affecting the host response to infection.

In addition to gE (US8), Meigner *et al.* (1988) examined the biological properties of 5 other genes in US namely US1, US2, US3, US4 (gG) and US11. Deletion of any of these genes was claimed to greatly reduce intracerebral neurovirulence. However, the pathogenicity of variants with single mutations in gG (US4) and US2 was not determined. In both cases the protein kinase gene (US3) (which was shown to be very important for neurovirulence) was also deleted. Deletion of US9, US10, US11 or US12 has no effect on pathogenicity (Taha, 1990; Nishiyama *et al.*, 1993). The affect of a US7 deletion on neurovirulence has not been described.

Any alteration in a virus gene which impairs replication *in vitro* will also affect the performance of the virus *in vivo* and so all essential genes could be considered 'virulence' genes. It is now clear however, that the virus possesses genes whose expression is not required for multiplication in tissue culture, but are required for a wild-type phenotype *in vivo*. In HSV-1 several genes are required for maximum demonstration of neurovirulence in various animal models; some of these are described below.

1.7.1. DNA polymerase

HSV-1 mutants which are resistant to antiviral drugs arise in the laboratory at a frequency of about 10^{-3} (Coen *et al.*,1982). Certain acyclovir (ACV)-resistant mutants and all known vidarabine (araC)-resistant mutants owe their resistance to mutations in the viral DNA polymerase gene (Crumpacker *et al.*, 1980; Coen *et al.*, 1982). Several early studies using HSV-1 drug resistant mutants (Darby *et al.*, 1984; Field and Darby, 1980) implicated a role for the DNA polymerase gene in pathogenicity for mice. Field and Coen (1986) looked at the pathogenicity of 3 distinct DNA polymerase mutants in a mouse ear model. These mutants exhibited no significant difference in replication kinetics compared to the wild-type virus at the periphery (ear pinna). No clinical signs suggesting neurological involvement were noted in any mice inoculated at the pinnae. Following intracerebral inoculation pol mutant viruses were found to be highly attenuated for pathogenicity. In a mouse flank model (Larder *et al.*,1986) DNA polymerase mutants were also found to be greatly reduced in neurovirulence.

HSV-2 strain 186 grows poorly in the eyes and sensory ganglia of mice, indicating that it is non-neuroinvasive following ocular infection (Oakes *et al.*, 1986). Marker rescue of the lesion to restore the wild-type phenotype (Day *et al.*, 1987) showed that a mutation in the DNA polymerase gene was responsible for the loss of neuroinvasiveness. Recently, Lausch *et al.* (1990) reported the failure of similar strains to grow in human lymphocytes indicating that the gene for HSV DNA polymerase may have an important role during infection of man. However, the significance of growth in lymphocytes, which are not likely to be infected by HSV-1 during a natural infection is questionable.

It is possible that the decrease in neuropathogenicity of DNA polymerase mutants is due to decreased affinities of mutant polypeptides for deoxynucleoside triphosphates which are presumed to be in lower concentrations in the nondividing cells of nervous tissues than in peripheral tissues.

1.7.2. Thymidine kinase

HSV-1 mutants that lack the ability to produce thymidine kinase (tk) have been studied in a

number of laboratories. Field and Wildy (1978), reported that tk^- mutants of HSV-1 and HSV-2, selected by treatment of infected cells with bromodeoxyuridene (BUdr) were less virulent than the parental tk^+ virus. Tenser *et al.* (1979, 1981) demonstrated that tk^- mutants do not replicate in the trigeminal ganglia.

Although tk^- mutants do not replicate in murine sensory ganglia, they do establish a latent infection (Coen *et al.*, 1989; Efstathiou *et al.*, 1989; Leist *et al.*, 1989; Tenser *et al.*, 1989). Efstathiou *et al.* (1989) demonstrated the reactivation of tk^- virus from latency following superinfection with wild-type virus, despite the inability to detect DNA from this virus in nervous tissue during acute or latent infection, or reactivate virus from explanted ganglia. This result confirmed an earlier study (Field and Wildy, 1978) which had shown that a tk^- variant produced a transient increase in titre 1 day after inoculation into the mouse ear pinnae with little infectious virus being found thereafter. Virus could not be isolated from the ganglia during acute infection, but was found to establish and reactivate from a latent infection following explant of ganglia.

Coen *et al.* (1989) similarly demonstrated the reactivation of tk^- virus from latency following superinfection with wild-type virus, although they were also unable to reactivate tk^- virus from explanted ganglia. Southern blotting of reactivated virus demonstrated *in vivo* complementation of the mutant virus with wild-type virus. Using *in situ* hybridization, they detected LAT in only ~2 to 5-fold higher frequencies in wild-type infected ganglia than in tk^- virus infected ganglia. These results confirmed the ability of tk^- virus to enter the PNS and establish a latent infection, and indicated that tk was specifically required for reactivation from latency.

1.7.3. Ribonucleotide reductase

Ribonucleotide reductase (RR) is a key enzyme in DNA biosynthesis of eukaryotic and prokaryotic organisms. By reducing ribonucleotides to their corresponding deoxyribonucleotides, the enzyme provides a major pathway in the formation of DNA precursors. HSV-1, like other herpesviruses, encodes its own RR (Dutia, 1983).

The existence of a *ts* mutant, *ts* 1207 with a lesion in RR suggested that this enzyme was

essential for virus replication (Preston *et al.*, 1984). In a mouse model, *ts* 1207 shows a reduction in neurovirulence of $>10^5$-fold compared to the wild-type virus following intracerebral or intraperitoneal injection (Cameron *et al.*, 1988). At the non-permissive temperature this mutant fails to induce reductase activity, and shows a reduction in yield of $\sim 10^2$ with respect to that at the permissive temperature following growth on BHK21/C13 cells. However, several reports indicated that RR was not absolutely required for virus growth in tissue culture. Concentrations of hydroxyurea that inhibited RR activity to undetectable levels in proliferating cells infected with HSV-2 reduced virus yield by only 6-fold (Nutter *et al.*, 1985), and an HSV-1 insertion mutant lacking most of R1 induces no detectable RR activity and produces only a 4- to 5-fold lower yield of progeny virus in exponentially growing tissue culture cells than does the parental virus (Goldstein and Weller, 1988a). However, in serum starved cells this mutant had an extremely small plaque size compared to the wild-type virus, and one-cycle growth analysis indicated a 30- to 40-fold reduction in yield of the mutant virus in these cells. A deletion variant , ICP6Δ, lacking 90% of the R1 coding sequences was found to have similar growth characteristics to *ts* 1207 in Vero cells at both the permissive and non-permissive temperature (Goldstein and Weller, 1988b) indicating that the temperature sensitivity of *ts* 1207 may not be due to the thermoliability of the viral RR at higher growth temperatures, but more likely is due to the inability of the host pathways to complement the defect at elevated temperatures. The absence of alternate pathways for obtaining deoxyribonucleotides in non-dividing cells explains the defect in serum starved cells.

In a mouse ocular model (Jacobson *et al.*, 1989), ICP6Δ showed reduced ability to replicate following peripheral inoculation, and was impaired in reactivation from latency. At 38°C ICP6Δ was impaired in growth in mouse cells relative to growth in Vero cells indicating that a mouse factor (eg. cellular RR) required for HSV growth in the absence of viral RR, is more sensitive to temperature than the corresponding factor in Vero cells. The extreme impairment of the RR mutant in the mouse eye is probably a result of the impairment of this mutant in mouse cells. The mutant replicated poorly in the cornea and unlike the wild-type virus KOS, and the wild-type rescuant it did not cause severe ocular disease (Brandt *et al.*,

1991). It also grew poorly in human primary corneal fibroblasts, suggesting that RR may be required for virulence in human infections.

1.7.4. dUTPase

A HSV-1 neurovirulence locus has been mapped to the genomic region which encodes the viral dUTPase (Thompson and Wagner, 1988). This gene is located between 0.69 and 0.70 m.u. on the viral genome (Preston and Fisher, 1984), corresponding to the UL50 ORF (McGeoch *et al.*, 1988a). In tissue culture, HSV-1 dUTPase is not required for viral replication, suggesting that a cellular counterpart is present in sufficient quantity and can be utilized by HSV-1 (Fisher and Preston, 1986; Williams, 1988; Barker and Roizman, 1990). Pyles *et al.* (1992) demonstrated that dUTPase negative mutants are approximately a 10-fold reduction in neurovirulence following intracerebral inoculation compared to the wild-type virus. In this study, the neuroinvasiveness of these mutants was assessed through determination of their ability to invade the CNS following peripheral (footpad) inoculation. The average LD50 value of HSV-1 strain 17^+ was $2.9x10^3$ following peripheral inoculation, whereas dUTPase mutants were at least 3 orders of magnitude less neuroinvasive using this assay. dUTPase mutants replicated with wild-type kinetics in nonneural tissue (footpad) and PNS, but were defective in entry or replication in the CNS. They reactivated from latency following explantation of ganglia at a frequency half that of the wild-type virus by day 7 post explantation (by day 7 100% of all wild-type infected ganglia had reactivated) but were more severely impaired in reactivation following hyperthermia induction (10% and 21% of ganglia infected with dUTPase mutant reactivating compared to 82% of wild-type virus infected ganglia).

Therefore the HSV-1 dUTPase is an important determinant of HSV-1 virulence, establishment and/ or reactivation from latency.

1.7.5. UL56

The HFEM strain of HSV-1 has a deletion of 4.1kb (compared to HSV-1 strain F) in its genome which completely abolishes its pathogenicity in tree-shrews by the intraperitoneal

route (Rosen and Darai, 1985). *In vivo* marker rescue of the deletion with a restriction enzyme fragment (np 113322-np 123464) from HSV-1 strain F which is highly pathogenic for tree shrews by the intraperitoneal route led to the isolation of a recombinant which regained intraperitoneal virulence for tree shrews (Rosen *et al.*, 1986). A 1.5kb RNA hybridizing to the DNA sequences of the HSV-1 strain F genome at np116659-116951 was found to be missing in cells infected with HSV-1 HFEM and other apathogenic HSV-1 strains (Rosen-Wolff *et al.*, 1988). The corresponding ORF in the HSV-1 genome was found to be the UL56 gene (Rosen-Wolff and Darai, 1991). In the HSV-1 HFEM genome, the promoter region of UL56, including the TATA box is deleted (Koch *et al.*, 1987) accounting for the absence of the 1.5kb transcript. At present the role of the UL56 gene in viral replication is unknown, but there is evidence that sequences in the deleted region of HFEM determine the suppression of humoral antibody formation by herpes simplex virus (Wollert *et al.*, 1991). Viruses deleted in UL56 of HSV-1 (strain 17^+) exhibit no impairment in pathogenicity by the footpad or intracerebral route in BALB/c mice and no impairment in reactivation from latency (Sinclair, 1992).

1.7.6. UL10

The HSV-1 UL10 gene is predicted to encode a membrane protein with eight potential transmembrane domains (McGeoch *et al.*, 1988a). It is a virion component which becomes associated with infected-cell membranes (Baines and Roizman, 1993; MacLean *et al.*, 1993) and is modified by N-linked glycosylation. Recently, the product of the UL10 gene has been somewhat inappropriately designated gM (Baines and Roizman, 1993); unlike other HSV-1 glycoproteins which have previously been described, the UL10 gene product is a type III membrane protein and spans the membrane more than once.

The UL10 gene product is non-essential for growth in tissue culture, but inactivation of the gene causes an impairment in growth (Baines and Roizman,1991; MacLean, C., *et al.*, 1991, 1993). *In vivo* characterization of a UL10 deletion variant (MacLean *et al.*, 1993) demonstrated that the UL10 gene product is required both for growth at the periphery and subsequent spread to the nervous system. Although still capable of establishing a latent

infection, its reactivation kinetics were impaired compared to the wild-type virus. Following intracerebral inoculation, the UL10 deletion variant was slightly less neurovirulent than the wild-type revertant. Although UL10 is not absolutely required for neurovirulence *in vivo*, it is apparently required for optimum virus growth and spread reflecting its impairment in tissue culture, rather than a specific *in vivo* defect.

1.7.7. ICP34.5

The existence of a neurovirulence locus in the long repeat region of the HSV-1 genome was first demonstrated by Thompson *et al.* (1983). They noted that the HSV-1 x HSV-2 intertypic recombinant, RE6, was completely non-neurovirulent following intracerebral inoculation of mice, with a LD_{50} of $3.2x10^7$ p.f.u./mouse, compared to a lethal dose of 10 p.f.u. for either HSV-1 strain 17^+ or HSV-2 strain HG52, the wild-type parental strains of this recombinant (Thompson and Stevens, 1983b). Restriction enzyme analysis and Southern blotting revealed that HSV-1 information from 0.71 to 0.83 m.u. had been incorporated into the RE6 genome (Thompson *et al.*, 1983) and was specifically associated with the decrease in the neurovirulence of this virus (Thompson *et al.*, 1985). Sequences mapping between 0.698 and 0.721 m.u. could partially rescue the non-neurovirulent phenotype of RE6 (Thompson and Wagner, 1988). However a cloned HSV-1 fragment spanning the region of the HSV-1 genome from 0.82 to 0.832 m.u. restored the virulence of RE6 to near wild-type levels (Thompson *et al.*, 1989).

At around the same time that this work was published, a non-neurovirulent variant of HSV-2 strain HG52 was described. This variant, JH2604, has previously been shown to have RL sequences spanning 0.00 to 0.002 m.u. and 0.81 to 0.83 m.u. in the viral genome deleted (Harland and Brown, 1985). The neurovirulent phenotype of JH2604 could be restored by recombination with HSV-2 sequences between 0.7 and 0.91 m.u. (Taha *et al.*, 1989a). Further analysis of this variant (Taha *et al.*, 1989b) revealed a 1488bp deletion which removes 1 complete copy of the 17bp DR1 element of the 'a' sequence and terminates 522bp upstream of the 5' end of IE-1. JH2604 was unable to replicate within neuronal cells of the CNS or produce necrotizing encephalitis in mouse brain following intracerebral inoculation

(Taha *et al.*, 1990).

In our laboratory, a non-neurovirulent variant of HSV-1 strain 17^+ has recently been described (MacLean, A., *et al.*, 1991a). This variant, 1716, has a 759bp deletion which removes 1 complete 18bp DR1 element of the 'a' sequence and terminates 1105bp upstream of the 5' end of IE-1. 1716 grows identically to the wild-type virus *in vitro*, but is non-neurovirulent following intracerebral inoculation of mouse brain with a LD_{50} of $7x10^6$ p.f.u./mouse. This absence of neurovirulence is due to an inability to replicate in mouse brain.

When the work described in this thesis began, the HSV-2 strain HG52 genome had not been sequenced and in HSV-1 strain 17^+, the region between IE-1 and the 'a' sequence was apparently non-protein coding (McGeoch *et al.*, 1988a). Initial sequencing data of the HSV-2 strain HG52 genome (Dr.D.J. McGeoch, personal communication) revealed a 63 amino acid coding sequence in this region of high homology, between HSV-1 strain 17^+ and HSV-2 strain HG52. In 1986, Chou and Roizman demonstrated that in HSV-1 strain F, the region between the 'a' sequence and IE1 is protein coding. Using a peptide antiserum which had been raised against a Pro-Ala-Thr trimer repeat found 10 times in the strain F sequence, Ackermann *et al.* (1986) detected a protein of 43.5K in HSV-1 strain F infected cell extracts. This protein had not previously been detected among electrophoretically separated proteins of HSV-1 and overlaps in part with the bands formed by ICP35. The promoter of the gene encoding ICP34.5 has several unusual features: it lies within the 'a' sequence which is G-C rich and contains numerous repeats and features, including a canonical TATA box characteristic of HSV promoters, are not present. The ORF ascribed to HSV-1 strain F ICP34.5 was not present in the published sequence of HSV-1 strain 17^+ (McGeoch *et al.*, 1988a). However, in 1990, when Chou and Roizman republished their sequence of HSV-1 strain F ICP34.5, they now predicted a protein of only 263 amino acids in size, but more importantly their revised sequence was now almost identical (with the exception of 1 frameshift) to the published sequence of the relevant region in HSV-1 strain 17^+.

Chou *et al.* (1990), confirmed the importance of HSV-1 strain F ICP34.5 in neurovirulence following intracerebral inoculation through the construction of 4 recombinant viruses. These

were (i) a virus in which both copies of the gene encoding ICP34.5 were deleted (ii) a virus into which a 6-frame stop-codon had been inserted in both copies of the gene (iii) a virus, containing after the first codon, an insert encoding a 16-amino acid epitope known to react with a specific monoclonal antibody and (iv) a wild-type rescuant. Both HSV-1 strain F and the wild-type rescuant were neurovirulent following intracerebral inoculation. The virus which contained the 6- amino acid epitope insert was only slightly reduced in neurovirulence compared to the wild-type virus, whereas the variants in which the gene encoding ICP34.5 had been deleted or truncated by insertion of a 6-frame stop codon were completely non-neurovirulent following intracerebral inoculation. These results implied that ICP34.5 plays a crucial role in neurovirulence following intracerebral inoculation of mice and indicated that homologous proteins must be present in HSV-1 strain 17^+ and HSV-2 strain HG52. Resequencing of HSV-1 strain 17^+ confirmed the presence of the homologue (Dolan *et al.*, 1992) and an equivalent ORF was also identified in HSV-2 strain HG52 (McGeoch *et al.*, 1991).

1.8. Virulence determinants in non-herpesviruses

A major focus for the study of virus-host interactions has been pathogenesis and the ability of viruses to cause disease. Viruses can initiate and cause disease by several means such as, (i) infection of susceptible cells causing molecular alterations which eventually lead to cell death, (ii) immunopathobiology eg. recognition and killing of virus infected cells and (iii) persistence and alteration of cellular function perhaps leading to oncogenesis. In HSV-1 virulence is multifactorial and several genes have been shown to be required for maximal display of neurovirulence in diverse animal models. In some other viruses however, the alterations in the viral genome which abolish pathogenicity have been well characterised; some of these are described below.

1.8.1. Poliovirus

Poliovirus, a human enterovirus belonging to the *Picornaviridae* family, is the causative

agent of poliomyelitis. It replicates in the human digestive tract and may induce paralysis by infecting and destroying motor neurons (Couderec *et al.,* 1989). Over the past 30 years, the disease has been controlled by the use of the Sabin live-attenuated vaccine (Assaad and Coburn, 1982). This vaccine is based on attenuated strains of poliovirus which were empirically derived by passage of wild-type viruses in monkey tissue *in vivo* and *in vitro* (Sabin, 1965; Sabin and Boulger, 1973). Although the Sabin vaccine against poliomyelitis is one of the safest and most effective in current use, in a very low percentage of recipients (less than 1 case per million recipients) poliomyelitis may occur as a consequence of vaccination (Minor, 1980; Minor, 1982; Notlay *et al.,* 1981).

The ability to clone and rapidly sequence the 7431 nucleotides of poliovirus has enabled the research groups headed by Jeffrey Almond of Leicester and Geoffrey Schild of London to determine what changes in sequence correlate with the acquisition of virulence.

Comparison of the complete nucleotide sequences of the genomes of the Sabin type 3 vaccine strain P3/Leon/12a_1b and its neurovirulent progenitor P3/Leon/37 indicates that they differ by just 10 point mutations (Stanway *et al.,*1984). Sequence analysis of revertant strains, vaccine virus (Cann *et al.,* 1984; Stanway *et al.,* 1984; Almond *et al.,* 1985; Evans *et al.,* 1985) and neurovirulence testing of recombinant viruses derived from infectious cDNAs of the Sabin vaccine strain P3/Leon/12a_1b and its neurovirulent progenitor (Westrop *et al.,* 1989) revealed that the attenuated phenotype of the poliovirus vaccine strain is the product of 2 point mutations which act additively. These mutations are at positions 472 (C-to-U) and 2034 (C-to-U) change in the virus structural protein VP3. The mutation at 472 in the 5' non-translated leader appears to contribute to the attenuation of Sabin type 3 poliovirus vaccine by decreasing the translation efficiency of the viral genome (Svitkin *et al.,* 1990) although the mechanism by which this occurs is unclear.

1.8.2. Coxsackie B4 virus

Coxsackieviruses are members of the enterovirus genus of the family *Picornaviridae.* Coxsackieviruses of the B group have been implicated in diseases such as pancreatitis, myocarditis, myositis and type 1 insulin-dependent diabetes mellitis (Grist *et al.,* 1978;

Melnick, 1985). Of the group B viruses, variants exist within a single serotype thereby contributing to the variability in pathogenicity of coxsackieviruses.

Coxsackieviruses have structural features common to all picornaviruses, namely a capsid comprised of 4 virus-encoded proteins (VP1 to VP4) enclosing a single-stranded positive sense RNA genome of approximately 7,500 nucleotides. The RNA is polyadenylated at the 3' terminus and has a small protein VPg, covalently attached to the 5' terminus. The primary translation product of picornavirus RNA is a single large polyprotein which is processed by virus encoded proteases to yield the mature viral proteins (reviewed by Rueckert, 1986).

Comparison of nonneurovirulent and highly neurovirulent coxsackie B4 virus recombinants in a mouse model (Ramsingh *et al.*, 1989; Ramsingh *et al.*, 1990) revealed that the virulence phenotype mapped to the P1 region of the genome. This region comprises the 5' untranslated region (UTR) and the genes encoding the structural proteins VP1, VP2, VP3 and VP4.

Comparison of the corresponding cDNA sqeuences of virulent and nonneurovirulent virus (Jenkins *et al.*, 1987; Ramsingh *et al.*, 1992) identified 13 nucleotide substitutions, 4 in the non-coding region, 4 silent and 5 resulting in amino acid substitutions in VP1, VP2 and VP4. Analysis of individual mutations in both VP1 and VP2 (Caggana *et al.*, 1993) revealed that a single residue (Thr-129 of VP1) determined the virulence phenotype. In contrast to other picornaviruses including poliomyelitis and Theiler's murine encephalomyelitis virus (Fu *et al.*, 1990; Westrop *et al.*, 1989) the 5' UTR does not appear to contribute significantly to neurovirulence.

Sequence and structure alignment (Caggana *et al.*, 1993) positions Thr-129 of coxsackie B4 on the DE loop of VP1 in a similar position to Ile-143 of poliovirus, an amino acid which has been shown to be of importance in attenuation of poliovirus type 2 (Ren *et al.*, 1991). Hence, the molecular mechanisms underlying virulence in both of these viruses may share common features mediated by the DE loop of the VP1 capsid proteins.

1.8.3. Louping ill virus

Louping ill (LI) virus, a tick-transmitted member of the *Flaviviridae* (Westaway *et al.*,

1985), causes an encephalitic or encephalomyelitic disease of sheep and grouse. The flaviviruses are ssRNA viruses with a positive-sense genome about 10.4kb in length. The viral proteins are encoded in one open reading frame and the entire gene order of both the structural and nonstructural (NS) proteins is core, pre-membrane (prM), membrane (M) , envelope (E), NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 (Bell *et al.*, 1985; Castle *et al.*, 1985; Speight *et al.*, 1988). From the biological standpoint, the viral E protein is considered particularly important because it agglutinates erythrocytes, induces antigenically cross-reactive neutralizing and protective antibodies in infected animals and probably defines the tropism of flaviviruses for their target cell, thus defining virus virulence (reviewed by Gould *et al.*, 1990; Heinz *et al.*, 1990).

To analyse the regions of the E protein which determines the virulent phenotype, Jiang *et al.* (1993) produced 7 LI virus immunological escape mutants. The nucleotide sequence of the E protein of each of these mutants was determined and the deduced amino acid sequence compared to that of the wild-type virus. A change from the amino acid aspartate to asparagine at position 308, which represented a potential glycosylation site was found to be the most effective at reducing neurovirulence. It is possible that this amino acid change affects the protein folding and hence the ability of the E protein to bind to its receptor. Antibody-resistant escape mutants in the E protein of both tick-borne encephalitis and Japanese encephalitis virus have previously been shown to exhibit reduced neurovirulence in mice (Holzmann *et al.*, 1990; Cecilia and Gould, 1991).

1.8.4. Lymphocytic choriomeningitis virus

Lymphocytic choriomeningitis virus (LCMV) is the prototype member of the *Arenaviridae* . The ssRNA genome consists of 2 segments L and S which give rise to a total of 5 structural proteins; an RNA polymerase (L), a nucleocapsid protein (NP), 2 structural proteins, GP-1 and GP-2 which are derived by cleavage of the precursor GP-C and a zinc-finger protein (Romanowski *et al.*, 1985; Salvato *et al.*, 1989; Salvato and Shimomaye, 1989).

In a normal host, LCMV introduced by a peripheral route is eradicated and the host is immune to subsequent challenge. However, in an immunocompromised host, the virus is

not cleared and establishes a life-long persistent infection. It has been clearly demonstrated that the outcome is dictated by the CTL response (reviewed by Buchmeier, 1980; Byrne and Oldstone, 1984). Adoptive transfer of CD8+ lymphocytes from an LCMV-immune mouse to a persistently infected animal results in clearance of the virus (Jamieson *et al.*, 1987; Oldstone *et al.*, 1986), while abolition of virus-specific CTL responses during acute infection changes the outcome from clearance to persistence (Gilden *et al.*, 1972).

Spontaneous variants have been isolated which cause a persistent infection in immunocompetent mice by suppression of the CTL response (Ahmed *et al.*, 1984). Early results (Ahmed *et al.*, 1988; Salvato *et al.*, 1988) indicated that the immunosuppressive phenotype mapped to the L RNA segment, but more recent analysis has demonstrated that this is not the case. Reassortment analysis with homologous LCMV strains implicated the S RNA segment as the major locus for the immunosuppressive phenotype (Matloubian *et al.*, 1990).

By sequencing oligo-primed DNA Salvato *et al.* (1991) demonstrated that a change in GP amino acid 260, located on the S RNA, was consistently associated with the immunosuppressive phenotype. It is most likely that the GP 260 mutation influences CTL induction by affecting early stages of viral replication ie. by altering virus entry or GP processing and stability. This mutation was found to be necessary, but not sufficient to cause the immunosuppressive phenotype with secondary mutations in the polymerase or transcriptional control regions on the L RNA also believed to be involved (Salvato *et al.*, 1991).

1.9. Aims of project

The work presented in this thesis had several aims; (i) to show that the HSV-1 strain 17^+ genome encodes an HSV-1 strain F ICP34.5 homologue, (ii) to demonstrate that in HSV-1 strain 17^+, this protein plays a crucial role in neurovirulence following intracerebral inoculation of mice, and (iii) to produce an ICP34.5 polyclonal antiserum which would specifically detect ICP34.5 in HSV-1 strain 17^+ infected cells and which might also detect

the semi-conserved protein in HSV-2 strain HG52.

The preliminary characterization of a U_S deletion variant which was isolated during the course of this work is also described.

Chapter 2- Materials and methods

2.1. Materials

2.1.1. Cells

Baby hamster kidney 21 clone 13 (BHK21/C13) cells were used throughout this study (MacPherson and Stoker, 1964).

2.1.2. Viruses

The wild-type parental viruses used in this study were HSV-1 strain 17^+ (Brown *et al.*, 1973) and HSV-1 strain F (Ejercito *et.al.*, 1968).

The HSV-1 strain 17^+ deletion variant 1716 was frequently utilized as an ICP34.5 negative control (MacLean, A., *et al.*, 1991).

2.1.3. Cell culture media

BHK21/C13 cells were grown in Eagle's medium (Gibco) supplemented with 10% newborn calf serum (Gibco) and 10% (v/v) tryptose phosphate broth (Busby *et.al.*, 1964). This will be referred to subsequently as ETC10.

Variations on the basic growth media were:

PIC	Phosphate-free Eagle's medium containing 1% newborn calf serum
Emet/$5C_2$	Eagle's medium containing one-fifth the normal concentration of methionine and 2% calf-serum.
EMC10%	Eagle's medium containing 1.5% methyl cellulose and 10% newborn calf serum.

2.1.4. Bacterial strains

The bacterial strains used for cloning were JM109 [supE, thi, (lac-proAB), (F'traD36, proAB, lac192 M15)] (Yanisch-Perron *et al.*, 1985) and NM522 [supE,thi, (lac-proAB), hsd5(r^-, m^-), (F' proAB, lac192 M15)], (Gough and Murray, 1983). For expression of ICP34.5 BL21(DE3) [F-ompT- $r^-_b m^-_b$] (Studier and Moffat, 1986) was used.

2.1.5. Bacterial growth media

The media used for bacterial growth were L-broth (170mM NaCl, 10g/l Difco bactotryptone, 5g/l yeast extract) and 2xYT (85mM NaCl, 16g/l Difco bactotryptone, 5g/l yeast extract).

Bacteria from glycerol stocks was generally plated out onto L-broth agar (L-Broth containing 1.5% w/v agar) plates.

Top agar - 1% (w/v) agar in water was used in the preparation of template DNA for sequencing.

2.1.6. Oligonucleotides

Synthetic oligonucleotides were synthesised in the department by Dr J.McLauchlan, using a model 8600 Biosearch multiple column DNA synthesiser.

2.1.7. Radioisotopes

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

[^{35}S]-methionine	>1000Ci/mmol
[^{32}P]-orthophosphate	3000Ci/mmol
5' [α -^{32}P] dNTPs	~3000Ci/mmol
[^{35}S] dATP	~5000Ci/mmol

2.1.8. Chemicals

The chemicals used were of analytical grade and most of these were supplied by either BDH chemicals UK or Sigma Chemical Co.. Exceptions were:

APS and Temed - Bio-Rad Laboratories

Ampicillin (Penbritin) - Beecham Research

Caesium Chloride - Koch Light Ltd. (Suffolk, England).

2.1.9. Enzymes

Restriction enzymes were obtained from either Bethesda Research Laboratories (BRL) or New England Biolabs. T4 DNA ligase and T4 polynucleotide kinase were obtained from Boehringer Mannheim Corporation.

Lysozyme, Ribonuclease A and Deoxyribonuclease 1 were obtained from Sigma Chemical Co..

2.1.10. Plasmids

The starting plasmid used for the construction of the ICP34.5 expression vector was pET8c (Studier *et al.*, 1990). This was kindly provided by Dr. Judy Furlong. For the construction of HSV-1 recombinant plasmids pGEM 3zf(-) (Promega) was used.

Wild-type HSV-1 strain 17^+ fragments cloned into pAT153 (ie. BamHI j and BamHI k) were kindly provided by Dr. A. Davison.

2.1.11. Commonly used buffers and solutions

Acrylamide gel tank buffer:	53mM glycine, 3.5mM SDS, 52mM Tris
Chloroform: isoamylalcohol:	This is a 24:1 mixture of chloroform and isoamylalcohol
Gel soak 1:	600mM NaCl, 200mM NaOH
Gel soak 2:	600mM NaCl, 1M Tris-HCl, adjusted to pH8.0 with HCl
Hybridization buffer:	7% SDS, 0.5M NaP (NaH_2PO_4; Na_2HPO_4),pH7.4.
Kinase buffer (10X):	100mM DTT, 1mM EDTA, 100mM $MgCl_2$, 10mM spermidine, 0.5M Tris-HCl, pH 7.6
Ligase buffer (10X):	200mM DTT, 100mM $MgCl_2$, 0.5M Tris-HCl, pH 7.8
T4 polymerase buffer (10X):	1M Tris-HCl, pH 7.6, 1M $MgCl_2$, 1M DTT
Phenol chloroform (1:1):	This is a 1:1 mixture of phenol and chloroform
PBS A:	170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH7.2

PBS complete:	PBS A plus 6.8mM $CaCl_2$, 4.9mM $MgCl_2$
PBS /calf:	PBS complete containing 5% newborn calf serum
RE stop:	100mM EDTA, 10% (w/v) Ficoll 400, 0.25% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol, 5xTBE
Saturated phenol:	Phenol saturated by mixing 2:1 with phenol saturation buffer, (10mM Tris-HCl, pH7.5, 10mM EDTA, 100mM NaCl)
1xSSC:	15mM trisodium citrate, 150mM NaCl
TBE:	2mM EDTA, 89mM boric acid, 89mM Tris-HCl pH8.0
Tris-saline:	140mM NaCl, 30mM KCl, 280mM Na_2HPO_4, 1mg/ml glucose, 0.0015% (w/v) phenol red, 25mM Tris-HCl (pH7.4), 100 units/ml penicillin, 0.1mg/ml streptomycin
Trypsin:	0.25% (w/v) Difco trypsin dissolved in Tris-saline
Trypsin-Versene:	1 volume trypsin + 4 volumes versene
Versene:	0.6mM EDTA dissolved in PBS containing 0.002% (w/v) phenol red.

2.1.12. Antisera

For the detection of ICP34.5 in HSV-1 infected cell extracts a peptide antiserum, 78, which had been raised against a ten times repeat of the Pro-Ala-Thr (PAT) sequence present in the published sequence of RL1 was utilized. The characterization of this antiserum has been previously described (MacKay *et al.*, 1993)).

Two other peptide antisera against ICP34.5 were provided by Dr.C. MacLean. These had been raised against peptides which corresponded to sequences within the 63 amino acid region of homology between HSV-1 and HSV-2 RL1 and were synthesized by Dr.A. Owsianka. These will be described more fully in a later section (Section 3.2.1.).

The monoclonal antibodies, 3104 and ZLF11 which recognise gI and 65KDBP respectively, were kindly provided by Dr.A.Cross.

Specific polyclonal antisera against 21K and Vmw110 were provided by Dr. Howard Marsden..

2.1.13. Miscellaneous

Phenyl sepharose	Pharmacia
S-sepharose	Pharmacia
Repelcote	BDH Chemicals
XS-1 film	Kodak
XK 16 column	Pharmacia
Giemsa stain	BDH
Neutralizing antibodies (human serum)	Gibco

2.2. Methods

2.2.1. Growth of cells

BHK21/C13 cells were grown in 80oz roller bottles within a humidified atmosphere of 95% air and 5% CO_2 at 37°C for 3 days. The yield from a confluent roller bottle is approximately $1x10^8$ cells.

Cells were harvested by washing the monolayers twice with 25mls of trypsin- versene, and resuspending the detached cells in 20mls of ETC10. Cells from 1 roller bottle could be used to seed a further 10 roller bottles. For some experiments cells were plated on 50mm or 30mm petri dishes or Linbro wells at a density of $4x10^6$, $2x10^6$ and $5x10^5$ cells per plate respectively.

2.2.2. Growth and harvest of HSV

Confluent roller bottles were infected with 0.003 p.f.u./cell of HSV in 20mls of ETC10, assuming that there were $1x10^8$ cells per roller bottle. These were incubated at 31°C for 3-4 days, until c.p.e. was complete, when the cells were shaken into the medium. The cells

were pelleted in 250ml plastic falcon tubes by spinning at 2K for 10 minutes at 4°C in a Fison's Coolspin. The supernatant and cell pellet were separated and two individual virus stocks prepared:

Supernatant stock: The supernatant was poured into 250ml centrifuge bottles and spun at 12K for 2hr (4°C) in a Sorvall GSA rotor. The supernatant was discarded, and the virus pellet resuspended in 1ml ETC10 or PBS/calf per roller bottle. The pellet was sonicated until homogeneous, before aliquoting into 2ml amounts and storing at -70°C.

Cell- associated stock: The cell pellet was resuspended in 0.5ml medium/ roller bottle and thoroughly sonicated before spinning at 2K for 10 minutes at 4°C in a Fison's Coolspin centrifuge. The supernatant was kept on ice while the process was repeated. The two supernatants were combined to give the cell-associated virus stock. This was aliquoted and stored as above.

2.2.3. Titration of virus stocks

Virus stocks were serially diluted 10-fold in PBS/calf. 0.1ml aliquots were added to 75% confluent monolayers of BHK21/C13 cells on 50mm petri dishes from which the medium had been removed. The plates were incubated at 37°C for 45min- 1hr, to allow absorption of the virus to the cells, before overlaying with 4ml EMC10%. Plates were incubated at 37°C or 38.5°C for 2 days, or for 3 days at 31°C. Monolayers were fixed and stained with Giemsa at RT for 1hr. After washing, plaques were counted on a dissection microscope and virus titres were calculated as p.f.u./ml.

2.2.4. Virus adsorption onto cells.

70% confluent monolayers of BHK21/C13 cells on 50mm plates were precooled to 4°C for 30-60 minutes, before infection with 400 p.f.u. virus/plate in 500ul ETC10. From 0, 15, 30, 45, 60, 80, 100, 120 and 240 minutes post-infection, unadsorbed virus was removed by washing the monolayers twice with PBS/calf. Plates were overlaid with 4ml EMC10%, incubated at 37°C for 2 days and stained. The number of plaques on each plate was counted and each time-point calculated as a percentage of the virus adsorbed at

T=240. The percentage virus bound at each timepoint was calculated as the mean of the plaque count from 3 plates.

2.2.5. Virus penetration into cells.

70% confluent monolayers of BHK21/C13 cells on 50mm plates were pre-cooled to 4°C for 30-60 minutes before infection with 400 p.f.u. virus/plate in 500ul ETC10. Following adsorption at 4°C for 1hr, unadsorbed virus was removed by washing the monolayers twice with PBS/calf. Plates were overlaid with 4ml ETC10 and transferred to 37°C (T=0). At 0, 5, 10, 20, 30, 45 and 60 minutes post-adsorption, medium was removed from the plates and 1ml citrate buffer (40mM citric acid, 10mM KCl, 135mM NaCl, pH 2.5) added. Plates were incubated at RT for 5 minutes, washed twice with PBS/calf and overlaid with 4ml EMC10%. In a control experiment, plates were incubated with 1ml PBS/calf at RT for 5 minutes, washed twice with PBS/calf and overlaid with 4ml EMC10%.

Following incubation at 37°C for 2 days, plates were stained and the number of plaques on each counted. Percentage virus bound at each timepoint represents the mean of the plaque count from 3 plates and was calculated as a percentage of the virus penetrated at T= 60.

2.2.6. Sterility checks on virus stocks.

Brain heart infusion agar (BHI) plates and BHI plates containing 10% horse blood (BHI blood agar) were obtained from the Cytology department.

To check for fungal contamination of stocks, a small aliquot was streaked onto BHI plates in duplicate, which were sealed with parafilm and incubated at RT. Yeast or bacterial contamination was detected by plating onto BHI blood agar and incubating at 37°C. If no colonies were visible after 7 days incubation, the stocks were considered sterile.

2.2.7. Preparation of HSV DNA.

To prepare a large scale HSV DNA stock, 10 roller bottles containing almost confluent monolayers of BHK21/C13 cells were infected with virus at a m.o.i. of 0.003 p.f.u./cell. The infection was continued at 31°C until c.p.e. was extensive (3-4 days). The cells were shaken into the medium and spun at 2K for 10 minutes in a Fison's Coolspin. The supernatant was kept on ice while the nuclei were extracted from the cell pellet by treatment with 0.5% (w/v) NP40 in RSB (10mM KCl, 1.5mM $MgCl_2$, 10mM Tris-HCl, pH 7.5) followed by centrifugation (2K for 10 minutes as before) to pellet the cell debris and nuclei. This was repeated before pooling the supernatants and spinning to pellet the virus at 12K for 2hr in a Sorvall SS34 rotor.

The virus pellet, containing cell released and cytoplasmic virus was resuspended in NTE buffer (10mM Tris-HCl, pH7.5, 10mM NaCl, 1mM EDTA) before adding EDTA and SDS to a final concentration of 10mM and 2% (w/v) respectively to cause lysis of the virus. Viral DNA was extracted 3-4 times with saturated phenol and once with chloroform: isoamylalcohol, prior to precipitating with 2 volumes of ethanol at RT for 5 minutes. DNA was pelleted at 2K for 10 minutes, washed with 70% ethanol, dried at 37°C and resuspended in a minimal volume of H_2O+ RNase A (50ug/ml).

2.2.8. Transformation of *E.coli.*

Ten ml of 2xYT broth was inoculated with 15ul of a glycerol stock of bacteria and incubated with shaking at 37°C overnight to produce a saturated culture.

One ml of this culture was used to inoculate 100ml of 2xYT broth which was shaken at 37°C for 2.5-3hr (when the bacteria should be in mid-log phase). The bacterial cells were pelleted by spinning at 2K for 10 minutes, in a Fison's Coolspin and resuspended in 1/10th volume of transformation and storage buffer (10mM $MgCl_2$, 10mM $Mg(SO)_4$, 10%(w/v) PEG 3,500, 5% (v/v) dimethyl sulphoxide). After 10 minutes on ice, the bacteria were considered competent for transformation.

Typically, 1ul and 10ul of a ligation mix or plasmid preparation were incubated for 30 minutes on ice with 100ul of competent *E.coli.* One ml of L-broth was added to the

transformation mix and incubation was continued for a further hour at 37°C. If the bacteria had been transformed with a plasmid which conferred a particular trait eg. antibiotic resistance, the 1hr incubation at 37°C allowed plasmid gene expression to occur.

One hundred ul of the transformed bacteria were plated onto L-broth agar plates containing, if appropriate 100ug/ml ampicillin. Plates were allowed to dry at room temperature, before incubating in an inverted position at 37°C overnight.

2.2.9. Small scale plasmid preparation.

Single, transformed, bacterial colonies were picked into 5ml of L-broth containing 100ug/ml ampicillin, and shaken at 37°C for 8-16hr. 1.5ml aliquots of each culture were spun at low speed (6500g) in a microfuge, the supernatant discarded and the cells resuspended in 100ul of solution I (50mM glucose, 25mM Tris-Cl , pH8.0, 10mM EDTA, 5mg/ml lysozyme added just prior to use). Following 5 minutes incubation at RT, 200ul of solution II was added (0.2M NaOH, 1% (w/v) SDS), and the cultures shaken vigorously. After a further 5 minutes at room temperature 150ul of solution III was added [5M KAc, pH4.8]. Following 5 minutes incubation at RT a white precipitate consisting of the cell debris was pelleted at high speed (13000g) in a microfuge .

Plasmid DNA was extracted from the supernatant using an equal volume of phenol: chloroform followed by ethanol precipitation at RT for 1 minute. The DNA was pelleted by spinning at 13,000g for 5 minutes in a microfuge, washed in 70% ethanol, dried in a Speedivac and resuspended in 100ul H_2O+ RNase A (50ug/ml).

2.2.10. Large scale plasmid preparation.

The method used was essentially as described by Birnboim and Doly (1979), and modified by Manniatis *et.al.* (1982). Single transformed bacterial colonies from a L-broth agar plate were inoculated into 5ml of L-broth containing the appropriate antibiotic and shaken at 37^{o}C for 8-16hr. The culture was transferred into 500ml L-broth containing the appropriate antibiotic in a.2 litre dimpled flask and shaken at 37^{o}C overnight.

The bacteria were pelleted by centrifugation at 8K for 10 minutes in a Sorvall GSA rotor, the pellet resuspended in 7ml solution I (see section 2.2.9.) and incubated at RT for 10 minutes. Freshly made solution II (14ml) was added and incubation continued for a further 10 minutes on ice. Ice-cold solution III (10.5ml) was added, incubation continued on ice for 10 minutes and the bacterial debris pelleted by centrifugation at 12K for 10 minutes in a Sorvall SS34 rotor. DNA was extracted twice with an equal volume of phenol:chloroform and once with an equal volume of chloroform. The DNA was precipitated by the addition of 2 volumes of ethanol, centrifuged at 12K for 30 minutes in a Sorvall SS34 rotor at RT, washed in 70% ethanol, pelleted as before, dried in the incubator and dissolved in water containing 50ug/ml RNase A.

In some instances, to remove residual host DNA and RNA, the DNA was further purified by isopycnic banding on caesium chloride gradients. In this case, the DNA was only extracted once with phenol:chloroform. Caesium chloride was added until the buoyant density was 1.56g/cm^{3} and ethidium bromide was added to a final concentration of 0.5 ug/ml. The mixture was pipetted into Oakridge tubes, which were capped, sealed and centrifuged at 45,000 r.p.m. overnight in Sorvall Ti50 rotor. The DNA was visualised on a long wave U.V. light box and the lower band which contained the supercoiled plasmid DNA was removed using a needle and syringe. After extracting at least 3 times with isoamyl alcohol, the DNA was ethanol precipitated as described above. The DNA concentration was quantitated by running a small quantity on an agarose gel alongside standards of known concentrations.

2.2.11 Restriction enzyme digestion of DNA

The manufacturer's recommended buffers and conditions were generally used for each individual restriction enzyme. To achieve complete digestion, 1ug samples of HSV or plasmid DNA were digested with 2-5 units of restriction enzyme for 4hr at the appropriate temperature. If the digested DNA was to be run on an agarose gel, 1/5- 1/6 volume of RE stop was added prior to loading, otherwise the digested DNA was recovered as described in section 2.2.13..

2.2.12. Recovery of DNA from agarose gels using DEAE-sephacel.

Generally the DNA to be recovered had been digested with restriction enzymes before running on an agarose gel. In order to cause the minimum possible damage to the DNA, it was visualised using a long wave (300-360nm) U.V. lamp and a gel slice containing the desired fragment cut out with a sharp scalpel.

This slice was placed in a length of dialysis tubing containing 1xTBE and the DNA was eluted at 100V for 2-3hr, using 1xTBE as a running buffer. Following this, the polarity of the current was reversed for 2 minutes to release any DNA which had attached to the inner wall of the dialysis tubing. The buffer surrounding the gel slice was carefully removed and the dialysis tubing was washed out with a small quantity of 1xTBE.

Disposocolumns (Bio-Rad) were packed with 0.6ml of DEAE-sephacel (sufficient to bind 20ug of DNA) which had been equilibrated with 10mM Tris-Cl, pH7.6, 1mM EDTA and 60mM NaCl. The packed columns were washed with 1.5ml of NTE (1mm EDTA, 100mM NaCl, 10mm Tris-HCl, pH7.4) before applying the sample to the top of the column. The eluate was collected, and reapplied to the top of the column, in case any DNA had not bound the first time. Bound DNA was washed with 2.5ml of NTE, before elution with 2x250ul aliquots of TE (10mM Tris-Cl, pH7.6, 1mM EDTA) + 1M NaCl. The DNA was extracted first with phenol and then chloroform, ethanol precipitated and washed with 70% ethanol. After drying in the incubator, the DNA was resuspended in 20-25ul of H_2O.

2.2.13. Phenol-chloroform extraction of DNA from restriction enzyme digestion mixtures.

If the digestion mixture was not at least 100ul, it was increased to this volume using dH_2O. An equal volume of phenol:chloroform was added, the mixture shaken, and spun at 13000g for 2 minutes in a microfuge. The top aqueous layer was removed into a separate eppendorf tube and the bottom layer was back extracted with an equal volume of dH_2O. To this 1 volume of chloroform was added and the mixture shaken and spun at 13000g for 2 minutes in a microfuge. The top layer was removed and the sample was back extracted as before.

To precipitate the DNA, 2 volumes of ethanol and 0.1 volumes of 3M sodium acetate were added. Following 10 minutes incubation on dry ice, the DNA was pelleted at 13000g for 5 minutes, washed with 70% ethanol and dried in a Speedivac, before resuspending in an appropriate volume of dH_2O.

2.2.14. Ligation.

Both vector and insert, were cut with the appropriate restriction enzymes. If the vector was cut with a blunt-cutting enzyme its digestion was usually carried out in the presence of 1 unit calf intestinal phosphatase to prevent recircularization during ligation. The vector was purified as described in section 2.2.13. and the fragment as described in section 2.2.12.. Three types of fragment ends could be generated:

i) Blunt or compatible ends: In this case various quantities of fragment were ligated to 100ng of vector DNA at 14^{o}C overnight. Ligation was carried out in 1x ligation buffer (10x ligation buffer contains 200mM DTT, 100mM $MgCl_2$, 0.5M Tris-HCl, pH 7.6) in the presence of 2 units of ligase.

ii) 5' overhangs: In the absence of a compatible site in the vector: if the fragment to be cloned had 5' overhangs these had to be filled in before cloning into the vector which had been cut with a blunt-cutting enzyme eg. Sma I. Prior to running on a gel, 0.1 volume of 2.5mM dNTPs and 1unit of Klenow was added to the digestion mixture. Following incubation at 37^{o}C for 30 minutes, the digest was run on a gel. The appropriate fragment

was purified as described in section 2.2.12. and ligated to the vector as described in (i) above.

iii) 3' overhangs: In the absence of a compatible site in the vector: if the fragment to be cloned into the vector had 3' overhangs, these had to be exonucleased. The digestion mixture was purified as described in section 2.2.13. and redissolved in an appropriate volume of dH_2O. To this was added 1 unit T4 DNA polymerase which has a strong 3'-5' exonuclease activity and 0.1 volume of T4 polymerase buffer. Following incubation at 37°C for 1hr, purification and ligation of the fragment was carried out as in (i) above.

2.2.15. Mini-gel electrophoresis.

This method was generally used to quantitate small volumes of fragment and vector DNA prior to ligation. Gels were prepared by boiling the appropriate concentration of agarose in 50ml 1xTBE. When cool, 0.5ug/ml ethidium bromide was added and the agarose was poured directly into the mini-gel kit (Bio-Rad). Once set, the comb was removed and 50ml 1xTBE was added to the kit. Samples (generally 5-7ul) were run at 50V for 1-2 hrs and visualised using a long wave U.V. lamp. A 1kb marker of known concentration was run alongside the fragments to enable confirmation of the vector/fragment size and quantitation of the concentration of each sample.

2.2.16. Purification of a synthetic oligonucleotide

Eighty ml of 6% acrylamide,1xTBE, 9M Urea sequencing gel mix was polymerized with 160ul of 25% (w/v) APS and 60ul TEMED. This was then poured between two 20x22cm glass plates separated by 1.5mm spacers and a 10 tooth comb inserted at the top.

The oligonucleotides were synthesized on a Biosearch 8600 DNA synthesiser, subsequently deprotected at 65°C for 5-6hr, frozen on dry ice and dried in the speedivac overnight. The oligonucleotide was resuspended in 50ul H_2O by vortexing. Fifty ul of sample buffer was added before boiling for 10 minutes and loading immediately onto the gel. Two ul of formamide dye mix was loaded in a separate well to act as a molecular weight marker and the gel was run at 10mA for 3-4hr in 1xTBE.

To visualise the DNA, the gel was removed, wrapped in cling-film and viewed against a white chromatographic plate by angled long-wave U.V. light. If the synthesis had been succesful, then a predominant band, with possibly a few lower molecular weight bands , was observed. The top band was cut out with a scalpel, mashed with a glass rod, and incubated at 42°C for 16hr in 1ml elution buffer (0.5M ammonium acetate, 1mM EDTA, 0.5% (w/v) SDS). This was filtered through glass wool to remove the acrylamide, phenol: chloroform extracted, ethanol precipitated, washed in 70% ethanol, dried and redissolved in dH_2O. To quantitate the DNA, the OD_{260} was read and the conversion factor: 1OD unit= 20ug/ml used.

2.2.17. Transfection of DNA into cells using the $CaPO_4$ technique.

This method is a modification of that described by Stow and Wilkie (1976). Four hundred ul HEBS, pH7.05, (130mM NaCl, 4.9mM KCl, 1.6mM Na_2HPO_4, 5.5mM D-glucose, 21mM HEPES) containing 10ug/ml calf thymus DNA , 0.2-2ug intact virus DNA, 1- 10ug plasmid DNA and 130mM $CaCl_2$, were added to semi- confluent monolayers of BHK21/C13 cells on 50mm petri dishes, from which the medium had been removed. Following incubation at 37°C for 45-60 minutes, cells were overlaid with 4ml ETC10. Four to eight hr later the media was removed from the plates and they were washed twice with ETC10. 1ml 25% (v/v) DMSO in HEBS was added and the plates incubated at RT for 4 minutes. The DMSO was removed and the plates washed twice and overlaid with, 4ml ETC10. Incubation was continued at 37°C until c.p.e. was complete.

2.2.18. Isolation of single plaques from transfection.

Once c.p.e. was complete, the cells were scraped into the growth media, transferred into a black-cap vial and sonicated until homogeneous. Serial 10-fold dilutions of each transfection were made in PBS/calf and the 10^{-3} to 10^{-7} dilutions from each plated onto 70% confluent BHK21/C13 cells. After absorption at 37°C for 1hr, the plates were overlaid with 4ml EMC10% and incubation continued at 37°C for a further 48hr. The plates were washed twice and overlaid with a few ml of PBS/calf. To prepare plate stocks,

single plaques were isolated and grown in Linbro wells containing BHK21/C13 cells . Plates were incubated for 3-4 days at 37°C, then stored at -70°C.

2.2.19. Preparation of ^{32}P labelled viral DNA.

This is a modification of the method described by Lonsdale (1979). Confluent monolayers of BHK21/C13 cells in Linbro wells were infected with either 100ul of a plate stock or $2x10^6$ p.f.u. of a virus stock. After absorption for 45 minutes at 37°C, the virus was removed and the cells washed with and maintained in PIC for 2hr at 31°C. One uCi of orthophosphate (^{32}P) in 50ul PIC was added per well and incubation continued at 31°C for 2-5 days.

Following incubation at 31°C for 2-5 days, 0.5ml of 5% (w/v) SDS was added to each well. Trays were incubated at 37°C for 5-10 minutes, before scraping the cells off the wells using a blue tip and adding them to 1ml of phenol. The samples were inverted and incubated at RT for 10 minutes, with a further inversion after 5 minutes. They were centrifuged at 2K for 10 minutes (RT) in a Fison's Coolspin before removing the top layer into 2 volumes (2ml) of ethanol. After gently inverting the tubes, they were spun as before. The ethanol was poured off and the DNA dried for 10 minutes at 37°C with the test-tubes in an inverted position. Two hundred ul of 50ug/ml RNAse A was added to each sample and the DNA left to dissolve for 2hr at 37°C. The DNA was then ready for digestion with the appropriate enzyme(s).

2.2.20. *In vitro* ^{32}P labelling of DNA.

Plasmid or gel purified fragments of virion DNA were ^{32}P labelled using the method of random priming. 10-100ng of DNA was boiled for 10 minutes before labelling. Five times random prime buffer comprises 3 solutions A, B, and C at a ratio of 10:25:15 respectively.

Solution A: 1ml 1.25M Tris-HCl, pH 7.8, 0.125M $MgCl_2$

18ul 2-mercaptoethanol

5ul 100mM dGTP

5ul 100mM dATP

5ul 100mM dTTP

Solution B: 2M HEPES, pH 7.6

Solution C: Hexadeoxyribonucleotides (Pharmacia) resuspended in TE at 90 OD units/ml.

The boiled DNA is added to 5ul of 5x random prime buffer, with 1ul of 1% BSA, 20uCi ^{32}P dCTP, 1 unit Klenow polymerase and made up to 25ul with water. The mixture was incubated at 37^{o}C for 30 minutes and boiled, prior to use for Southern blotting.

2.2.21. Southern blotting.

Purified virus, infected cell DNA or plasmid DNA was digested with the appropriate restriction enzyme(s) before running on an agarose gel. The gel was visualised under short-wave U.V. light to confirm DNA digestion and to partially fragment larger DNA fragments and so aid their transfer to membranes.

The gel was placed in 500ml Gel Soak I (see section 2.1.11.) for 1hr, rinsed with deionised water and transferred to Gel Soak II for 1hr. After rinsing as before, it was transferred to 500ml 10xSSC for a further 1hr. The gel was now ready for blotting onto either 1 sheet or 2 sheets simultaneously of Hybond N nylon membrane (Amersham). For each transfer 1 sheet of nylon membrane and 5 sheets of Whatmann 3MM paper, all cut to the exact size of the gel were required.

A bundle of 'Hi-Dry' towels was placed on the bench followed by 3 sheets of dry then 2 sheets of 10xSSC soaked 3MM filter paper. The sheet of nylon membrane, soaked in 10xSSC was placed on top of the filter paper followed by the gel, thus ensuring that there were no air bubbles between the gel and the nylon membrane. A glass plate and heavy weight were placed on top and left for at least 4hr during which time the DNA was drawn out of the gel and onto the adjacent side of the nitrocellulose. To transfer DNA to two membranes this procedure was repeated on top of the gel.

The DNA was cross-linked to the nylon membrane using a U.V. Stratalinker, and hybridized to the labelled probe in a sealed bag containing 20ml hybridization buffer. Hybridization was generally carried out overnight at 65^{o}C. The nylon membrane was

washed for 3x30 min with 1 litre 2xSSC, 0.1%(w/v)SDS before drying and setting up for autoradiography against Kodak XS-1 film.

2.2.22. Construction of M13 recombinant plasmids

M13mp18 and mp19 double-stranded DNA was commercially available from Pharmacia. Typically 10ug DNA was digested with the appropriate restriction enzyme. Following extraction as described previously (section 2.2.13.) the DNA was resuspended in 10ul H_2O. One ug aliquots of M13 and a wide range of fragment amounts (0.1-10ug) were generally used in each ligation mix. Standard cloning methods were employed (Manniatis et.al.1982).

2.2.23. Transformation of *E.coli* cells with M13

E.coli strain JM109 were prepared as described in section 2.2.8.. Typically 1ul and 10ul of ligation mix was added to 200ul of competent cells and incubated on ice for 30 minutes. Three ml melted top agar at 42°C, containing 25ul of 25mg/ml IPTG in water and 25ul of 25mg/ml 5-chloro-4-bromo-3-indoyl-2-D-galactoside (X-gal) in dimethyl formamide was added and the mixture poured onto 90mm L-broth agar plates and incubated at 37°C overnight.

2.2.24. Preparation of M13 single stranded DNA.

Single, colourless, transformed M13 colonies were used to inoculate 3ml 2xYT broth containing 1/100 dilution of an overnight saturated bacterial culture. Following 4.5-6hr incubation with shaking at 37°C, 1.5ml culture was spun at 13,000g in a microfuge to pellet the bacteria. The supernatant, which contained the M13 bacteriophage, was retained and respun to ensure no bacterial contamination. M13 was precipitated by the addition of 200ul of a solution containing 2.5% (w/v) PEG 6000 and 3M ammonium acetate. Following incubation at RT for 10 minutes, bacteriophage were pelleted by centrifugation at 13,000g in a microfuge. The supernatant was discarded and the pellet respun before removing any excess liquid with an eppendorf tip. After resuspending the pellet in 200ul

H_2O, the DNA was extracted once with an equal volume of phenol-chloroform, before precipitating with 2 volumes of ethanol and 0.1 volumes of 3M sodium acetate on dry-ice for 10 minutes. The DNA was pelleted by centrifugation at 13,000g for 5 minutes in a microfuge, washed with 70% ethanol, dried and resuspended in 15ul H_2O containing 50ug/ml RNase A.

2.2.25. Sequence analysis of recombinant M13 clones.

To facilitate the sequencing of regions of DNA with a high G-C content a Sequenase Version 2.0 kit (Amersham), was used in accordance with the manufacturer's instructions, otherwise the following protocol was used.

Five ul of each single-stranded DNA template was annealed to 3ng of commercial oligonucleotide primer (Pharmacia) (which hybridizes upstream of the multicloning site and is known as the universal sequencing primer) in a buffer containing 40mM Tris.HCl , pH7.5, 10mM $MgCl_2$ and 50mM NaCl in a total volume of 10ul, by first heating to 55°C for 5 minutes in a 1.5ml eppendorf tube, then allowing to cool to room temperature over a period of 15-30 minutes. The templates were briefly chilled on ice, before being labelled and extended using a buffer which contained 0.19uM dCTP, 0.19uM dGTP, 0.19uM dTTP, 10uCi ^{35}S dATP, 6mM DTT and 2 units of Klenow polymerase in a total volume of 16ul and labelling carried out at RT for 2-5 minutes. Each template was extended and terminated by addition of 3ul of labelled template to 2.5ul of each of the 4 ddNTP termination mixes in microtitre wells, which had been pre-warmed to 37°C. Each ddNTP termination mix contained one ddNTP at a concentration of ddATP: 300uM, ddCTP: 100uM, ddGTP: 150uM, ddTTP: 500uM. The dNTPs were at a concentration of 250uM except for the one corresponding to the ddNTP where the concentration was 25uM. The plate contents were mixed by centrifugation in a Beckman benchtop centrifuge and the reactions carried out at 37°C for 10-30 minutes. Three ul formamide dye mix (95% formamide, 20mM EDTA, 0.5% (w/v) bromophenol blue, 0.5%(w/v) xylene cyanol) was added to each sample. The samples were boiled for 5 minutes before loading onto a gel for electrophoresis.

2.2.26. Electrophoresis and autoradiography of sequencing gels.

Electrophesis was carried out at 70W through vertical gels 42x34x0.04 cm in size. Gels consisted of 6% acrylamide (cross-linked with 5%(w/v) N,N'-methylbisacrylamide) and 9M urea in 1xTBE. Polymerization was achieved by addition of 0.05%(w/v) APS and 0.1%(v/v) TEMED. Spacers and gel combs were supplied by Gibco-BRL. Both plates were treated with repelcote enabling the gel to be transferred to Whatmann 3MM chromatography paper following electrophoresis and dried under vacuum. Dried gels were then exposed to XS-1 film (35x43 cm) and developed using a X-omat processor.

2.2.27. Animal studies.

a) Neurovirulence.

Three week old female BALB/c mice (Bantin and Kingman) were inoculated intracerebrally with individual virus stocks. Mice were anaesthetized with halothane and 25ul of the appropriate virus dilution in PBS/calf was inoculated into the central region of the left cerebral hemisphere. Four mice were inoculated with each virus dilution. Depending on the known, or postulated neurovirulence phenotype of the viruses being tested, doses were in the range 10^1 to 10^7 p.f.u./mouse. Stocks were always retitrated on the day of inoculation to ensure that the correct dose had been administered.

Mice were observed daily up to 21 days post inoculation for signs of illness or death, and the LD_{50} for each virus was calculated according to the formula of Reed and Muench (1938).

b) *In vivo* growth

The ability of mutant and wild-type virus to replicate in mouse brains following intracerebral inoculation was assessed using a mouse model. Twenty-five ul aliquots of wild-type virus (10^2 p.f.u./mouse) and mutant virus (10^5 p.f.u./mouse) were injected into the left cerebral hemisphere of 3-week old BALB/c mice. At 0, 1, 2, 3, 4 and 5 days post-inoculation, 2 mice per virus were killed by cervical dislocation, their brains removed and homogenized in 1ml PBS/calf. The final volume was measured and recorded, before storing at -70°C. When all samples had been collected, they were thawed, briefly

sonicated and progeny virus titrated separately on BHK21/C13 cells. Final titres were calculated as mean p.f.u./mouse.

c) *In vivo* marker rescue.

A neurovirulent phenotype was restored to non-neurovirulent variants using the method of *in vivo* marker rescue. One ug of DNA from a non-neurovirulent virus was transfected onto BHK21/C13 cells with a 1-, 10- and 100- fold molar excess of a wild-type fragment known to restore neurovirulence (section 2.2.17.). After 2 days, the monolayer in which c.p.e. was most extensive was harvested and sonicated briefly. 10^{-1} and 10^{-2} dilutions were prepared in PBS/calf and 25ul aliquots of each were injected intracerebrally into 3-week old BALB/c mice.

If the mice died, their brains were removed, homogenised in PBS/calf, and resultant virus titrated on BHK21/C13 cells. Single plaques were isolated and their genomes analysed to identify wild-type rescuants. Generally 95-100% of isolated plaques had a wild-type DNA profile as non-neurovirulent virus did not replicate *in vivo* and neurovirulent recombinants were selectively amplified.

d) Latency.

As has been described by Clements and Subak-Sharpe (1983, 1988), 4 week old BALB/c mice were inoculated in the right rear footpad with various doses of virus in PBS/calf. Following inoculation, the virus stocks were retitrated to ensure that the correct dose had been administered. After 6 weeks, all surviving mice were dissected and the 9 ipsilateral dorsal root ganglia supplying the lower limb of each mouse were separately cultured in microtitre plates. They were screened every second day for the presence of infectious virus by transferring the culture supernatant to control BHK21/C13 cells. These cells were then incubated for 2 days at 37°C, before staining and examination for the presence of virus plaques or c.p.e..

2.2.28. Generation of E.coli containing the expression vector pET34.5.

The intact ICP34.5 open reading frame (ORF) from HSV-1 strain 17^+ was cloned into pET8c using standard procedures. This recombinant plasmid, pET34.5, was used to transform *E.coli* BL21(DE3) cells containing the T7 RNA polymerase gene under the control of the inducible *lac* UV5 promoter. A glycerol stock was prepared by picking a transformed colony into 5ml 2xYT containing 250ug/ml ampicillin and growing overnight with shaking at 37°C. An equal volume of sterile glycerol was added and mixed thoroughly before aliquoting into 1ml amounts and storing at -70°C.

For each experiment a small amount of frozen bacteria was used to inoculate 1ml 2xYT. One hundred ul was plated onto a L-broth agar plate containing 250ug/ml ampicillin, which was incubated at 37°C overnight. The following day single colonies were used to inoculate larger cultures.

2.2.29. Analysis of ICP34.5 solubility over a range of temperatures.

To ensure that a soluble form of ICP34.5 was produced, solubility was examined over a range of growth temperatures. As described above, (section 2.2.28.), a small amount of frozen bacteria was plated out to give single colonies. The following day, single colonies were used to inoculate 5ml L-broth containing ampicillin. Following growth at 37°C for 4hrs, T7 RNA polymerase expression and hence ICP34.5 production was induced by the addition of IPTG to the growing cultures. These were then either maintained at 37°C for a further 1 hr or transferred to 31°C, 28°C or 26°C for 1hr. The bacteria were pelleted and lysed with lysozyme. The bacterial debris was then pelleted by centrifugation and equivalent volumes of pellet and supernatant were analysed by Western blotting for the presence of ICP34.5 in the soluble supernatant but not in the pellet which contained all the insoluble material.

2.2.30. Expression of ICP34.5 in *E.coli.*

A 500 ml overnight culture of *E.coli* BL21(DE3) containing pET8c was diluted 20-fold into ten 1litre cultures which were incubated with shaking at 37°C. On reaching an optical

density of 0.5 at 600nm, the temperature of the cultures was rapidly decreased to 28°C, IPTG added and incubation continued for 1hr at 28°C. Cells from the 10 litre culture were collected by centrifugation at 5K for 10 minutes in a GSA rotor and resuspended in 100ml of 50mM-Hepes, pH7.2, 50mM NaCl.

Bacteria were lysed by addition of lysozyme to a final concentration of 500ug/ml, followed by 30 minutes incubation on ice. Cell debris was pelleted by centrifugation at 15K for 20 minutes in a SS34 rotor, at 4°C and the supernatant, which contained the expressed protein, was stored at -70°C.

Protein concentrations were determined using a Bio-Rad protein assay kit according to the manufacturers recommended instructions using known concentrations of BSA (in the range 0.01-2mg/ml) as standards.

2.2.31. Analytical and quantitative ammonium sulphate fractionation.

To look at ICP34.5 solubility over a range of ammonium sulphate concentrations, increasing amounts of a saturated solution of ammonium sulphate were added to 100ul aliquots of crude extract. After 30 minutes on ice, samples were clarified by centrifugation at 13.5K for 30 min, at 4°C. Pellets were redissolved in 300ul 50mM-Hepes, pH7.2, 50mM NaCl, and supernatants were brought to a volume of 300ul using the same buffer. Pellet and supernatant fractions were analysed by SDS-PAGE.

For purification of ICP34.5, the crude extract was salt-fractionated using ammonium sulphate powder. Salt was slowly added to the extract with constant stirring, at room temperature, followed by incubation on ice for at least 30 minutes. The protein precipitate was collected by centrifugation at 12K for 30 minutes, in an SS34 rotor at 4°C.

2.2.32. Anion-exchange chromatography.

The salt-fractionated material was resuspended in 50mM Hepes, pH7.6, 50mM NaCl and applied onto a FPLC Mono Q HR 5/5(1ml) or 10/10(8ml) anion-exchange column, which had been equilibrated with the same buffer. At this pH the expressed protein did not bind to the column, whereas the vast majority of the *E.coli* proteins did. The flowthrough was

collected and stored at -70°C and the column was thoroughly washed with buffer B (50mM Hepes pH7.2, 1M NaCl) to remove the bound *E.coli* proteins, which were subsequently discarded.

2.2.33. Purification of ICP34.5 using phenyl sepharose or S-sepharose.

This step was carried out at 4°C. A XK 16 column with adjustable adaptors (Pharmacia), was packed with 50ml of the appropriate matrix. This was equilibrated with 1 column volume of Buffer A [1M NaCl or 1.5M$(NH_4)_2SO_4$, with 50mM Tris-HCl, (pH range 7-8) or 50mM (2-[N-Morpholino]ethanesulfonic acid) (pH range 5.5-6.5)] before loading the sample which had been dialysed overnight into the same buffer. After washing the column with a further 1 column volume of Buffer A, the protein was eluted using a 500ml decreasing salt gradient from either 1.5M $(NH_4)_2SO_4$ or 1M NaCl and 50mM appropriate buffer to 50mM buffer alone. Five ml fractions were collected and analysed by SDS-PAGE.

2.2.34. Production of polyclonal ICP34.5 specific antisera.

Rabbit antisera to ICP34.5 were produced using bacterially expressed ICP34.5 as the antigen. New Zealand White rabbits were immunized intramuscularly first with 2mg of protein containing approximately 100ug ICP34.5 in Freund's complete adjuvant followed by 4 boosts, each at a 14 day interval, using the same amount of antigen but in Freund's incomplete adjuvant. Animals were bled 10 days after each boost and the antisera tested at different dilutions against HSV-1 strain F and HSV-1 strain 17^+ infected cell extracts for reaction with ICP34.5 specific bands.

2.2.35. Pre-incubation of antiserum prior to use in immunofluoresence.

Before use in immunofluoresence studies, the protein antiserum was pre-incubated against *E.coli* BL21(DE3) cell extracts to cut down non-specific binding. The extracts were prepared by inoculating a single colony of *E.coli* BL21(DE3) cells lacking the plasmid pET34.5 (section 2.2.28.) from an L-broth agar plate into 5ml L-broth and growing at

37°C for 8hr. After pelleting the bacteria, they were resuspended in 5ml boiling mix and as much extract as possible was loaded prior to SDS-PAGE. Proteins were transferred onto nitrocellulose which was blocked as normal for Western blotting (section 2.2.38.) and incubated with the protein antiserum at a 1/100 dilution in PBS / 1% BSA. Following incubation at 37°C for 2hrs the antiserum was removed and used directly for immunofluoresence.

2.2.36. Immunofluoresence.

Washed, sterilized coverslips were placed at the bottom of 35mm plates (2 or 3 coverslips per plate), which were seeded with BHK21/C13 cells at one-half the normal density and grown for 16hr at 37°C. The medium was poured off and the cell monolayers washed twice with PBS. Cells were infected with 0.2ml virus dilution at a m.o.i. of 0.1 p.f.u./cell. After absorption for 1hr at 37°C, the cells were overlaid with 3ml ETC10. Following incubation at 37°C for 24hr the medium was removed and the plates washed twice with 3ml PBS. Three ml methanol was added to each plate and incubation carried out at RT for 4 minutes. Specific antibodies were then added to the plates at a dilution of 1/100. For the peptide antiserum, control reactions were carried out using sera which had been pre-incubated with the specific peptide. The plates were shaken at 37°C for 30 minutes, then washed twice with PBS, before the second antibody - anti-rabbit IgG (whole molecule) Fluorescein Isothiocyanate conjugate- was added at 1/80 dilution. This was again shaken at 37°C for 30 minutes. Plates were rinsed twice with PBS, before removing the coverslips, polishing the underside and mounting them on microscope slides using a small amount of fluorcolor. The edges of the coverslips were sealed with clear nail polish and the slides were stored in the dark.

2.2.37. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE analysis of proteins was performed in 5 to 12.5% gradient gels cross-linked with 5% (w/w) N,N'-methylenebisacrylamide, or 10% gels using 2.5% (w/w) crosslinker. Slab gels were cast vertically in a sandwich consisting of 2 glass plates separated by

1.5mm thick perspex spacers. Typically, 60ml gel mix was prepared using a running gel buffer (RGB) which contained 375mM Tris-HCl, pH8.9 and 0.1% (w/v) SDS. Polymerization was achieved by the addition of 0.06% (w/v) ammonium persulphate (APS), and 0.04% (v/v) N,N,N',N', tetramethylenediamine (TEMED), just prior to pouring. A thin layer of butan-2-ol (3-5ml), was poured on top to exclude air and enable polymerization of the gel. Prior to adding the stacking gel, the butan-2-ol was removed and the surface of the running gel was rinsed several times with distilled water. It was then thoroughly dried using filter papers to allow good adhesion between it and the stacking gel.

The stacking gel was composed of 5% acrylamide crosslinked with the same ratio of N,N'-methylenebisacrylamide used in the resolving gel, in a buffer (SGB), composed of 0.11M Tris.HCl, pH 6.7 and 0.1%(w/v) SDS. As previously, APS and TEMED were added to the gel just prior to pouring and a teflon comb was used to form the wells. Samples were boiled for 5 minutes in sample buffer (151mM Tris-HCl, pH7.6, 6.28%(w/v) SDS, 0.15%(v/v) 2-mercaptoethanol, 0.31%(v/v) glycerol) before loading on the gel, and were run either for 3-4hr at 50mA, or overnight at 8mA (Marsden *et al.*, 1976, 1978).

Gels were fixed or stained in a solution of methanol: acetic acid: water, 50:7:50, with or without 0.2% (w/v) Commassie Brilliant Blue R250, for 1hr at RT, then destained for a minimum of 3x 30 minutes in a 5:7:88 solution of methanol: acetic acid: water. Gels were either dried down immediately under vacuum and exposed to Kodak XS-1 film, or were enhanced in 3 volumes of En^{3}Hance (New England Nuclear) for 1hr, washed in water for 30 minutes, before being dried under vacuum and exposed for fluorography at -70^{o}C.

2.2.38. Western blotting .

SDS-PAGE was carried out as previously described (section 2.2.37.) and Western blotting carried out essentially as described by Towbin (1979). In general two types of protein sample were used for Western blotting. The first was infected cell proteins which had been harvested into boiling mix at a density of 10^7 cells/ml, and the second was small aliquots of fractions which had been obtained from protein purification columns. After separation of the proteins, they were transferred onto nitrocellulose using a Bio-Rad blotting apparatus. The foam pads, sheets of Whatmann 3MM paper and the nitrocellulose to be used for the transfer were pre-soaked in transfer buffer (192mM glycine, 25mM Tris.HCl pH8.3, 20% methanol). The gel was placed on top of the nitrocellulose, 3 sheets of 3MM paper and one foam pad. Three further sheets of 3MM paper and foam pad were placed on top . The plastic folder was then closed, placed in the transfer kit and blotted at 250mA for a minimum of 3hrs. After this time the nitrocellulose was removed, placed in a plastic 'tupperware' tub and blocked for 2x30 minutes using 2% dried milk in PBS/ complete, 0.05% Tween 20. The nitrocellulose was washed for 3x10 minutes in PBS/complete, 0.05% Tween 20, before addition of the first antibody diluted appropriately in PBS/complete, 0.05% Tween 20, 1% BSA. Following incubation for 2hr at 37^{o}C or overnight at 4^{o}C or RT the nitrocellulose was washed as before. The second antibody Protein A coupled to HRP was added at a 1/1000 dilution in PBS/complete, 0.05% Tween 20, 1% BSA and incubated at 37^{o}C for 1hr, after which time the nitrocellulose was washed twice with PBS/Tween, and once with PBS alone. To visualize the proteins, 60ul 4-chloro-1-naphthol was dissolved in a universal of ice-cold methanol and 60ul hydrogen peroxide was added to 100ml PBS/complete. These were mixed and poured into the tub containing the nitrocellulose, from which the wash buffer had been removed. The tub was covered with tin-foil to exclude light, and checked periodically . When the reaction was complete the nitrocellulose was washed in dH_2O and dried.

In some cases, proteins were visualized using ECL (Amersham) in accordance with the manufacturer's recommended instructions.

2.2.39. Immunoprecipitation of gI.

Infected BHK21/C13 cells on 50mm plates were labelled between 3-7hrs post-infection with 100uCi/ml [^{35}S] L-methionine in 4ml Emet/5C$_2$. The media was removed, the plates washed with PBS and the cells lysed by the addition of 1ml extraction buffer (10mM Tris-HCl, pH8.0, 10%(v/v) glycerol, 0.5%(v/v) NP40, 0.5%(w/v) sodium deoxycholate) (Showalter *et al.*, 1981). Extracts were incubated at 4^{o}C for 1hr, sonicated and clarified by centrifugation at 13,000g for 5 minutes in a microfuge.

Extracts (100ul) were incubated with 5ul gI mAb or control ascites, at 37^{o}C for 1hr and for a further 1hr at 4^{o}C with 70ul of a 50%(w/v) suspension of protein-A-sepharose in extraction buffer. Unbound proteins were removed by washing the protein-A-sepharose beads 3-4 times with 1ml extraction buffer and proteins were subsequently eluted from the pellet by boiling in sample buffer before separation by SDS-PAGE (section 2.2.37).

Chapter 3- Characterization and overexpression of RL1.

3.1. Characterization of the HSV-1 strain 17$^+$ neurovirulence locus RL1.

3.1.1. Introduction

In 1986 Chou and Roizman demonstrated that the 'a' sequence contains the promoter-regulatory region and transcription initiation sites of a gene located in the 'b' region of the HSV-1 strain F genome. From sequence studies and RNA analysis, they predicted that the gene encoded a protein of 358 amino acids in size. Using an antipeptide serum raised against a Pro-Ala-Thr (PAT) repeat, found 10 times in the HSV-1 strain F sequence, they identified a protein of M_r 43,500 in HSV-1 strain F, but not in HSV-2 or mock infected cells (Ackermann *et al.*, 1986). No equivalent open-reading frame (ORF) was present in the published sequence of HSV-1 strain 17$^+$ (Perry and McGeoch, 1988).

In 1990, Chou and Roizman resequenced the HSV-1 strain F ICP34.5 locus. Their data now predicted a protein of 263 amino acids in size, but more importantly, their republished sequence was almost identical to the published sequence of HSV-1 strain 17$^+$, with the only major difference being a 2bp insert in the strain 17$^+$ sequence relative to the strain F sequence. The insert resulted in 60% of the strain 17$^+$ sequence including the PAT repeat being out of frame which did not correlate with results which had clearly shown that HSV-1 strain 17$^+$ carries a neurovirulence determinant at the ICP34.5 locus (Thompson *et al.*, 1989; MacLean, A. *et al.*, 1991a). It was therefore crucial to resequence HSV-1 strain 17$^+$ in the relevant region in order (i) to resolve this anomaly and verify that HSV-1 strain 17$^+$ encoded an ORF for ICP34.5, and (ii) to show that lack of ICP34.5 expression alone results in loss of neurovirulence of HSV-1 strain 17$^+$.

3.1.2. Reinvestigation of the HSV-1 strain 17$^+$ RL1 sequence.

As a prerequisite to any further work on HSV-1 strain 17$^+$ ICP34.5, it was necessary to resequence the HSV-1 strain 17$^+$ RL1 gene. For this purpose, a 1.46kb AluI/RsaI fragment (np 125074-np 126530; Perry and McGeoch, 1988) encompassing the entire IR_L copy of

the RL1 ORF, was subcloned into the SmaI site of M13mp18 and single-stranded templates prepared as described in section 2.2.24. The source of this 1.46kb subfragment was HSV-1 strain 17^+ BamHI k, which had been previously cloned in the vector pAT153. This BamHI k clone had previously been demonstrated to restore neurovirulence to the non-neurovirulent variant 1716 (MacLean, A., *et al.*, 1991a). Sequence analysis was carried out using an oligonucleotide primer (supplied by Mr.A.Dolan) corresponding to HSV-1 residues 718 to 736 in the TR_L copy of the RL1 ORF (all further sequence numbers refer to the TR_L copy of RL1). The sequence of interest is shown in figure 3.1.. Because of the high G-C content of this region of the genome the gel shown was run by Mr.A.Dolan on a sequencing kit designed to maintain the gel at a constant temperature of 80^oC and so aid in the resolution of compressions and other structural abnormalities which could not be resolved on a normal gel.

Two differences from the published HSV-1 strain 17^+ sequence were found: (i) residue 818 was G instead of C and (ii) more importantly, residues 823 and 824 were absent. Correction of the HSV-1 strain 17^+ sequence to that of the newly sequenced clone at residues 818 to 824 opens the reading frame proposed by Chou and Roizman (1990) showing that HSV-1 strain 17^+ contains an ORF equivalent to that of HSV-1 strain F (Dolan *et al.*, 1992) (fig.3.2.). This ORF has been designated RL1 (McGeoch *et al.*, 1991) and was proposed to encode ICP34.5.

Having confirmed that HSV-1 strain 17^+ contains a gene, RL1, which encodes an ICP34.5 homologue, we wished to demonstrate the importance of this protein in neurovirulence following intracerebral inoculation of mice. To do this, it was decided to insert an in-frame stop-codon close to the initiating ATG of RL1 by site-directed mutagenesis using the M13 clone described above as a starting template. Marker transfer of this mutated RL1 into the HSV-1 strain 17^+ genome should result in a variant which is completely non-neurovirulent following intracerebral inoculation of mice. However, it soon became clear that site-directed mutagenesis was not the best approach to use, due to non-specific hybridization of

Figure 3.1. DNA sequence of the RL1 M13 clone.

An autoradiograph of the DNA sequence of HSV-1 strain 17^+ RL1, in the region where the expected frame-shift relative to the HSV-1 strain F sequence occurred. Sequences were obtained by the dideoxynucleotide sequencing method using T7 DNA polymerase. Products were fractionated by electrophoresis in a 6% polyacrylamide gel containing 9M-urea which was maintained at approximately 80^oC. The sequence refers to the TR_L copy of RL1. The sequence around the variable region described in the text and figure 3.2., is indicated.

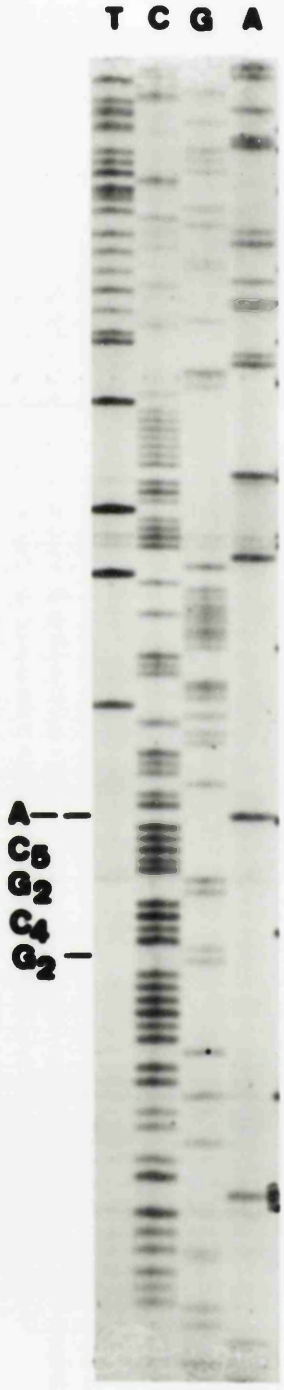
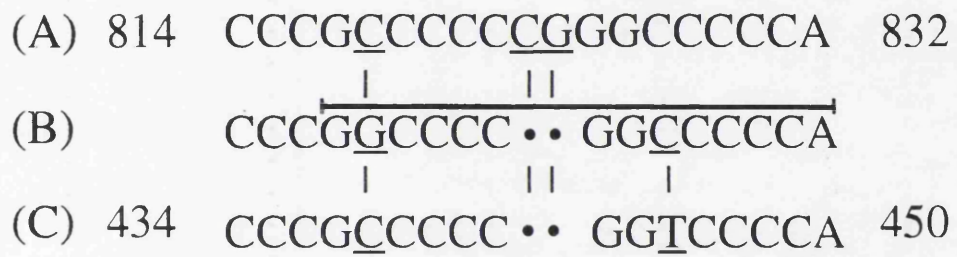

T C G A
A
C5
G2
C4
G2

Figure 3.2. Aligned versions of the 'frameshifting region' in the ICP34.5 coding sequence

(A) Original sequence for HSV-1 strain 17^+ (Perry and McGeoch, 1988), shown as residues 814 to 832 of the rightward 5' to 3' strand of genomic DNA. (B). Sequence of the strain 17^+ XhoI c and new BamHI k fragment clones. The bases marked in the sequence gel (fig.3.1.) are highlighted by a bar (⊢—⊣). The alteration of G-to-C at np 818 and more importantly the absence of CG at np 823 and 824 can be clearly seen in fig.3.1..(C) Strain F version of the ICP34.5 sequence from Chou and Roizman (1990) with their numbering. The dots in the second and third sequences represent introduced padding characters. Differences between successive sequences are underlined and marked by vertical lines.


(A) 814 CCCGCCCCCCGGGCCCCCA 832
(B) CCCGGCCCCC•• GGCCCCCA
(C) 434 CCCGCCCCC•• GGTCCCCA 450

the oligonucleotide to the HSV-1 sequences within the starting template, and an alternative approach, described below, was adopted.

3.1.3. Construction of the recombinant plasmid pEA10.

The 1.46kb AluI/RsaI sub-fragment of HSV-1 BamHI k, which had been cloned into M13 for sequencing was also cloned into the SmaI site of pGEM 3zf(-), to produce the recombinant plasmid pGEM34.5 (fig.3.3.).

The RL1 gene contains a unique NcoI restriction enzyme site which contains the initiating ATG of the gene and ~95bp downstream from this there is a unique BstEII restriction enzyme site. The plasmid, pGEM34.5, was digested with NcoI and BstEII (recognition sites for these enzymes are not found in pGEM 3zf(-)) and a synthetic double-stranded oligonucleotide was cloned in (fig.3.4.). This oligonucleotide had overhangs complementary to BstEII and NcoI restriction enzyme sites and was identical to the RL1 sequence with the exception of a 6bp insert which introduced an in-frame stop-codon in the ICP34.5 ORF leading to the potential production of a truncated protein of 4 amino acids. The insert also contained a recognition site for the restriction enzyme XbaI, so that recombinant plasmids and viruses could be easily identified.

To ensure that the stop-codon in pEA.10 was in the correct place a fragment of approximately 760bp in size which ran from the EcoRI site in the polylinker of pGEM 3zf(-) to the unique BstEII site at the 5' end of RL1 was cloned from pEA.10 (fig 3.3.). The fragment was blunt-ended and cloned into the SmaI site of M13mp18, and sequenced using the universal M13 primer (section 2.2.25.) to confirm the position of the stop-codon relative to the initiating ATG of the gene. The sequence of the plasmid pEA.10 in the region around the initiating ATG of RL1 is shown in fig.3.5.. The fragment was inserted into the vector in an orientation such that the BstEII site is adjacent to the M13 universal primer hybridization site. Thus the non-coding strand of RL1 is read. The positions of the 6bp insert and initiating ATG (CAT as the non-coding strand is being read) of the gene are

Figure 3.3. Construction of pGEM34.5

A 1.46kb AluI/RsaI fragment (np 125074-np 126530) was isolated from the plasmid pBamk. This was cloned into the SmaI restriction enzyme site, in the multicloning site of pGEM 3zf(-), creating the plasmid pGEM34.5.

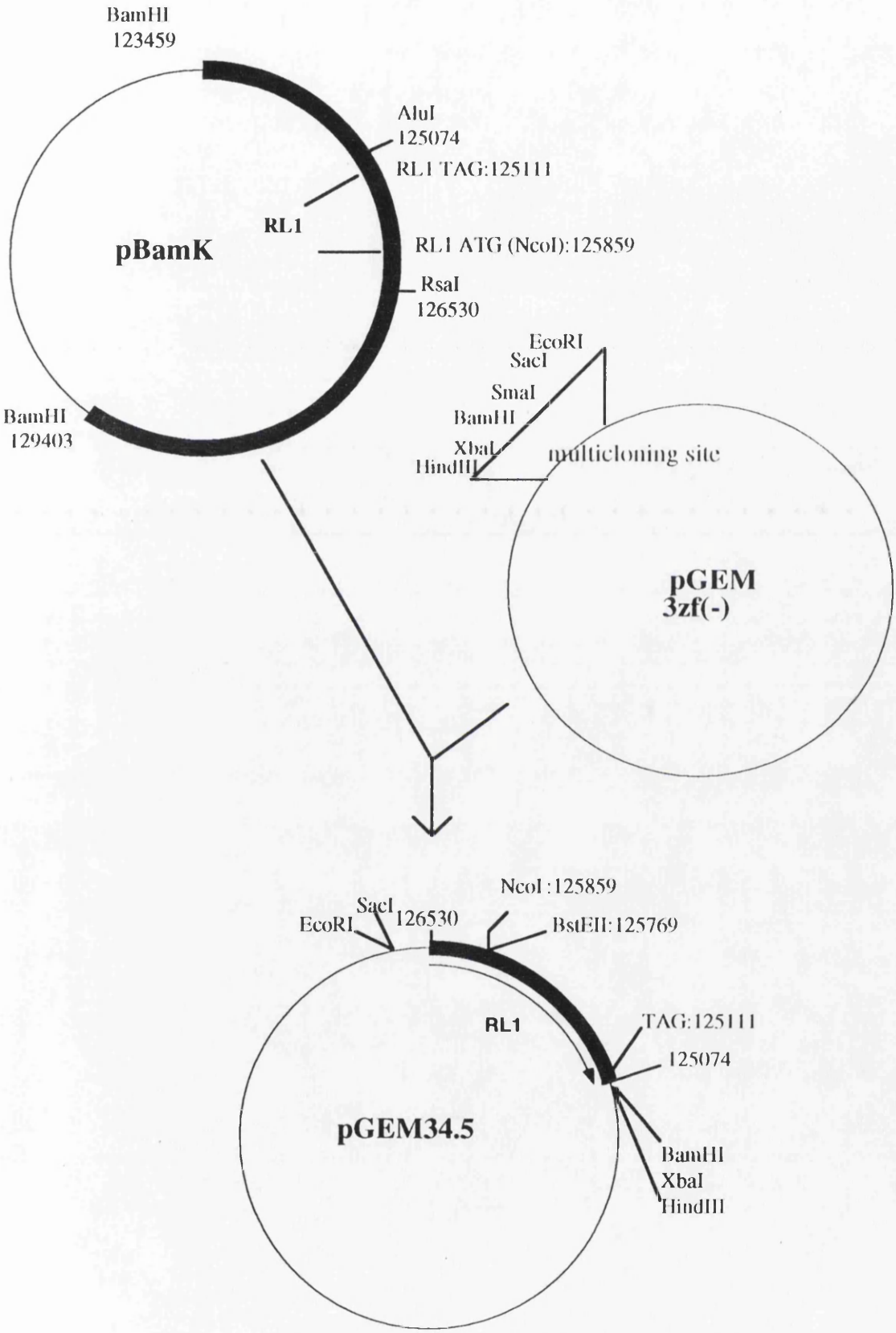

BamHI
123459
AluI
125074
RL1 TAG:125111
RL1
pBamK
RL1 ATG (NcoI):125859
RsaI
126530
BamHI
129403
EcoRI
SacI
SmaI
BamHI
XbaI
HindIII
multicloning site
pGEM
3zf(-)
NcoI :125859
SacI
EcoRI
126530
BstEII:125769
RL1
TAG:125111
125074
pGEM34.5
BamHI
XbaI
HindIII

Figure 3.4. Sequence of the synthetic double-stranded oligonucleotide used to construct pEA.10.

pGEM34.5 was digested with NcoI and BstEII and a synthetic double-stranded oligonucleotide was cloned in. This oligonucleotide had overhangs complementary to BstEII and NcoI restriction enzyme sites as indicated and was identical to the RL1 sequence except that it contained a 6bp insert (underlined in bold) which introduced an in-frame stop-codon in the RL1 ORF and contained a recognition site for the enzyme XbaI (T'CTAG_A). The initiating ATG and inserted stop-codon are marked with a bar (—).

NcoI 'sticky ends'

5' C-ATG-GCC-CGC-CTC-TAG-AGC-CGC-CGC-CAT-CGC-GGC-CCC-CGC-CGC-

3' CGG-GCG-GAG-ATC-TCG-GCG-GGG-GTA-GCG-CCG-GGG-GCG-GCG-

CCC-CGG-CCG-CCC-GGG-CCC-ACG-GGC-GCC-GTC-CCA-ACC-GCA-CAG-TCC-

GGG-GCC-GGC-GGG-CCC-GGG-TGC-CCG-CGG-CAG-GGT-TGG-CGT-GTC-AGG-

CAG 3' (91 mer)

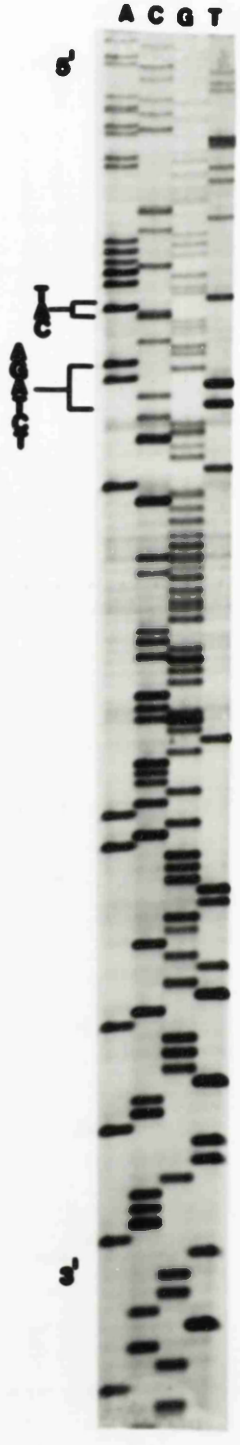
GAC-CAT-TG 5' (92 mer)

BstEII 'sticky ends'

Figure 3.5. DNA sequence of pEA.10 in the region of the initiating ATG of RL1.

A BstEII/EcoRI subfragment of pEA.10 containing the 92bp replaced sequence was purified, blunt-ended and cloned into the SmaI site of M13mp18. It was then sequenced using a M13 universal primer to confirm the position of the 6bp insert relative to the initiating ATG (CAT as the non-coding strand is being read) of the gene. The entire sequence of the 92bp oligonucleotide was identical to the wild-type sequence with the exception of the 6bp insert. This gel shows the sequence of the non-coding strand.

The sequence of the non-coding strand is read here, thus the 3' end of RL1 is at the bottom of the gel and the 5' end at the top.


A C G T
5'
3'

indicated, confirming correct insertion of the stop codon. The remainder of the gel shows that the oligonucleotide insert is identical to the wild-type sequence which it has replaced.

3.1.4. Construction of a HSV-1 strain 17$^+$ variant, 1771, containing a stop codon at the start of RL1.

The plasmid, pEA.10, was linearised for transfection by digestion with the restriction enzyme BamHI which cuts once in the multi-cloning site of the vector and was transfected onto BHK21/C13 cells with intact HSV-1 strain 17$^+$ DNA as described in section 2.2.17.. One hundred and forty-four single plaques were isolated and analysed by preparation of ^{32}P labelled viral DNA (section 2.2.19.). DNA from each plaque isolate was screened by digestion with the restriction enzyme XbaI. Recombinant virus will contain 2 extra XbaI restriction enzyme sites, one 511bp from the L terminus and the second 511bp from the L-S junction. (fig.3.6.). This will result in digestion of the 0.5M g and d bands to 1M bands, g' and d', 511bp smaller than the respective wild-type bands and 2, 0.5M bands each of 511bp. The corresponding L-S junction fragments disappear being digested into the 1M bands g' and d' and 2 new equivalent 0.5M bands a' and b', [S]+ 511bp, running between c and e (see fig.3.7.). Due to the large size of the fragments, these alterations, with the exception of the loss of a will not significantly alter the XbaI profile. Because mixed plaques would not result in the loss of the a band it became obvious that the use of XbaI alone was not the best choice for analysis of potential recombinants. As no positive clones were identified from the first 144 plaque isolates , the transfection was repeated. This time DNA from plaque isolates was double-digested with XbaI and BamHI where recombinant virus will have a more obvious restriction enzyme profile.

Digestion of HSV-1 strain 17$^+$ with BamHI and XbaI gives essentially a BamHI restriction enzyme profile with the following minor alterations: BamHI c is cleaved into 2 fragments, 1 of 7728bp (e^o)which runs just below BamHI g and another of 1185bp which runs between BamHI c' and d'. BamHI i is cleaved into 2 fragments, 1 of 6059bp (i^o)which runs between j and k and another of 581bp which runs between j' and k'. In

Figure 3.6. Position of the XbaI restriction enzyme sites in the genome of 1771.

This figure shows the HSV-1 strain 17^+ genome in the prototype orientation (A). The wild-type XbaI restriction enzyme sites are marked and the two additional sites which have been introduced into the 1771 genome are indicated (X). (B) HpaI map of HSV-1. (C) BamHI map of HSV-1. The dotted vertical line indicates the position of the L-S junction.

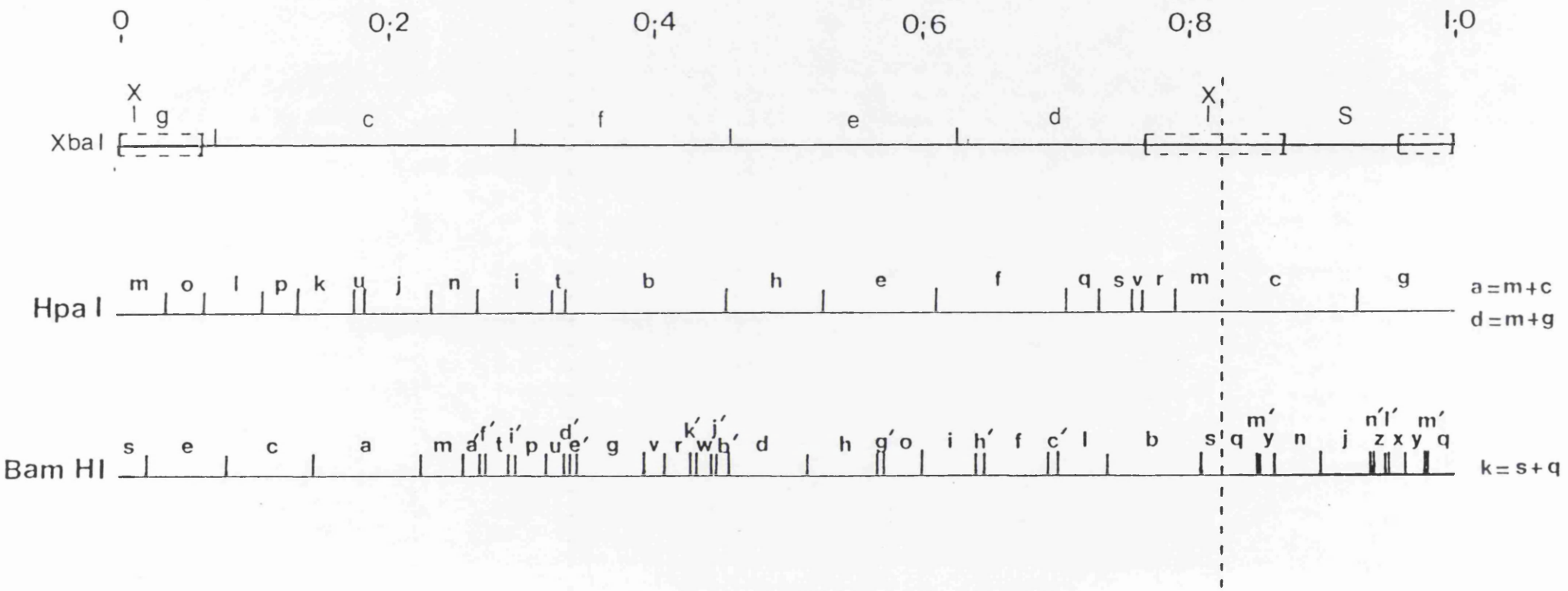
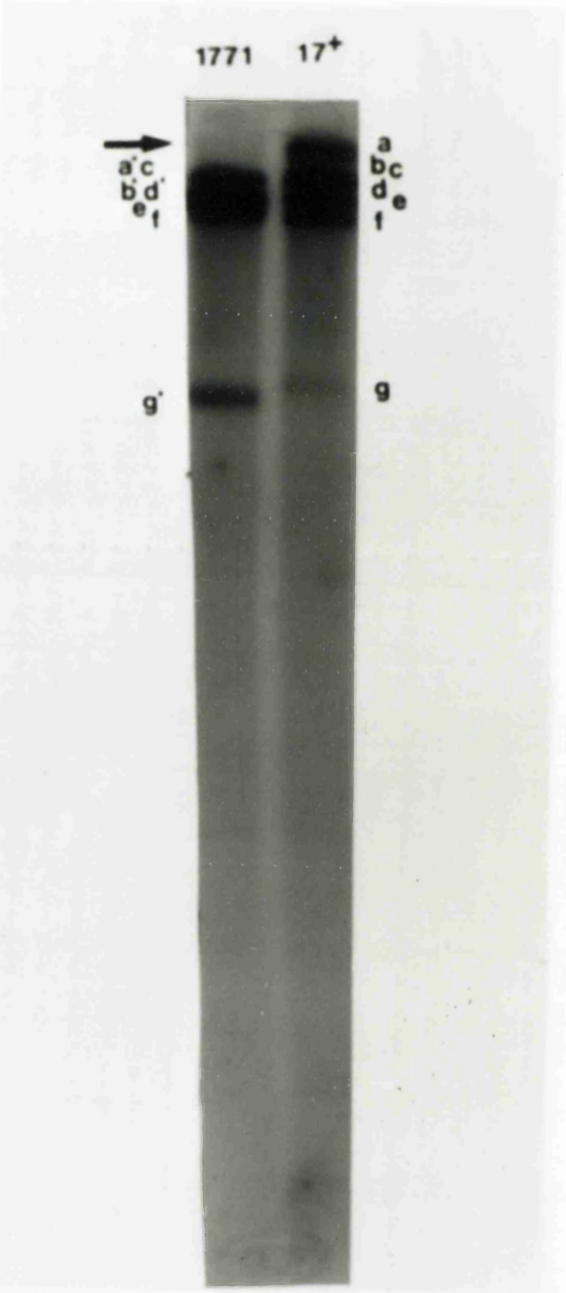

0
0;2
0;4
0;6
0;8
1;0
X
l
g
c
f
e
d
X
l
S
Xba I
m
o
l
p
k
u
j
n
i
t
b
h
e
f
q
s
v
r
m
c
g
Hpa I
a=m+c
d=m+g
s
e
c
a
m
a′
f′
t
i′
p
u
d′
e′
g
v
r
k′
j′
w
h′
d
h
g′
o
i
h′
f
c′
l
b
s
q
m′
y
n
j
n′
l′
z
x
y
m′
q
Bam HI
k=s+q

Figure 3.7. XbaI restriction enzyme profile of 1771.

Autoradiograph of XbaI DNA digest of HSV-1 strain 17^+ and variant 1771 with 2 extra XbaI sites 511bp from the L terminus and L-S junction. Novel bands are designated by the letter of the band from which they were derived plus a prime symbol. Lanes are labelled at the top of each track.

The most obvious difference between the 2 digests is the loss of XbaI a in 1771 (indicated by an arrow).


1771
17+
a'
c'
b'
d'
e'
f
g'
a
b
c
d
e
f
g

addition BamHI i' and Bam HI b' are cleaved into 2 fragments (107+555bp and 921+392bp respectively), but these alterations can not be seen on the 0.8% agarose gels used.

Digestion of a recombinant containing the additional XbaI sites at np 511 and np 125850 with BamHI and XbaI gives a similar profile, except that, because of the additional XbaI site, BamHI s is digested into 2 bands of 511bp and 2391bp (s'). The s containing L-S junction BamHI k is digested to give 2 fragments, one which is also 2391bp in size and the other, the new L-S junction, which is 3598bp (q') in size and migrates above BamHI q. One such isolate was identified (fig.3.8.) and its structure confirmed by Southern blotting a BamHI/XbaI digest, using random primed BamHI k as a probe (fig.3.9.). Confirmation of the correct XbaI profile (see above) was also determined (fig. 3.7.)

This isolate, which was designated 1771 was plaque-purified a further 3 times before growing a stock

3.1.5. Western blotting confirms that 1771 does not produce ICP34.5.

Confirmation that 1771 does not produce ICP34.5 was provided by Western blotting (fig 3.10.).

Three wild-type HSV-1 strain 17^+ and 3 1771 plaque isolates were isolated at random, protein extracts prepared from infections with each of the plaques and Western blotted (section 2.2.38.). ICP34.5 production was assayed using the polyclonal antiserum against ICP34.5 (section 3.3.5.) at a 1/15 dilution. ICP34.5 was detected in wild-type HSV-1 strain 17^+ infected cell extracts, whereas ICP34.5 was absent in cells which had been infected with 1771. This confirmed the sequencing data that the stop-codon had been inserted in-frame in the RL1 ORF.

3.1.6. Neurovirulence of 1771

The neurovirulence of 1771 was determined by estimation of its LD_{50} value in BALB/c mice compared to the wild-type parental HSV-1 strain 17^+ and the RL1 deletion mutant

Figure 3.8. BamHI/XbaI restriction enzyme profile of 1771.

Autoradiograph of BamHI/XbaI digest of viral DNA. 1771 contains two novel XbaI sites 511bp from the L terminus and L-S junction. Missing bands are designated (*). Novel bands are indicated (▶) and are designated by the letter of the band from which they were derived plus a prime symbol (‘). The largest bands produced following digestion of BamHI e and i with XbaI are designated e^{o} and i^{o} respectively. Lanes are labelled at the top of each track.

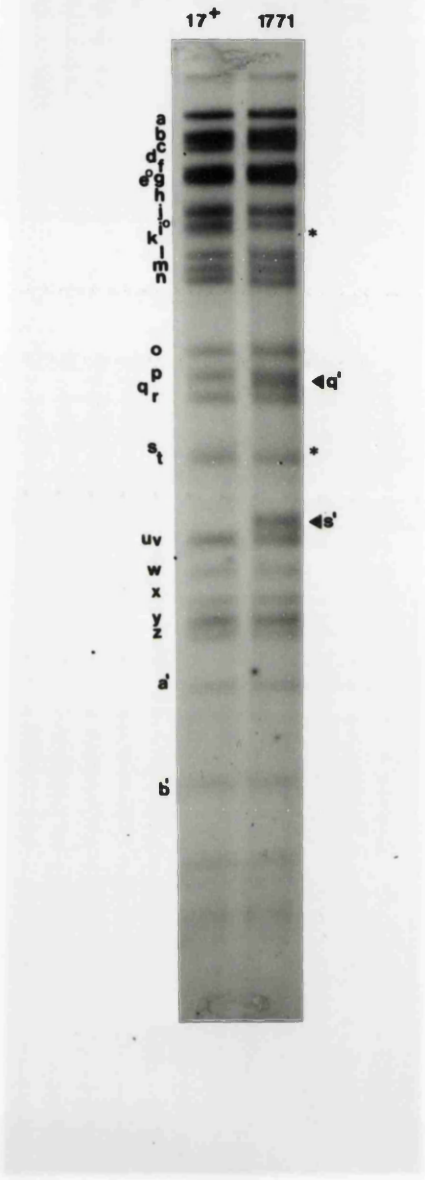
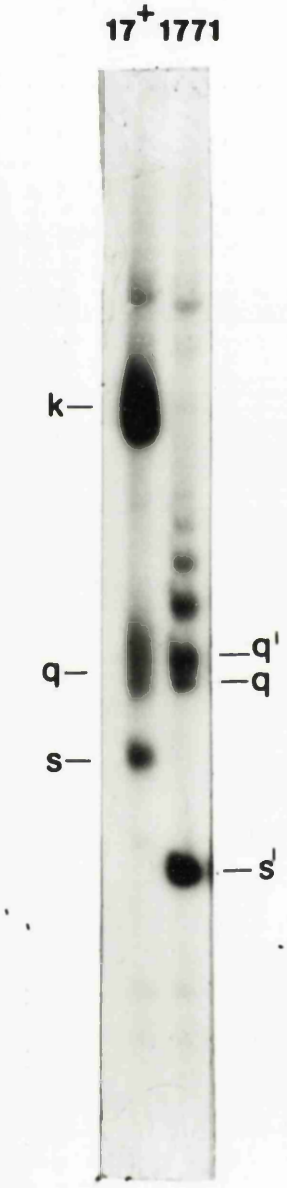

17+
1771
q'
s'
*
*

Figure 3.9. Southern blot of 1771 using wild-type HSV-1 strain 17^+ BamHI k as a probe.

Autoradiograph of a Southern blot in which wild-type HSV-1 strain 17^+ BamHI k was labelled by random priming and used as a probe. The DNA was digested with BamHI and XbaI and the fragments separated on a 0.8% agarose gel. Lanes are labelled at the top of each track.

Digestion of 17^+ with BamHI/XbaI gives an identical profile to that seen with BamHI alone, namely detection of k, q and s. On digestion of 1771 with BamHI and XbaI, BamHI k disappears and two novel bands appear; one migrates 511bp above BamHI q (q') and the other migrates 511bp below s (s'). In addition BamHI s is cleaved into 2 fragments, one of 511bp, which cannot be detected on the percentage gel used and the other s'.

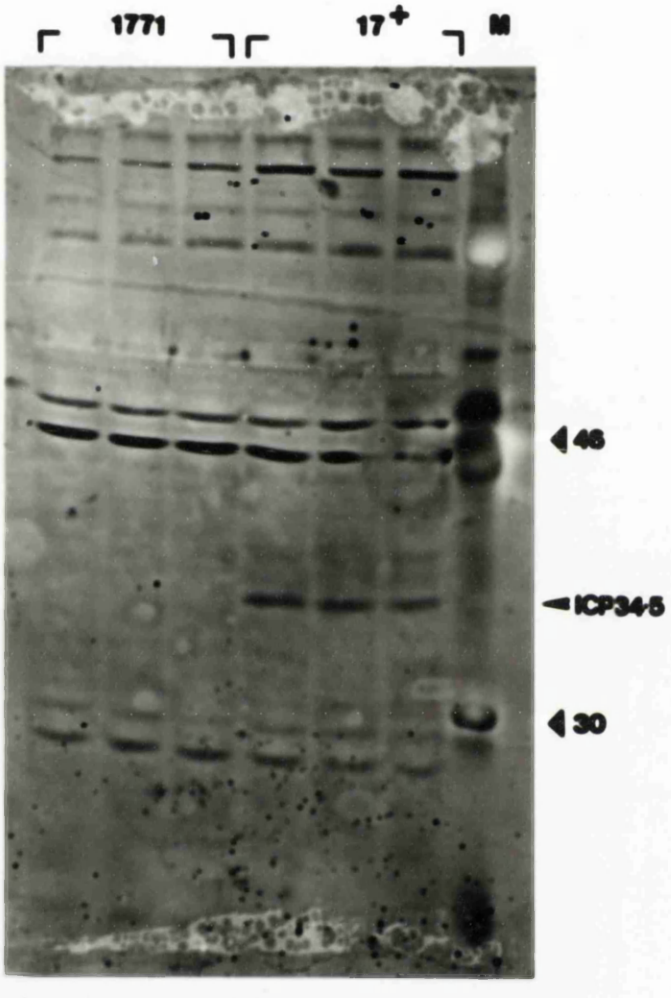
The presence of additional 'a' sequences in the novel L-S junction leads to the laddering above q'. This is a consistent finding with L-S junction fragments (Davison and Wilkie,1981). The background band running above BamHI k in both 17^+ and 1771 is consistently seen when probing BamHI digests with BamHI k (MacLean, A., *et al.*, 1991).


17+1771
k—
q—
s—
—q'
—q
—s'

3.10. Western blot analysis of 1771.

Detection of ICP34.5 by Western blot analysis, using the polyclonal antiserum (at a dilution of 1/15) which had been raised against the *E.coli* -expressed ICP34.5. Molecular weight markers in (kDa) and the position of ICP34.5 are shown on the right hand side. Lanes are labelled at the top of the gel.

In HSV-1 strain 17^{+}, ICP34.5 corresponds to a band of 37kDa in size.


1771
17+
M
46
ICP34.5
30

1716. Twenty-five ul aliquots of different doses of strain 17^+, 1771 and 1716 were inoculated into the left cerebral hemisphere of 3-week old BALB/c mice. Deaths from encephalitis were scored up to 21 days post inoculation and the results are shown in table 3.1. The wild-type parental HSV-1 strain 17^+ was inoculated at doses of 10^1 and 10^2 p.f.u./mouse. At a dose of 10^2 no mice survived, whereas, at a dose of 10^1, there were 2 surviving mice giving a LD_{50} value of 10 p.f.u./mouse. The RL1 deletion variant 1716 was inoculated at doses of 10^5 and 10^6 p.f.u./animal. As anticipated, (MacLean, A. *et al.*, 1991a) no animals died at any of the doses, giving a LD_{50} value of $>10^6$ p.f.u./animal. The recombinant virus 1771 did not produce ICP34.5, as demonstrated by Western blotting (section 3.1.5.), it was therefore anticipated that this mutant would be essentially non-neurovirulent compared to the wild-type virus, HSV-1 strain 17^+ and was thus inoculated at doses of 10^5 and 10^6 p.f.u./mouse. At these doses no animals died, giving a LD_{50} value of $>10^6$ p.f.u./animal ,the same as 1716.

3.1.7. Growth of 1771 *in vivo.*

The HSV-1 strain 17^+ variant 1716, and the HSV-2 strain HG52 variant JH2604 have been shown to be non-neurovirulent following intracerebral inoculation of mice, due to an apparent failure to replicate in mouse brain (Taha *et al.*, 1989a, MacLean, A. *et al.*, 1991a) and, in the case of JH2604, failure to produce necrotizing encephalitis (Taha *et al.*, 1990). To determine if this inability to replicate was due solely to a failure to produce ICP34.5, the *in vivo* growth properties of 1771 were examined. Samples of strain 17^+ (10^2 p.f.u.) and 1771 (10^5 p.f.u.), were separately injected into the left cerebral hemisphere of 3-week old BALB/c mice. At various times post-inoculation, two mice per virus were killed, their brains removed, homogenized and sonicated in PBS/calf. The final volume was recorded, before storing at -70°C. The virus titre in each brain was calculated separately and the mean obtained. On day 5, only 1 mouse inoculated with HSV-1 strain 17^+ was alive. Thus, the HSV-1 strain 17 day 5 timepoint represents the titre of virus from only 1 animal. The final titres (fig.3.11.) were calculated as p.f.u./mouse. The parental HSV-1 strain 17^+

Table 3.1. Neurovirulence of 1771 following intracerebral inoculation of 3-week old BALB/c mice.

virus \ dose	10^1	10^2	10^3	10^4	10^5	10^6	LD50 (p.f.u./ mouse)
17 +	2/4 *	4/4	ND	ND	ND	ND	10
1716	ND	ND	ND	ND	0/4	0/4	$>10^6$
1771	ND	ND	ND	ND	0/4	0/4	$>10^6$

*no. of deaths/no. injected
ND= not done.

Figure 3.11. *In vivo* growth of 1771

The replication kinetics of HSV-1 strain 17^+ and 1771 were examined *in vivo*..Three week old BALB/c mice were inoculated intracerebrally into the left cerebral hemisphere with 10^2 (HSV-1 strain 17^+) or 10^5 (1771) p.f.u./mouse in 25ul PBS/calf. At the times indicated post inoculation 2 mice/timepoint were sacrificed (except for HSV-1 strain 17^+ at day 5- where only 1 mouse had survived) their brains removed, homogenised and the resulting homogenate titrated for virus on BHK21/C13 cells. Titres are expressed as p.f.u./mouse. Each timepoint represents the mean titre of two animals (except HSV-1 strain 17^+ at day 5, which represents only 1 animal).

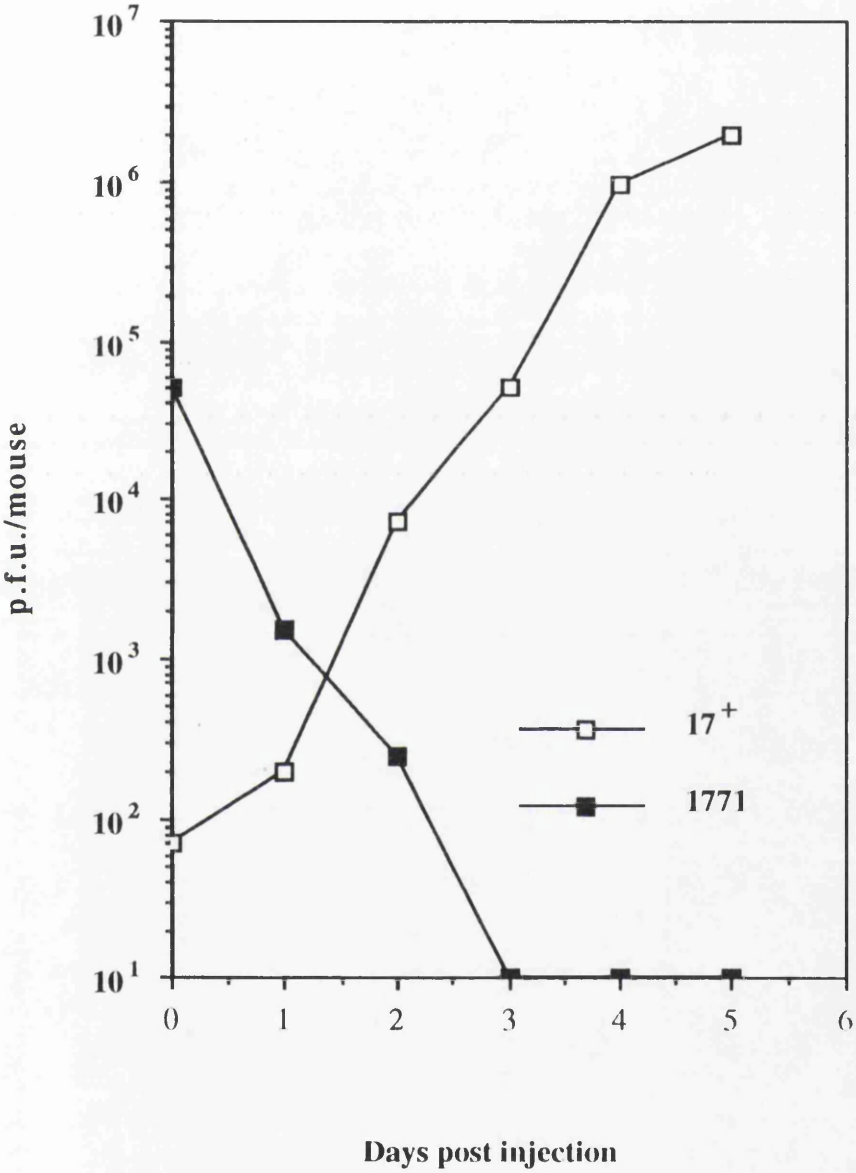

p.f.u./mouse
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
1
2
3
4
5
6
17+
1771
Days post injection

Figure 3.12 (a). One-cycle growth curve of 1771

The one-cycle growth characteristics of 1771 were compared to wild-type strain 17^+ on BHK21/C13 cells. Cells were infected at a multiplicity of 10 p.f.u./cell, and following absorption at 37°C for 1hr, the monolayers were washed twice with PBS/calf , overlaid with ETC10 and incubation continued at 37°C. Plates were harvested at 0, 2, 4, 6, 8, 12 and 24hrs post-infection and titrated on BHK21/C13 cells.

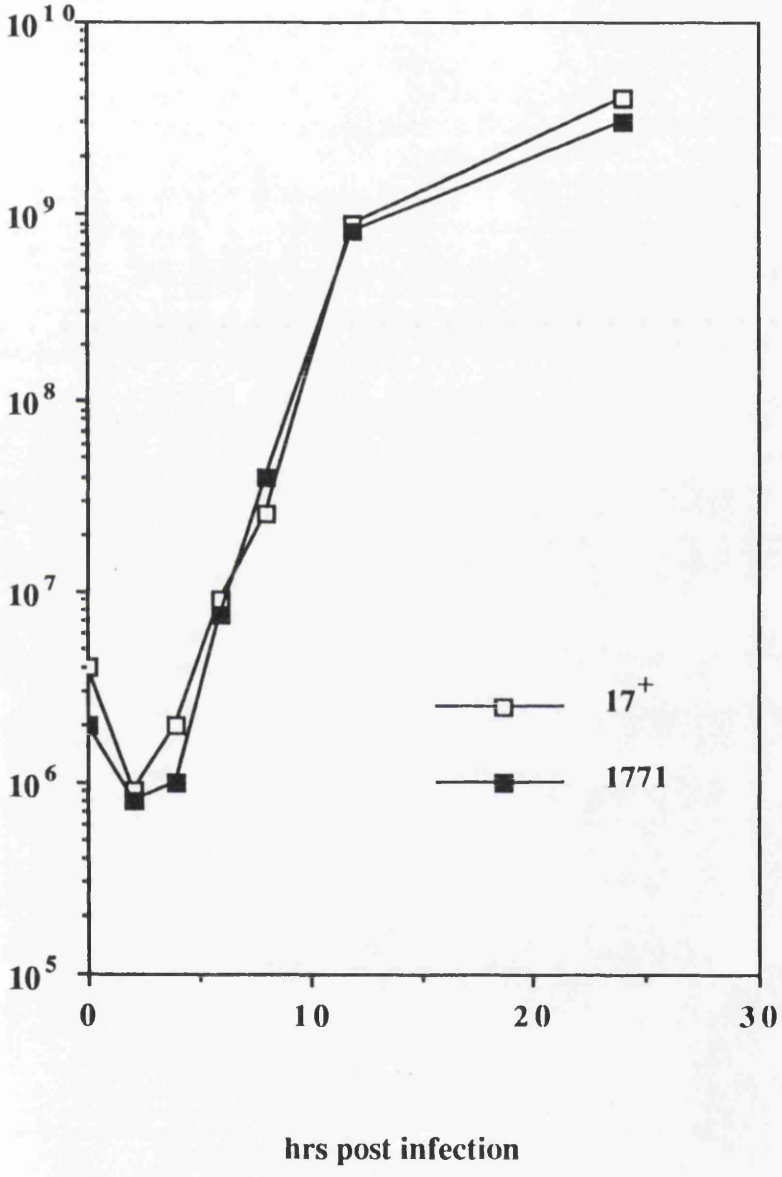
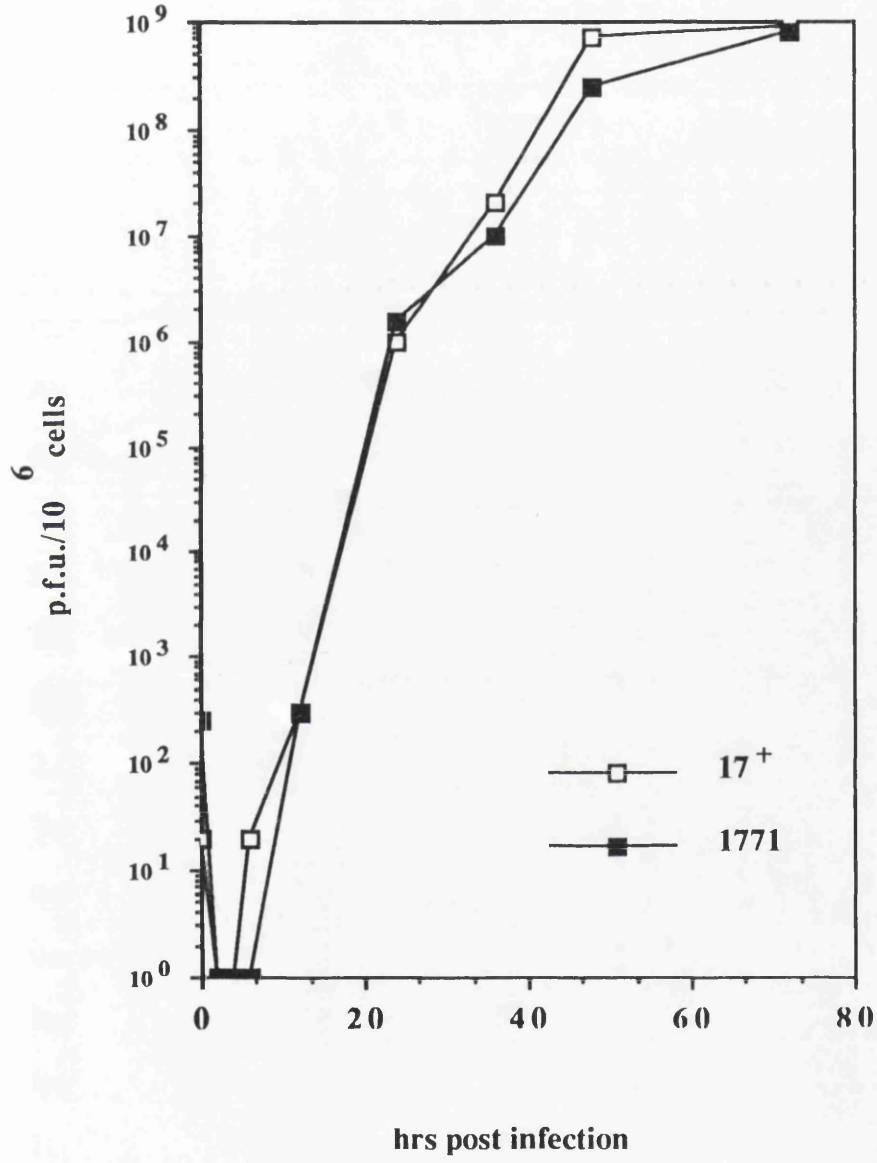

p.f.u./10 cells
10 10
10 9
10 8
10 7
10 6
10 5
0
10
20
30
17+
1771
hrs post infection

Figure 3 12 (b). Multi-cycle growth curve of 1771.

The multi-cycle growth characteristics of 1771 were compared to the wild-type parental strain 17^{+} on BHK21/C13 cells. Cells were infected at a multiplicity of 0.001 p.f.u./cell, and following absorption at 37°C for 1hr, the monolayers were washed twice with PBS/calf and incubation continued at 37°C. Plates were harvested at 0, 2, 4, 6, 12, 24, 36, 48 and 72 hrs post-infection and titrated on BHK21/C13 cells.


p.f.u./10^6 cells
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
20
40
60
80
17+
1771
hrs post infection

titre rose rapidly following intracerebral inoculation, reaching a titre of $1x10^6$ p.f.u./animal by day 4-5. In contrast, no replication was detectable in 1771 inoculated animals and the input virus was rapidly cleared until by day 3 post-inoculation there was no detectable virus (<10p.f.u.).

3.1.8. Growth of 1771 *in vitro*.

Analysis of the *in vitro* growth characteristics of 1771 relative to wild-type HSV-1 strain 17^+ was carried out on BHK21/C13 cells. Cells were infected with either 10 p.f.u./cell (one-cycle growth experiment), or 0.001 p.f.u./cell (multi-cycle growth experiment) of HSV-1 strain 17^+ and 1771. At various times post-infection plates were harvested and virus titres calculated by titration on BHK21/C13 cells.

As can be seen from figure 3.12., 1771 grew identically to the wild-type virus in both one-cycle (fig.3.12a.) and multi-cycle growth experiments (fig.3.12b.).

3.1.9. Marker rescue of the lesion in 1771.

To show that neurovirulence could be restored to 1771 by rescuing back the wild-type RL1 gene, rescuants were produced by the method of *in vivo* marker rescue (section 2.2.17.)

To produce a revertant of 1771 (1771R) 1ug 1771 DNA was transfected onto BHK21/C13 cells with 1-, 10- and 100- fold molar ratios of the wild-type BamHI k fragment, containing RL1. After 2-3 days, the plate in which c.p.e. was most extensive was harvested and 25ul of 10^{-1} and 10^{-2} dilutions (approximately 10^4 to 10^5 p.f.u.) injected intracerebrally into groups of 4, 3 week old BALB/c mice. Four to six days post-inoculation, most of the mice began to show signs of neurological disorder (ruffled fur, hunched back, tendency to sit alone, lack of interest in food). At this point they were killed to prevent any further unnecessary suffering and their brains removed. The brains from 2 mice were homogenised in PBS/calf, titrated on BHK21/C13 cells and single plaques isolated. Small scale stocks of virus were grown in Linbro wells and ^{32}P labelled DNA

prepared and analysed as has been previously described (section 2.2.19.). One isolate with a wild-type restriction enzyme profile was chosen (data not shown). Three further rounds of plaque purification took place before growing up a virus stock. A parallel control experiment was carried out using virus from a transfection carried out with 1771 DNA alone. In this case no mice died.

3.2 Construction of a HSV-1 strain F ICP34.5 deletion variant.

3.2.1. Introduction.

In an attempt to detect ICP34.5 from HSV-1 strain 17^+ infected cells, antisera were raised against peptides corresponding to different regions of the predicted RL1 open reading frame encoded protein (fig 3.13.). Until recently (MacKay *et al.*, 1993), none of these peptide antisera were successful in identifying ICP34.5 in HSV-1 strain 17^+ infected cell extracts , although one directed against a tenmer of the PAT trimer repeat strongly recognised ICP34.5 in HSV-1 strain F infected cell extracts. To confirm the specificity of this antiserum, to show that loss of ICP34.5 correlated with a loss of neurovirulence and to help study cellular localization of ICP34.5, we decided to construct a HSV-1 strain F mutant with a deletion in RL1.This mutant will subsequently be referred to as F11.

3.2.2. Construction of F11.

Construction and characterization of the HSV-1 strain 17^+ RL1 deletion mutant 1716 has been previously described (MacLean, A., *et al.*, 1991a). Dideoxynucleotide sequence analysis of this mutant revealed that it was deleted by 759bp in both copies of the long repeat region of the genome (fig.3.14.). The deletion removes one complete copy of the 18bp DR1 element of the 'a' sequence and terminates 1105bp upstream from the 5' end of IE gene 1. The novel L-S junction fragment BamHI k of 1716 has previously been cloned into pGEM 3zf(-) using standard procedures (Maniatis *et al.*, 1982) and was supplied by Dr.A. MacLean. The RL1 deletion from 1716 was marker transferred to HSV-1 strain F by

3.13. Antisera raised against peptides from HSV-1 RL1.

Antisera were raised against synthetic oligopeptides from 7 different regions of the predicted RL1 encoded protein in an attempt to detect HSV-1 strain 17^+ ICP34.5. The amino acid sequences of these peptides are listed in the table. Their position in relation to the RL1 ORF are indicated in the diagram underneath. Only one, which was raised against a tenmer of the PAT repeat detected ICP34.5 in HSV-1 strain F and HSV-1 strain 17^+ infected cell extracts (MacKay *et al.*, 1993). 1B (Br.212) and 2A (L.211) also recognised *E.coli* -expressed ICP34.5 (fig.3.23) although they did not detect ICP34.5 in HSV-1 infected cell extracts. No sera recognised an ICP34.5 homologue in HSV-2 strain HG52.

Antipeptide sera	Sequence	Sera tested and result [a]	
		HSV-1 strain F	HSV-1 strain 17
1A	YAARLARRGSWARE	-	-
1B	(YAARLARRGSWARE)$_8$K$_7$A	-	-
2A	YEAVIGPCLGPEAR	-	-
2B	(YEAVIGPCLGPEAR)$_8$K$_7$A	-	-
3A[d]	[(PAT)$_7$]$_8$K$_7$A	-	-
3B[e]	[PAT)$_7$]$_8$K$_7$A	+/- [b]	-
4	(ATP)$_{10}$C	+	+ [c]
5	(ARARALARGAGPANSV)$_8$K$_7$A	-	-
6	(MARRRRHRGPRRRPRP)$_8$K$_7$A	-	ND
7	(PGPTGAVPTAQSQVT)$_8$K$_7$A	-	ND

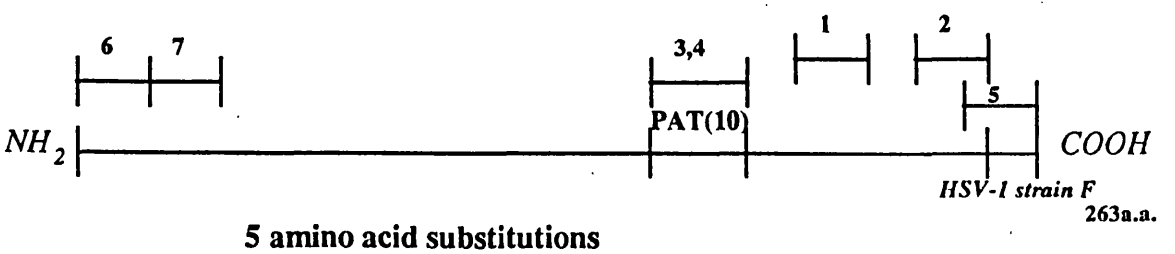
a) tested in Western blots and immunoprecipitation.

b) faint, the better of the two sera works at 1:10 dilution on Western blot only.

c) these antisera recognise HSV-1 strain 17$^+$ ICP34.5 by Western blot only.

d) these antisera was raised in Half Sandylop rabbits.

e) these antisera were raised in New Zealand White rabbits.



5 amino acid substitutions

conserved region

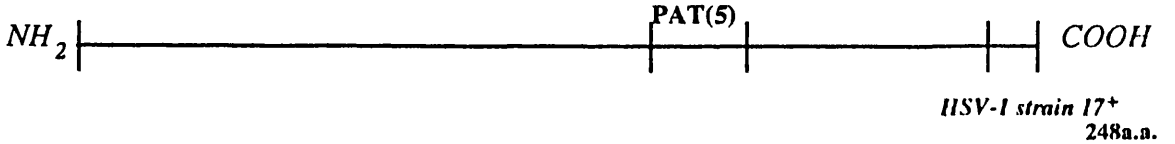
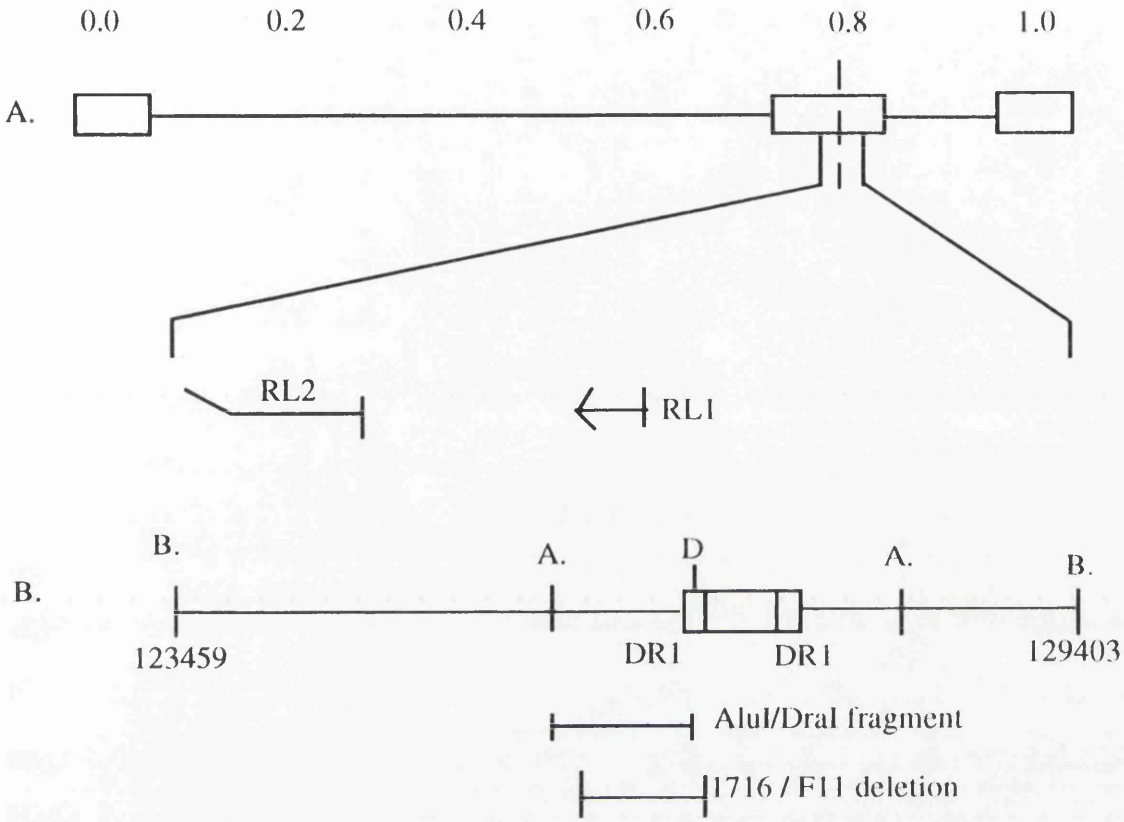


Figure 3.14. Construction of F11, 1716a and F11a.

(A) HSV genome in the inverted long (I_L) orientation. (B) An enlargement of Bam HI k. The DR1 element of the 'a' sequence, RL1, RL2, AluI (A) BamHI (B) and DraI (D) restriction enzyme sites are marked. The position of the AluI/DraI fragment used as a probe in fig.3.18 is indicated below the line, as is the deletion in 1716/F11. (C) The 1716/F11 deletions were rescued using Xba I g. The dashed line is used to indicate that the Xba I g extends beyond the Bam HI site shown.


0.0
0.2
0.4
0.6
0.8
1.0
A.
RL2
RL1
B.
B.
A.
D
A.
B.
123459
DR1
DR1
129403
AluI/DraI fragment
1716 / F11 deletion
C.
10.6 kb
strain F / strain 17+
XbaI g

co-transfection of intact HSV-1 strain F DNA with 1716 BamHI k, linearized by digestion with XbaI which cuts in the vector multi-cloning site. Single plaques were isolated and positive clones identified by restriction enzyme analysis of ^{32}P labelled viral DNA. Plaque isolates were first screened by looking for a deletion in the terminal fragment XbaI g (fig.3.6., 3.15). The deletions in the much larger terminal fragment XbaI d and L-S junction fragments a and b are not obvious. Six positive clones were identified one of which was designated the prototype F11.. Confirmation that the deletion in F11 was in the correct region of XbaI g was provided by fine mapping with several other restriction enzymes. For later analysis 2 wild-type HSV-1 strain F plaque isolates were also chosen and analysed by restriction endonuclease digestion to show they had no obvious genomic alterations.

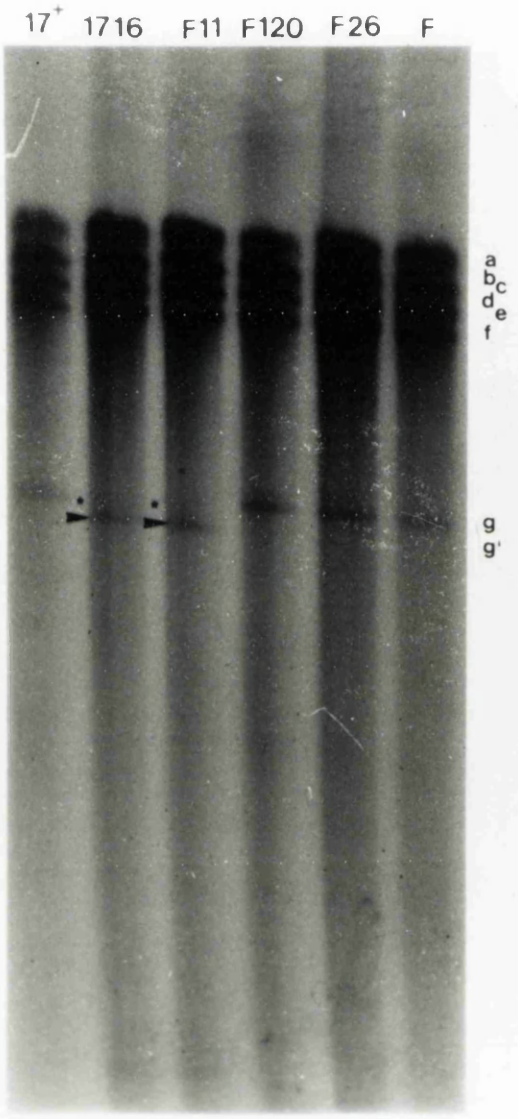
Figure 3.16. shows the HpaI restriction enzyme profile of F11. HpaI l is missing and a novel band is observed, running just below HpaI m. Because of site alterations HpaI l of HSV-1 strain F corresponds to HpaI m of HSV-1 strain 17^+. Again the deletions in the L-S junction fragments a and d are not obvious. The size of the deletion in HpaI l of F11 and HpaI m of 1716 as expected is the same.

The BamHI restriction enzyme profile of F11 is shown in fig.3.17. In this case the most noticable difference between the mutant and wild-type virus is the loss of the L-S junction fragment BamHI k and the appearance of a novel band corresponding to a deleted k which runs just above BamHI l. Bands running just above BamHI k and the novel k are due to the presence of additional 'a' sequences. The L terminal fragment BamHI s is also missing and is replaced by a novel band corresponding to deleted s which co-migrates with the u/v doublet. BamHI s and k of HSV-1 strain F have a slightly higher M_r than those of HSV-1 strain 17^+. (Refer to fig.3.6. for HpaI and BamHI restriction enzyme maps of HSV-1 strain 17^+).

F11 was plaque purified a further 3 times before growing up a virus stock. The two wild-type isolates - F26 and F120 - were also plaque purified 3 times prior to preparation of virus stocks.

Figure 3.15. Xba I restriction enzyme profile of F11.

Autoradiograph of Xba I digest of viral DNA. F26 and F120 are plaque-purified wild-type strain F isolates; F11 is the strain F ICP34.5 deletion mutant. Missing bands are designated (*). Novel bands are indicated by an arrowhead, and are designated by the letter of the band from which they were derived plus a prime symbol ('). Lanes are labelled at the top of each track.


17+
1716
F11
F120
F26
F
a
b
c
d
e
f
g
g'

3.16. HpaI restriction enzyme profile of F11.

Autoradiograph of HpaI digest of viral DNA. F26 and F120 are plaque-purified wild-type isolates; F11 is the strain F ICP34.5 deletion variant. Missing bands are indicated by an arrowhead. Novel bands are marked (●). HSV-1 strain F bands are labelled on the left-hand side and HSV-1 strain 17^+ bands are labelled on the right-hand side. Lanes are labelled at the top of each track.

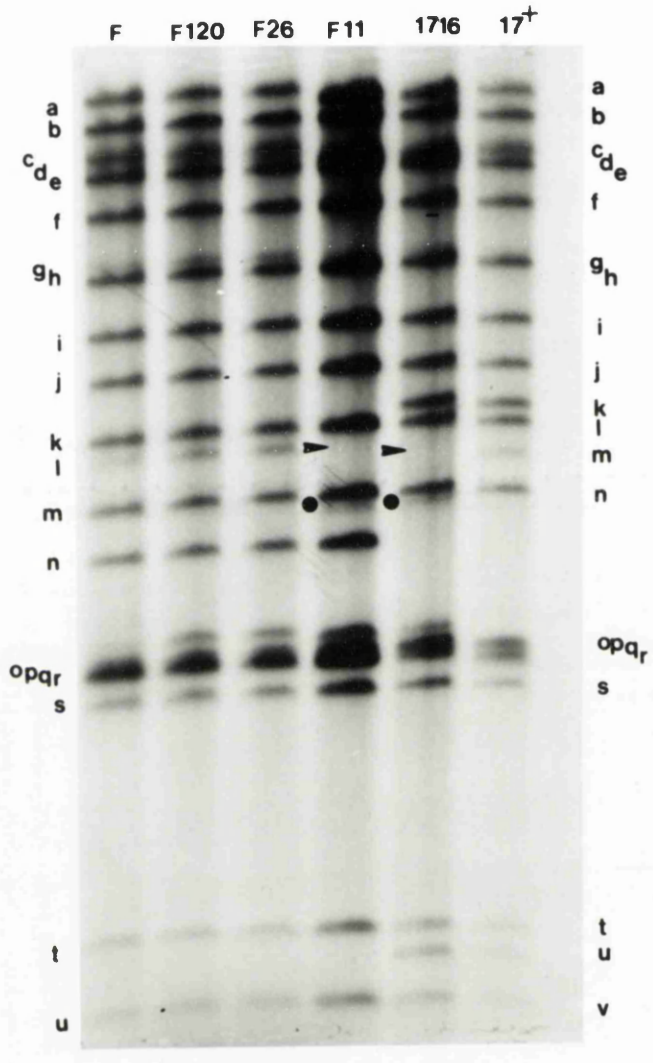
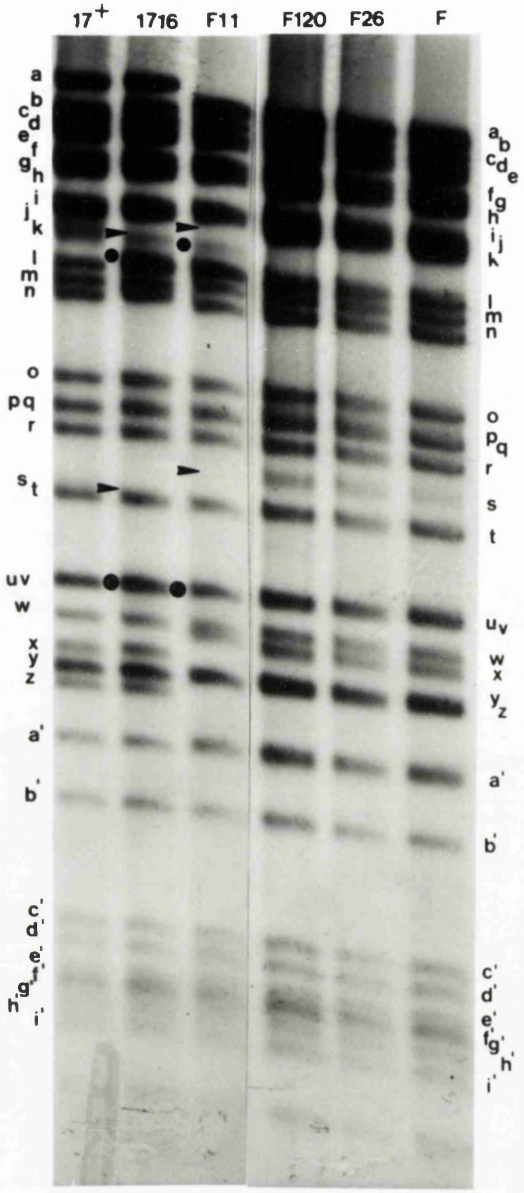

F
F120
F26
F11
1716
17+
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
u
v

Figure 3.17. BamHI restriction enzyme profile of F11.

Autoradiograph of a BamHI digest of viral DNA. F26 and F120 are plaque-purified wild-type isolates; F11 is the strain F ICP34.5 deletion variant. Missing bands are indicated by an arrowhead. Novel bands are marked (●). HSV-1 strain 17^+ bands are marked on the left-hand side, and HSV-1 strain F bands are marked on the right-hand side. Lanes are labelled at the top of each track.


17+
1716
F11
F120
F26
F
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
u
v
w
x
y
z
a'
b'
c'
d'
e'
f'
g'
h'
i'

3.2.3 Detection of RL1 by Southern Blotting.

Confirmation that F11 and 1716 did not contain RL1 coding sequences was further provided by Southern blotting HSV-1 DNA digested with AluI and DraI (fig.3.18.). A gel-purified AluI/DraI sub-fragment of HSV-1 strain 17^+ BamHI k (np 125074-np125989) (fig.3.14.) which encompasses the entire RL1 ORF was used as a probe. The strain 17^+ RL1 containing AluI/DraI fragment is smaller than that of HSV-1 strain F and the HSV-1 strain F isolates F26, F120 and F11R (section 3.2.5). This is in agreement with the smaller size of RL1 which leads to a lower M_r protein product (MacKay *et al.*, 1993). As expected F11 and 1716 do not hybridize to the AluI/DraI probe confirming they lack the RL1 ORF. Recombinants 1716a and F11a are discussed in 3.2.5..

3.2.4. Neurovirulence of F11.

Experiments to determine the neurovirulence of F11 compared to its parent HSV-1 strain F, HSV-1 strain 17^+ and 1716, by estimation of their LD_{50} values in BALB/c mice were carried out. Twenty-five ul aliquots of F, F11 17^+ and 1716 were inoculated into the left cerebral hemisphere of BALB/c mice. Deaths from encephalitis were scored up to 21 days post- inoculation and the results are shown in table 3.2.. HSV-1 strain F and strain 17^+ had LD_{50} values of $<10^2$ p.f.u./animal. However, none of the mice injected with either 1716 or F11 died even with an inoculum as high as 10^7 p.f.u./mouse, giving a LD_{50} value of $>10^7$ p.f.u./mouse. It has previously been shown (MacLean, A. *et al.*, 1991a), that the non-neurovirulent phenotype of 1716 is caused by its inability to grow, when injected intracerebrally into the mouse brain. As F11 contains exactly the same deletion in its genome, it would be reasonable to assume that it also is unable to replicate when injected intracerebrally into the mouse brain and hence is unable to cause encephalitis.

3.18. Detection of RL1 by Southern blotting

HSV-1 DNA of wild-type and recombinant viruses was digested with AluI/DraI and fragments separated on a 1.2% agarose gel. RL1 coding sequences were detected using a random primed AluI/DraI (np 125074-np 125989) sub-fragment of HSV-1 strain 17^+ (fig.3.13.)· Molecular weight markers are on the left-hand side. Lanes are labelled at the top of each track. The position of the AluI/DraI fragment hybridizing to the probe is indicated by an arrow.

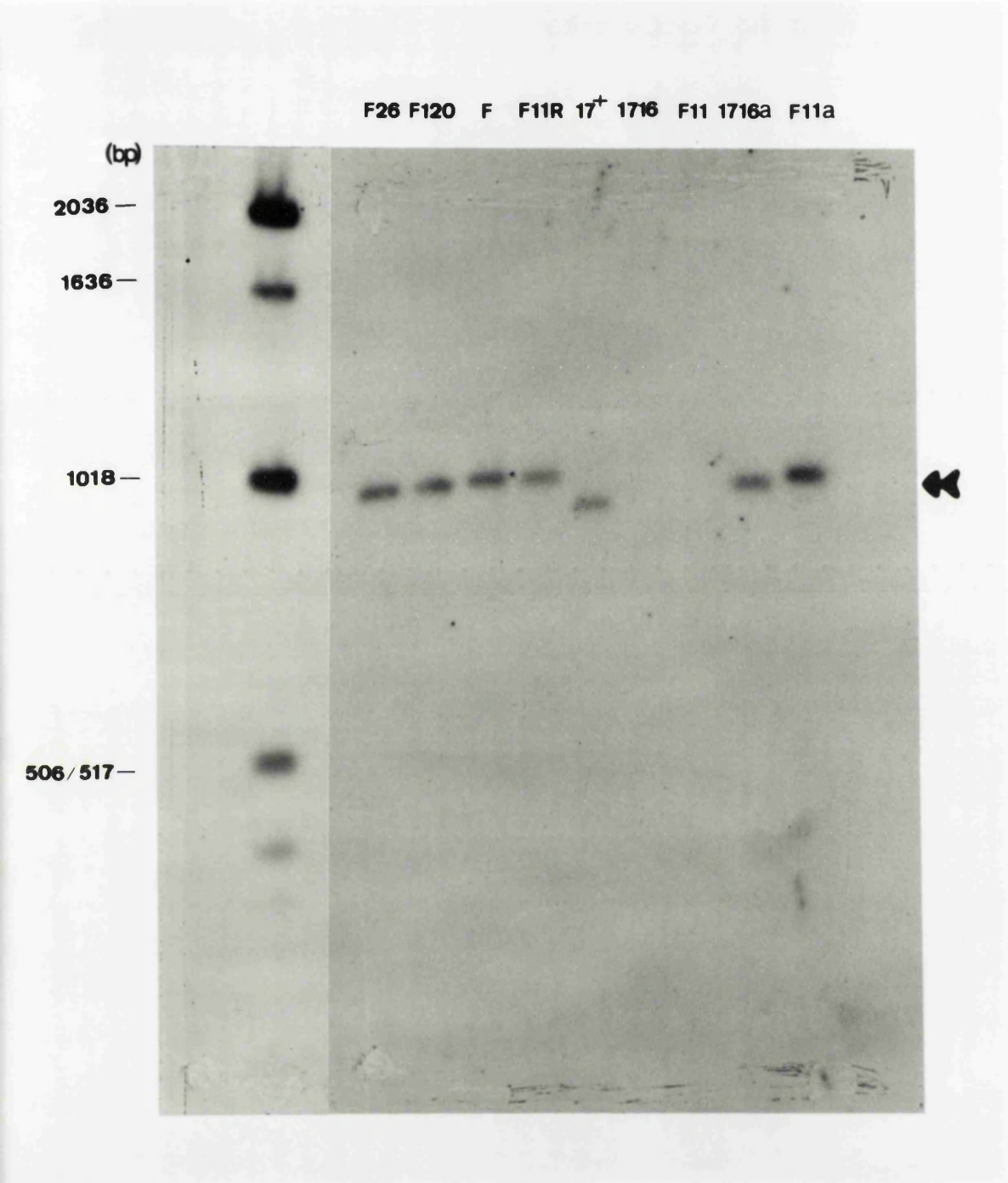

F26 F120 F F11R 17+ 1716 F11 1716a F11a
(bp)
2036
1636
1018
506/517

Table 3.2. Neurovirulence of F11 following intracerebral inoculation of 3-week old BALB/c mice.

virus \ dose	10^2	10^3	10^4	10^5	10^6	10^7	LD50(p.f.u./ mouse)
17 +	4/4 *	4/4	ND	ND	ND	ND	$<10^2$
1716	ND	ND	ND	0/4	0/4	0/4	$>10^7$
F	4/4	4/4	ND	ND	ND	ND	$<10^2$
F11	ND	ND	ND	0/4	0/4	0/4	$>10^7$

* no. of deaths/no. injected.
ND= not done.

3.2.5. Marker rescue of the lesion in F11.

To show that neurovirulence could be restored to F11, by rescuing the RL1 gene, wild-type rescuants were produced by *in vivo* marker rescue as described in section 2.2.27c., except that to produce the F11 rescuant (F11R), 1ug F11 DNA was transfected onto BHK21/C13 cells with 1-, 10- and 100- fold excess amounts of the wild-type HSV-1 strain F XbaI g restriction enzyme fragment, which contains the intact RL1 gene. After 2-3 days the plate in which c.p.e. was most extensive was harvested and serial 10-fold dilutions made. The 10^{-1} and 10^{-2} dilutions (containing approximately 10^4-10^5 p.f.u.) were then injected intracerebrally into groups of 4, 3-week old BALB/c mice. When the mice died the brains from 2 were homogenised in 1ml PBS/calf, single plaques isolated and ^{32}P labelled viral DNA prepared and analysed. One isolate with a wild-type profile (figs.3.15.-3.18.) was chosen for further plaque purification, before preparation of a stock.

MacKay *et al.* (1993) have previously observed, using an antiserum raised against a tenmer of the PAT repeat contained in the ICP34.5 sequence, a significant difference (>30 fold) between the sensitivity of detection of ICP34.5 of HSV-1 strain F compared to HSV-1 strain 17^+. This could be due to different antibody affinities for ICP34.5 of the 2 strains, or different levels of protein accumulation in infected cells. If the result of a differential level of accumulation it could be due to either a *cis* -acting effect of the RL1 sequence (eg. enhanced expression or polypeptide stability) or a *trans* -acting effect of other virus proteins. In an attempt to at least partially answer this question, 2 other recombinant viruses were also produced using the method of *in vivo* marker rescue (fig.3.14.).

F11a was generated by co-transfecting F11 DNA with a HSV-1 strain 17^+ fragment (XbaI g) spanning the RL1 gene and isolating a plaque isolate with a wild-type DNA profile. Similarly, 1716a arose from a cotransfection of 1716 DNA with the same HSV-1 strain F fragment (XbaI g) used to construct F11R. So, essentially, F11a has a strain F background, but contains the strain 17^+ RL1 promoter and coding sequences, whereas 1716a contains the strain F ICP34.5 promoter and coding sequences in a strain 17^+ background. The profile of these recombinants was analysed by restriction enzyme analysis and Southern

blotting. Figure 3.18 shows a Southern blot of 1716 and F11a digested with AluI/DraI and probed with the appropriate random primed AluI/DraI fragment (fig.3.14.). As expected the RL1 coding fragment is detected.

The question of differential detection of ICP34.5 of HSV-1 strain F compared to HSV-1 strain 17^+ will be referred to again in a later section (3.3.7.)

3.2.6. Neurovirulence of 1716a and F11a

Experiments to confirm that a wild-type phenotype had been restored to 1716a and F11a through recombination with RL1 sequences from HSV-1 strain F and HSV-1 strain 17^+ respectively were carried out by estimation of their LD_{50} values in BALB/c mice (table 3.3). It was anticipated that both 1716a and F11a would be neurovirulent following intracerebral inoculation of mice. In our laboratory we routinely find that the LD_{50} value of mice injected with HSV-1 strain F is around 10-fold higher than that of mice injected with HSV-1 strain 17^+, therefore, F11a, which has a HSV-1 strain F background was injected at doses of 10^2, 10^3 and 10^4 p.f.u./mouse, whereas 1716a, which has a HSV-1 strain 17^+ background was injected at doses of 10^1, 10^2 and 10^3 p.f.u./mouse. However, both 1716a and F11a had similar LD_{50} values of $3x10^2$ and 10^2 p.f.u./animal respectively, although these were slightly higher than the value obtained for HSV-1 strain 17^+ (7 p.f.u./animal). In this experiment 4 animals were injected with HSV-1 strain F at a dose of 10^2, however none of the animals died. Subsequent titration of the virus inoculum revealed that only 10^1 p.f.u. had been injected per animal. It has previously been found in our laboratory (Dr L. Robertson, personal communication) that this dose of HSV-1 strain F is not consistently lethal for mice, which has a LD_{50} value between 10^1 - 10^2 p.f.u./mouse.

3.2.7. Latency characteristics of F11.

The ability of F11 to reactivate from latency was examined using the mouse footpad model previously described by Clements and Subak-Sharpe (1983, 1988). Groups of 4, 4

Table 3.3. Neurovirulence of 1716a and F11a following intracerebral inoculation of 3-week old BALB/c mice.

virus \ dose	10^1	10^2	10^3	10^4	LD50 (p.f.u./mouse)
17 +	3/4 *	4/4	ND	ND	7
F11a	ND	1/4	4/4	4/4	$3x10^2$
1716a	0/4	2/4	4/4	ND	10^2
F	0/4	ND	ND	ND	$>10^1$

*no. of deaths/no. injected
ND= not done.

week old BALB/c mice were inoculated in the right rear footpad with varying doses of F, F11, 1716 and 17^+ and were monitored daily for signs of illness or death. Six weeks post inoculation, mice were killed and the 9 ipsilateral ganglia supplying the footpad screened as described in section 2.2.29d.. The results are summarised in figure 3.19.

At a dose of 10^5 p.f.u./mouse, HSV-1 strain 17^+ was found to be the most efficient at reactivating from latency, with a maximum frequency of ~55% explanted ganglia reactivating by 8 days post explant. Comparatively HSV-1 strain F showed poor reactivation when injected at an equivalent dose- reaching a maximum reactivation frequency of ~14%, 9 days post explantation (fig. 3.19a.). Because of this difference in reactivation frequency between the parental wild-type viruses 17^+ and F, it was considered more valid to compare F11 with F and 1716 with 17^+.

Taking this into account, F11 did not show any significant difference in reactivation kinetics compared to HSV-1 strain F. At a dose of 10^5 p.f.u., F11 reached a maximum reactivation frequency of ~7.5%, 10 days post explantation. The reactivation frequency was found to be dose dependent. When injected at a dose of 10^6 p.f.u., F11 reached a maximum reactivation frequency of ~10% 9 days post explant, and at 10^7 p.f.u., reached a maximum of 25% 8 days post explant. Similarly, the reactivation of 1716 from latency is dose-dependent (Robertson *et al.*, 1992). As has been previously shown, 1716 was impaired (15% at a dose of 10^7 p.f.u./animal) in reactivating from latency compared to the wild- type virus (55% at a dose of 10^5 p.f.u./animal) HSV-1 strain 17^+ (fig. 3.19a.) (Robertson *et al.*, 1992). In fig. 3.19a. the reactivation characteristics of F, F11, 17^+ and 1716 at the highest doses of each inoculated are compared. In fig. 3.19b., the dose dependent reactivation frequency of F / F11 is shown.

3.2.8. *In vitro* growth characteristics of F11.

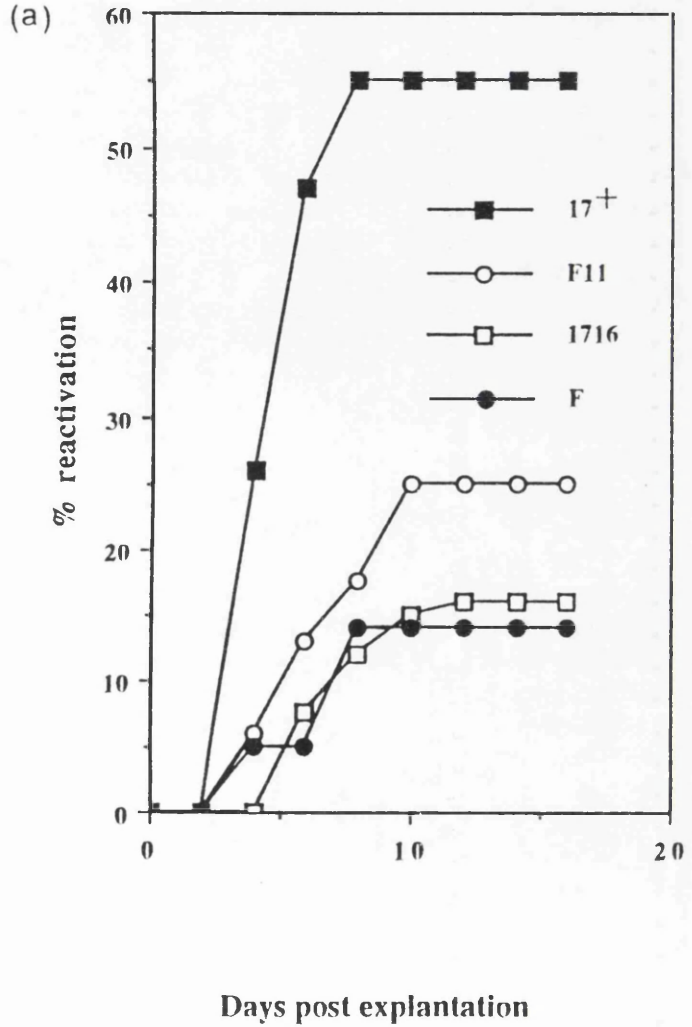
Analysis of the *in vitro* growth characteristics of the HSV-1 strain F variant F11 and the rescuant F11R was carried out on BHK21/C13 cells, using HSV-1 strain 17^+ and 1716 as controls. In both one- cycle (fig. 3.20(a).) and multi-cycle (fig.3.20(b).) growth

3.19. Latency characteristics of F11.

The latency characteristics of F11 were examined using the mouse footpad model. Groups of 4, 4-week old BALB/c mice were inoculated in the right rear footpad with varying doses of HSV-1 strain F, F11 1716 and HSV-1 strain 17^+. Six weeks post-inoculation, mice were killed and the 9 ipsilateral ganglia supplying the footpad removed and screened every second day for the presence of infectious virus. The frequency of ganglia reactivating at each time-point was calculated as a percentage of the total number of ganglia explanted, for each virus at each dilution.

(a). This shows the reactivation frequency of each virus at the highest dose used (10^5 p.f.u. HSV-1 strain F and HSV-1 strain 17^+; 10^7 p.f.u. F11 and 1716).

(b). The dose dependent reactivation of HSV-1 strain F and F11.


(a)
% reactivation
60
50
40
30
20
10
0
0
10
20
17+
F11
1716
F
Days post explantation

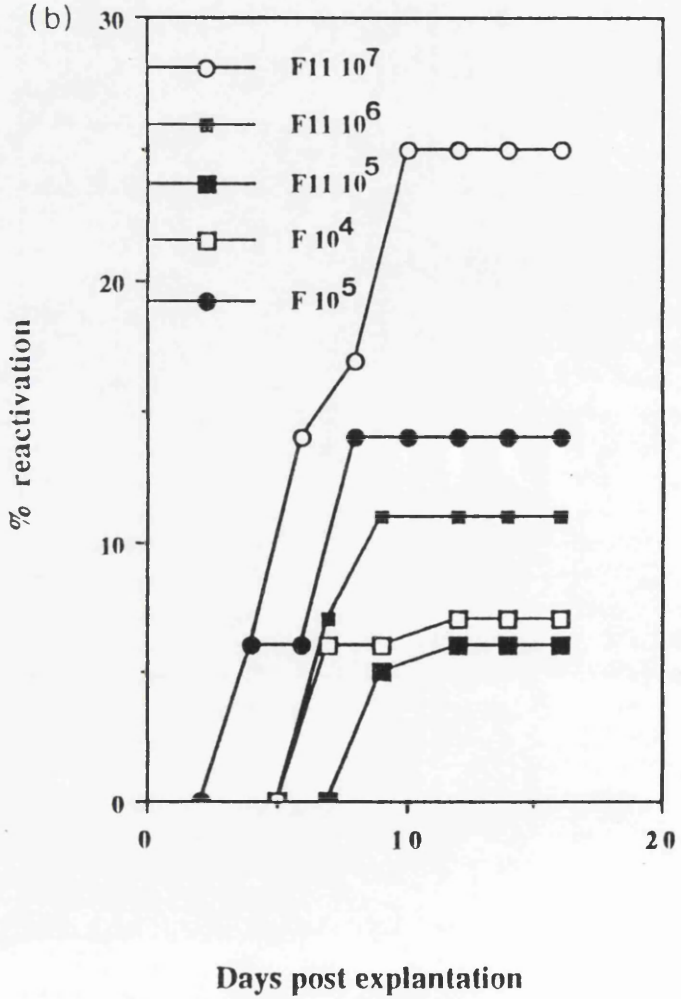

(b)
% reactivation
30
20
10
0
0
10
20
F11 10^7
F11 10^6
F11 10^5
F 10^4
F 10^5
Days post explantation

Figure 3.20 (a). One-cycle growth curve of F11

One-cycle growth experiments of HSV-1 strain 17^+, 1716, the wild-type rescuant F11R and F11 were carried out in BHK21/C13 cells. Cells were infected at a multiplicity of 10 p.f.u./cell, and following adsorption at 37°C for 1hr, the monolayers were washed twice with PBS/calf , overlaid with ETC10 and incubation continued at 37°C. Plates were harvested at 0, 2, 4, 6, 8, 12 and 24 hrs post-infection and titrated on BHK21/C13 cells.

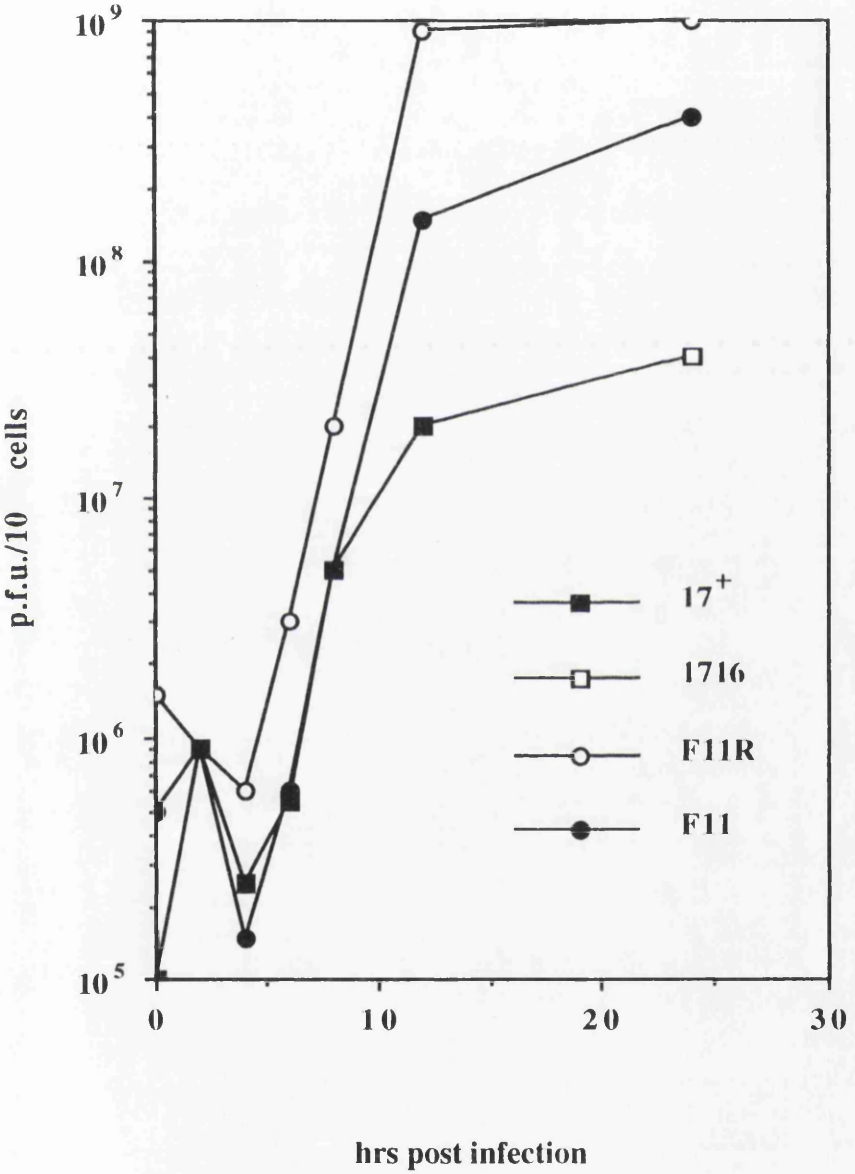
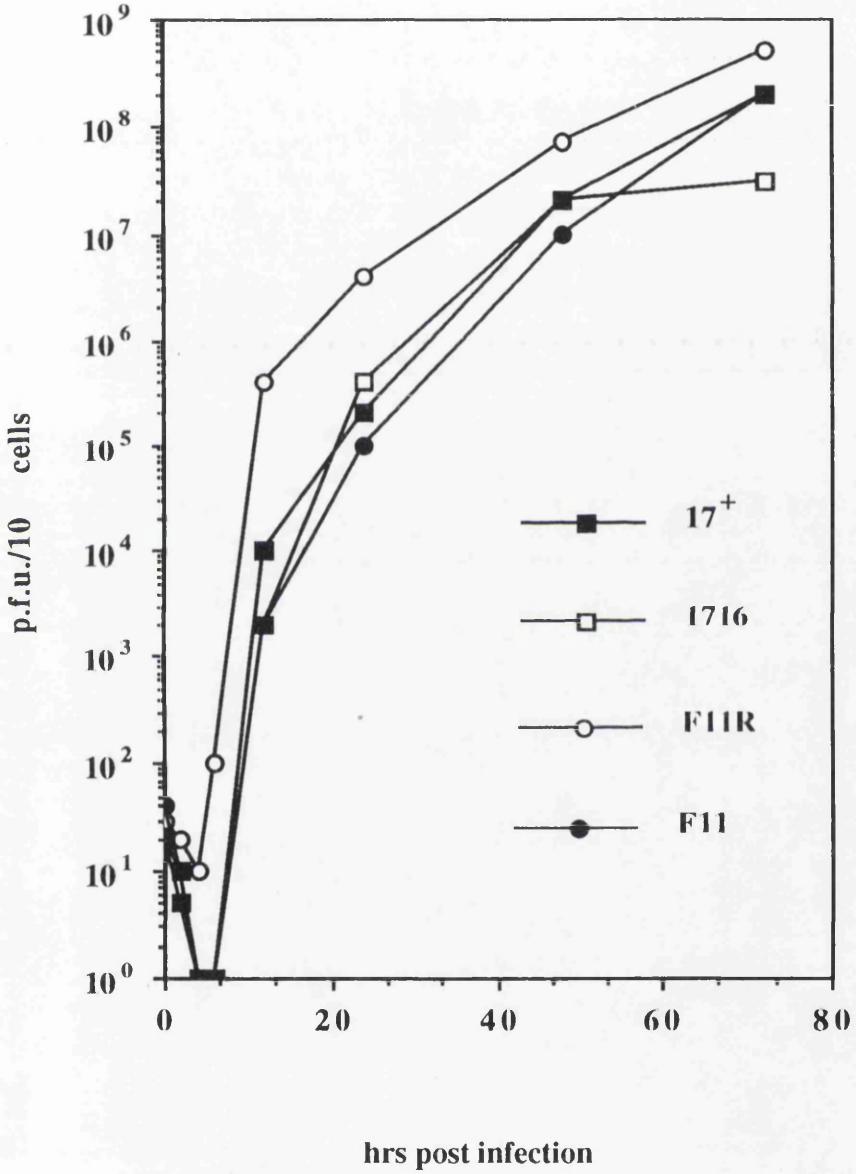

10^9
10^8
10^7
10^6
10^5
p.f.u./10^6 cells
0
10
20
30
hrs post infection
17^+
1716
F11R
F11

Figure 3.20 (b). Multi-cycle growth curve of F11

Multi-cycle growth experiment of HSV-1 strain 17^{+}, 1716, the wild-type rescuant F11R and F11 were carried out on BHK21/C13 cells. Cells were infected at a multiplicity of infection of 0.001 p.f.u./cell, and following adsorption at 37°C for 1hr, the monolayers were washed twice with PBS/calf and incubation continued at 37°C. Plates were harvested at 0, 2, 4, 6, 12, 24, 48 and 72hrs post-infection and titrated on BHK21/C13 cells.


10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
p.f.u./10^6 cells
0
20
40
60
80
hrs post infection
17^+
1716
F11R
F11

experiments 17^+ and 1716 grew identically as expected (MacLean, A., *et al.*, 1991a). F11 showed no significant impairment in growth compared to the wild-type rescuant F11R, although in the one-cycle growth experiment F11 had an extended lag phase This extended lag phase was not evident in the multi-cycle growth experiment. Both F and F11 reached final titres approximately 10- fold higher than that of strain 17^+ or 1716- in the one-cycle growth experiment, however all 4 viruses reached comparable final titres in the multi-cycle growth experiment.

3.2.9. Western blotting to check for ICP34.5 production.

One of the main aims of this study was to show that a loss of ICP34.5 specifically correlated with a loss of neurovirulence. Identification of ICP34.5 was carried out by Western blot analysis (fig.3.21.).

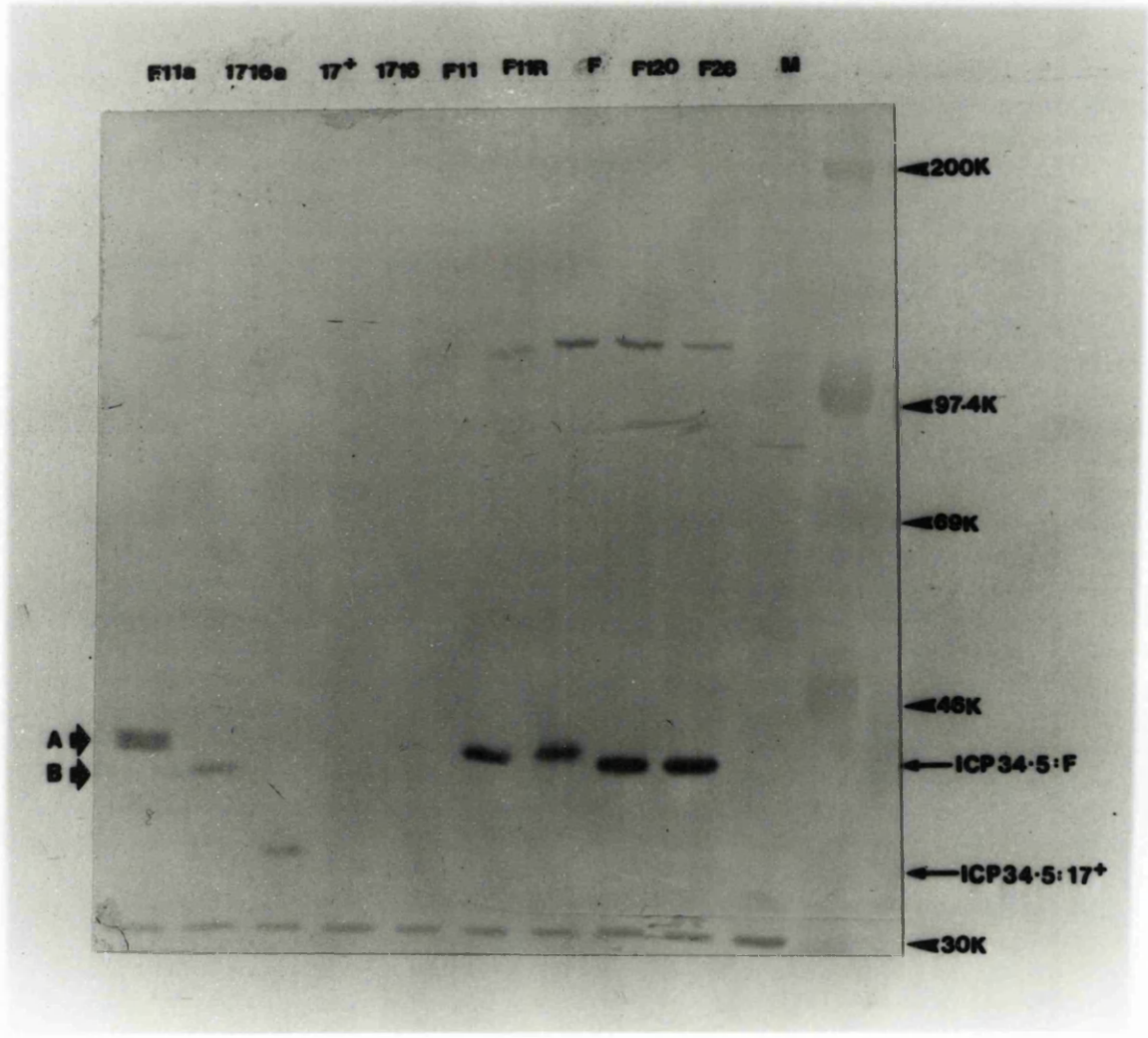
Infected cell extracts were prepared and Western blotted as described in section 2.2.38. to assay for ICP34.5 expression using peptide antiserum 78 at a 1/50 dilution.

The parental wild type HSV-1 strain F stock, two individual plaque isolates of F ie.F26 and F120 and the rescuant, F11R, produce ICP34.5, although there is variation in M_r amongst individual HSV-1 strain F isolates. As was described previously (section 3.2.3.) HSV-1 strain 17^+ produces a smaller version of the protein which is apparently present in lesser amounts than in HSV-1 strain F extracts. As expected 1716 and F11 do not produce ICP34.5.

F11a and 1716a are the two wild-type recombinants. F11a has a HSV-1 strain F background, but contains the strain 17^+ RL1 promoter and coding sequences. This variant was expected to produce ICP34.5 with the same apparent M_r as that of HSV-1 strain 17^+, but the apparent M_r is the same as the strain F protein. The band is a doublet, suggesting heterogeneity in the size of the RL1 gene within the virus stock. This heterogeneity within a virus population is also seen later (fig. 3.32.) in a Western blot of HSV-1 strain 17^+ using a protein antiserum and may be due to variation in the number of copies of the DNA sequence coding for the PAT repeat.

Figure 3.21. Western blot analysis of ICP34.5 from F11 infected cells.

Detection of ICP34.5 by Western blot analysis, using the anti-peptide serum (at a dilution of 1/50) which had been raised against the PAT trimer repeat. Molecular weight markers are shown on the right hand side. A and B refer to ICP34.5 of F11a and 1716a respectively. Lanes are labelled at the top of each track.


F11a
1716a
17+
1716
F11
F11R
F
F120
F26
M
200K
97·4K
69K
46K
A
B
ICP34·5:F
ICP34·5:17+
30K

1716a contains the strain F RL1 promoter and coding sequences in a HSV-1 strain 17^+ background. This virus produces a protein with a similar M_r to that of HSV-1 strain F, but in amounts similar to that of HSV-1 strain 17^+. 1716a and F11R were marker rescued using the same fragment of DNA, but in 1716a, with a strain 17^+ background, ICP34.5 production is reduced.

The relative sizes of the F11a and 1716a ICP34.5 correlate with those obtained for the RL1 containing fragment by Southern blotting (section 3.2.8.) indicating again, that the variation in polypeptide size is probably due to variation in the copy number of the DNA coding for the PAT repeat in the polypeptide.

3.2.10. Localization of ICP34.5 to the cytoplasm of infected cells.

Ackermann *et al.* (1986) have reported the accumulation of ICP34.5 largely in the cytoplasm of Hep-2 cells. The availability of a peptide antiserum which strongly recognises HSV-1 strain F ICP34.5 and is specifically inhibited by preincubation with the relevant peptide (MacKay *et al.*, 1993), and the deletion variant F11, which provides an ideal negative control allowed us to confirm these findings by immunofluoresence studies. Samples were prepared as described in section 2.2.36. and viewed using a Nikon Microphot- SA microscope.

When cells were infected with wild- type HSV-1 strain F(fig. 3.22(c)), dense punctate patches of fluoresence could be seen in the cell cytoplasm, with minimal signs of perinuclear staining. Cells which were mock infected or infected with the mutant F11 (fig. 3.22(a&b)), tended to show a general low level of background fluoresence, with no intense patches of staining as observed with the wild- type virus infected cells. A similar result was seen when using strain F if the antiserum was preincubated with the peptide (data not shown). These results were consistent with those obtained in cell fractionation studies (MacKay *et al.*, 1993).

Figure 3.22. Localization of ICP34.5 in HSV-1 strain F infected cells.

Immunofluoresence of mock (top), F11 (middle) and HSV-1 strain F (bottom) infected cells incubated with peptide antiserum 78 at a 1/100 dilution. Anti-rabbit IgG FITC conjugate was used as the second antibody.

3.3. Expression of ICP34.5 in *E.coli.*

3.3.1 Introduction.

The observation that peptide antisera had been raised against 7 different regions of RL1- but only 1 had detected ICP34.5 from HSV-1 strain F (MacKay *et al.*, 1993) suggested that the homologous protein of HSV-1 strain 17^+ may not be detected by this method. To show that the strain 17^+ RL1 ORF was capable of expressing a protein we decided to express it under the control of a strong promoter. One method used successfully in this Institute for the production of R1 and R2 (Lankinen *et al*, 1991) is the pET system (Studier *et al.*, 1990), which allows high expression of foreign proteins in *E.coli;* this method was chosen for the expression of ICP34.5. Furthermore, it was intended to use purified ICP34.5 to raise a polyclonal antiserum, which hopefully would recognise both ICP34.5 in HSV-1 strain 17^+ infected cells and the homologous protein in HSV-2 strain HG52.

3.3.2. Construction of the expression vector pET34.5.

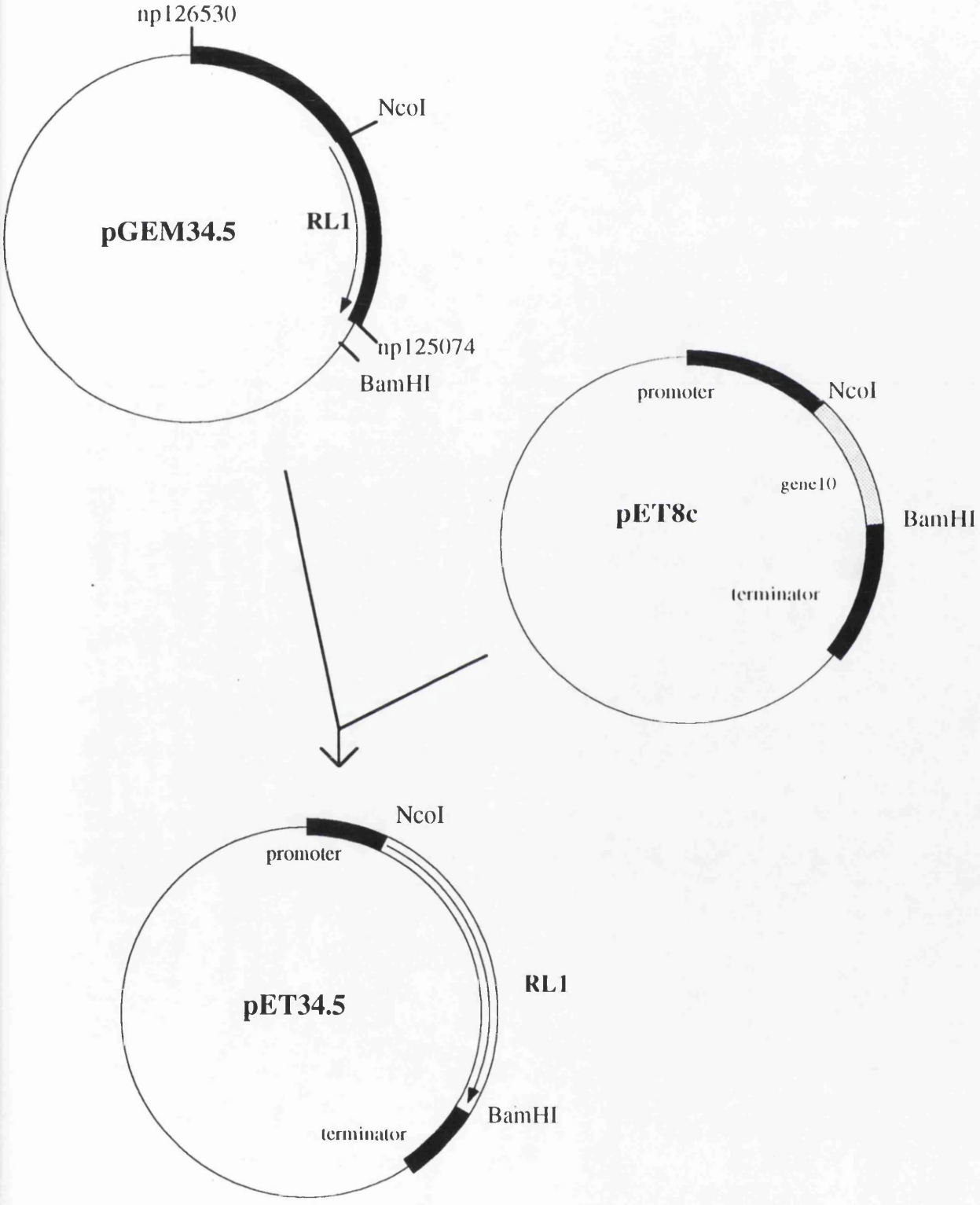
The intact RL1 ORF from HSV-1 strain 17^+ was cloned into pET8c (fig.3.23.). pET8c encodes the T7 gene 10 promoter including the Shine-Dalgarno sequence and initiating ATG. The starting plasmid was pEA.10, which contained a 1.46kb fragment (np 125074-np 126530) off HSV-1 strain 17^+ cloned into pGEM 3zf(-). Following digestion with NcoI and BamHI (in the vector polylinker) the entire RL1 ORF was released from the initiation codon, to ~40bp beyond the stop codon of the gene. This fragment was inserted into pET8c, which had been digested with NcoI and BamHI generating the plasmid pET34.5.

3.3.3. Induction of ICP34.5 protein synthesis using IPTG.

The recombinant plasmid pET34.5 was used to transform *E.coli* BL21(DE3) cells containing the T7 RNA polymerase gene under the control of the inducible *lac* UV5 promoter as described in section 2.2.28.. Expression was first monitored in 5ml cultures using 0.05mM IPTG. *E.coli* BL21(DE3) cells which had not been transformed with pET34.5 were used as a control. One ml aliquots were removed for sampling at various

Figure 3.23. Construction of pET34.5

pGEM34.5 contains a 1.46kb fragment of HSV-1 strain 17^+ (np 125074-np 126530) cloned into pGEM3zf(-). This was digested with NcoI and BamHI and the entire RL1 gene sequence isolated. pET8c was also digested with NcoI and BamHI and the gene 10 fragment removed. Purified RL1 and vector fragments were ligated together thus placing the entire RL1 ORF immediately downstream of the T7 gene 10 promoter. This plasmid was designated pET34.5


np126530
NcoI
pGEM34.5
RL1
np125074
BamHI
promoter
NcoI
gene10
pET8c
BamHI
terminator
NcoI
promoter
pET34.5
RL1
BamHI
terminator

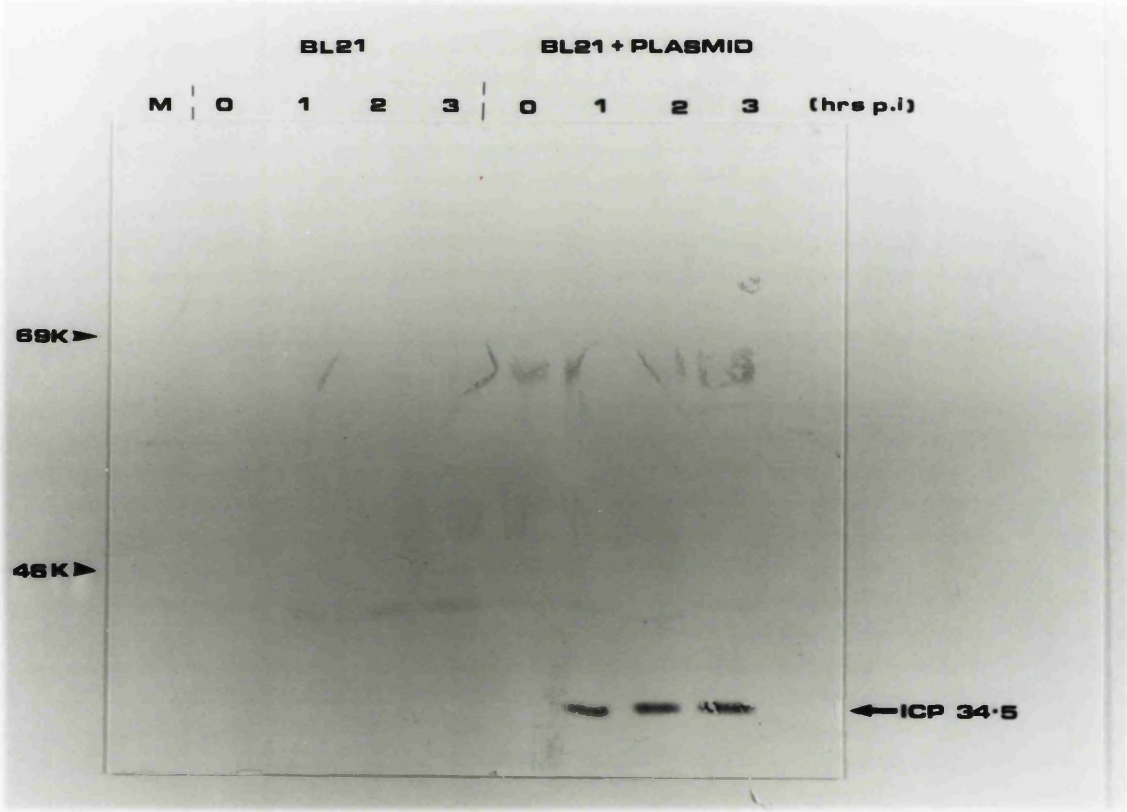
times post induction, pelleted in a microfuge and resuspended in 500ul of extraction buffer. After boiling, 50ul aliquots of each sample were run on 7.5% SDS-PAGE and Western blotted as described in section 2.2.38.. Antiserum 78 was used as the first antibody at a 1/50 dilution. As can be seen in figure 3.24, the *E.coli* BL21(DE3) which have not been transformed with the plasmid pET34.5 fail to produce a protein which is recognised by the ICP34.5 specific antiserum. In contrast a band of the expected size from HSV-1 strain 17^+ ICP34.5 was detected 1hr post-induction in the bacteria transformed with pET34.5 The amount of protein produced did not appear to increase significantly with time.

To optimise ICP34.5 production 500ml cultures were induced with concentrations of IPTG ranging from 0.to 0.4mM. As before, 1ml aliquots were removed for sampling at various times post-induction, the bacteria pelleted, and proteins run on 10% SDS-PAGE and Western blotted using the ICP34.5 specific peptide antiserum. Figure 3.25. shows samples which were removed 1, 2 or 3hrs post-induction with 0.2 or 0.4 mM IPTG. The first 4 lanes are the uninduced control. It is apparent that ICP34.5 production occurs even in the absence of IPTG, suggesting that the protein is relatively non-toxic to the cells. Up to a concentration of 0.2mM IPTG ICP34.5 production was relatively constant. Levels of ICP34.5 appeared maximal 1hr after induction with IPTG. Thus in all future experiments bacteria were harvested 1hr post-induction with 0.05mM IPTG. There appeared to be fainter ICP34.5 specific bands of lower molecular associated with ICP34.5 production (most noticably ~25-28K and ~30-35K) and these may represent breakdown products due to proteases in the bacteria. These were not observed in extracts from the smaller cultures which had been run on a lower percentage gel.

To further purify ICP34.5 from crude bacterial extracts (section 2.2.30.) the lysed bacteria were separated into a soluble cytosolic fraction and an insoluble bacterial pellet with the purpose of further purifying the soluble fraction . Unfortunately, when induction took place at 37°C, ICP34.5 was retained in the insoluble fraction. Similar problems have been found in the production of R2 protein of HSV-1 ribonucleotide reductase (Lankinen *et al.*,

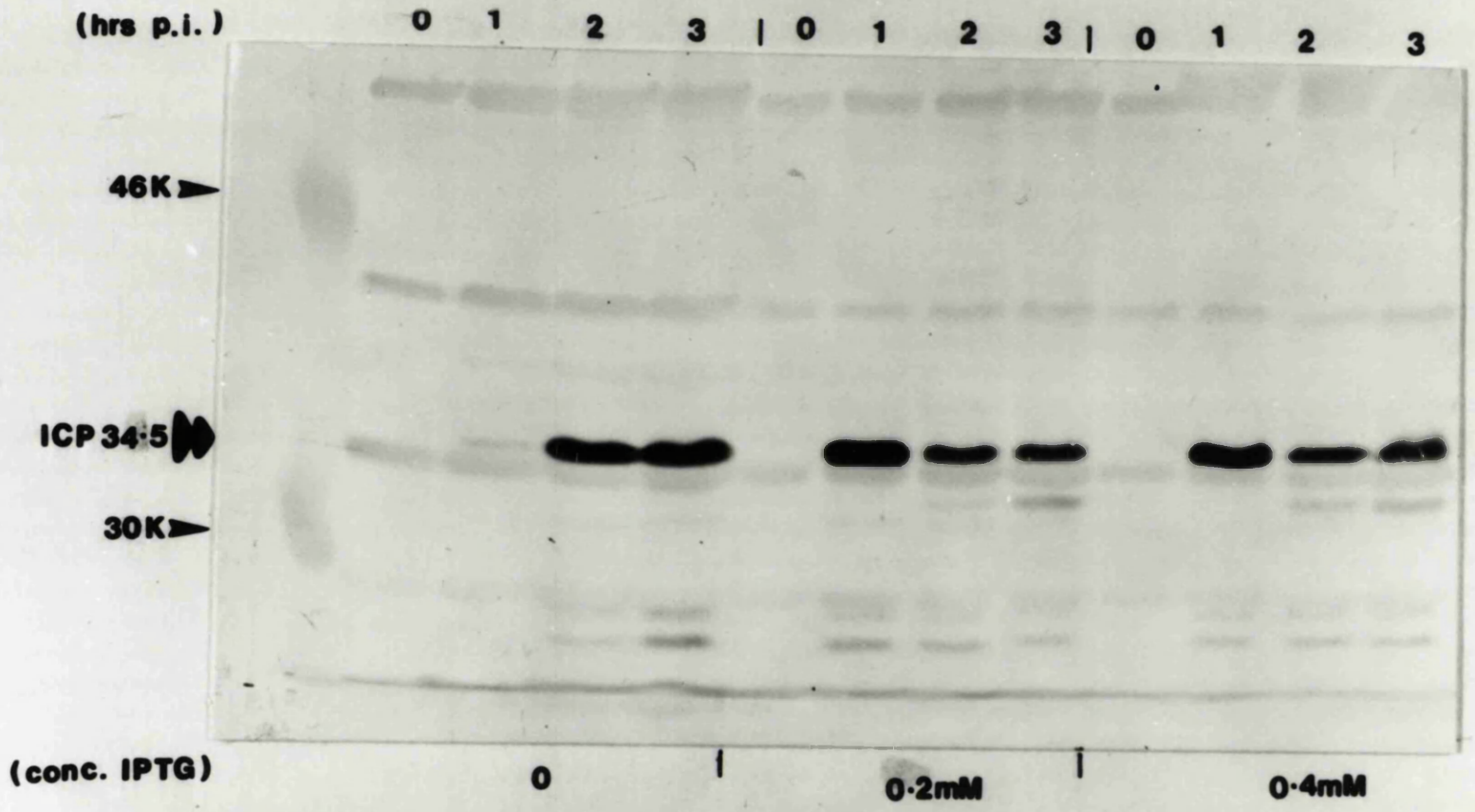
3.24. Detection by Western blotting, of a band equivalent in size to HSV-1 strain 17$^+$ ICP34.5, in *E.coli*. BL21 cells transformed with pET34.5.

ICP34.5 expression in BL21 cells transformed with pET34.5 at various times post-induction with 0.05mM IPTG was assayed by Western blotting using the peptide antiserum at a 1/50 dilution. Molecular weight markers are on the left-hand side; Lanes are labelled at the top of the gel.


BL21
BL21 + PLASMID
M
0
1
2
3
0
1
2
3
(hrs p.i)
69K
46K
ICP 34·5

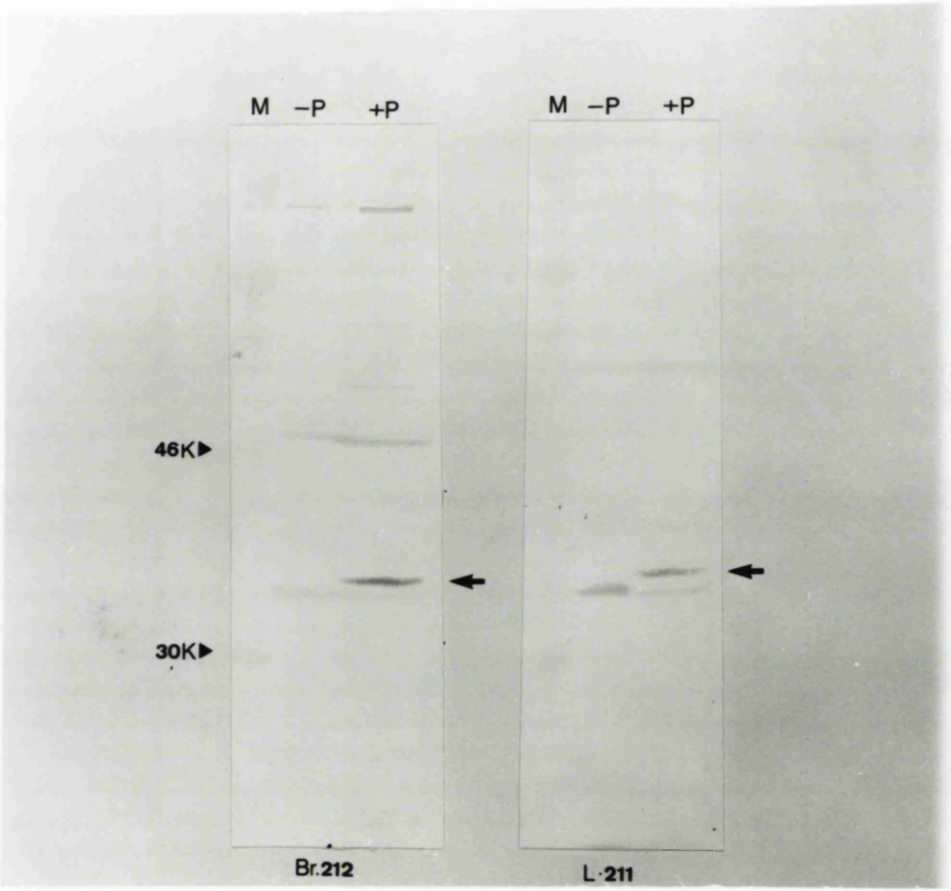
3.25. ICP34.5 expression with various concentrations of IPTG.

The level of expression of ICP34.5 was examined using various concentrations of IPTG. Western blotting with the peptide antiserum at a dilution of 1/50 was used to assay ICP34.5 expression. Molecular weight markers are on the left-hand side ; Lanes are labelled at the top of each track. The concentrations of IPTG used are indicated at the bottom of the gel.


(hrs p.i.)
0 1 2 3 | 0 1 2 3 | 0 1 2 3
46K
ICP 34·5
30K
(conc. IPTG)
0
0·2mM
0·4mM

3.26. Recognition of *E.coli* - expressed ICP34.5 by antisera which fail to recognise ICP34.5 in HSV-1 infected cells.

This figure demonstrates specific recognition of *E.coli* -expressed ICP34.5 by 2 antisera, Br.212 and L.211. Both antisera were used at a 1/15 dilution. No band equivalent to ICP34.5 is detected in bacteria which have not been transformed with pET34.5 (-P). +P indicates *E.coli* BL21(DE3) cells transformed with pET34.5. Molecular weight markers are on the left-hand side; Lanes are labelled at the top of each track. *E.coli* BL21(DE3) cell extracts were prepared 1hr post-induction with 0.05mM IPTG. The arrows indicate the position of ICP34.5.


M
−P
+P
M
−P
+P
46K
30K
Br.212
L·211

1991). In the case of R2, following induction with IPTG, the cultures were rapidly reduced to 26^{o}C. This procedure produced soluble expressed protein. By investigating various induction temperatures (table 3.4.) it was deduced that induction at 28^{o}C, produced soluble ICP34.5.

3.3.4. Detection of *E.coli* - expressed ICP34.5 by Western blotting, using antisera which fail to detect ICP34.5 in infected cells.

Two other peptide antisera- Br.212 and L.211 (fig.3.13.) also detected ICP34.5 expressed in *E.coli* by Western blotting (2.2.38.), although they had failed to detect ICP34.5 in HSV-1 or HSV-2 infected cell extracts (Dr.C. MacLean, personal communication). *E.coli* BL21(DE3) cells which either had (+P), or had not (-P) been transformed with pET34.5 were grown and harvested as described in section 2.2.30.. Western blotting was carried out as described previously (section 2.2.38.) using both antisera at a 1/15 dilution (fig.3.26.). A band of the expected size of HSV-1 strain 17^{+} ICP34.5 was detected in *E.coli* BL21 cells which had been transformed with pET34.5, but not in those which had not been transformed with the plasmid. In some experiments, the fainter ICP34.5 specific bands, detected using antiserum 78 were also seen.

3.35. Ammonium sulphate fractionation of expressed ICP34.5.

Crude bacterial extracts were prepared as described in section 2.2.30.. At this stage there was about 25mg of total soluble protein per litre of bacterial culture. ICP34.5 solubility was examined at ammonium sulphate concentrations ranging from 10-50%. Increasing amounts of a saturated solution of ammonium sulphate were added to 100ul aliquots of the crude extract (section 2.2.29.). Examples of volumes added per 100ul crude extract are shown in table 3.5.. After 30 minutes on ice precipitated protein was pelleted at 4^{o}C. Pellet and supernatant fractions were analysed by 10% SDS-PAGE to determine at which ammonium sulphate concentration ICP34.5 could be fully recovered from the crude extract. ICP34.5 could not easily be identified by Commassie blue staining of gels as there

Table 3.4. Solubility of *E.coli* -expressed ICP34.5 when expressed at a variety of temperatures.

solubility / induction temperature	ICP34.5 detected in pellet (insoluble)	ICP34.5 detected in supernatant (soluble)
37 °C	+	–
31 °C	+/-	+/-
28 °C	–	+
26 °C	–	+

+ : protein mainly detected in this fraction
+/- : relatively equal amounts of protein found in both fractions
- : very little protein detected in this fraction

Table 3.5. Precipitation of crude extract using a saturated solution of ammonium sulphate.

volume crude extract	final % ammonium sulphate	volume 4M solution added
100ul	5	5.3ul
100ul	10	11.1ul
100ul	15	17.7ul
100ul	20	25ul
100ul	25	33.4ul
100ul	30	42.8ul
100ul	35	53.9ul
100ul	40	66.7ul
100ul	45	81.9ul
100ul	50	100ul

were many host bacterial bands of a similar size and intensity. Figure 3.27. shows Commassie blue stained ammonium sulphate supernatant and pellet fractions from 42-55%. As explained above ICP34.5 is not apparent in the gel but it serves to show the number of other contaminating *E.coli* bands in the sample following ammonium sulphate fractionation. The expected position of ICP34.5, based on Western blotting, is indicated. It soon became clear that this was not the best way of detecting ICP34.5 in the pellet fractions, therefore it was detected by Western blot using peptide antiserum 78 (fig.3.28). Analysis of supernatant and pellet fractions showed that ICP34.5 started to precipitate at an ammonium sulphate concentration between 10 and 20%, and that increasing amounts precipitated up to 40% salt. At this point there was still small amounts of ICP34.5 in the supernatant fractions, however, at 50% ammonium sulphate, all ICP34.5 was precipitated. Therefore, the procedure was scaled up and all further preparations of ICP34.5 were precipitated with 50% ammonium sulphate. The protein detected by antiserum 78 was confirmed as ICP34.5 by its absence when the antiserum was preincubated with the relevant peptide (fig.3.29.). In all gels where ammonium sulphate fractions are shown, saturated ammonium sulphate, to a final concentration equal to that of the highest concentration used for analysis (50% or 60%) has been added to the pellet fractions, prior to running on the gel. This was done so that the osmotic pressure in each track is even, hence preventing distortion of the gel. Following precipitation with 50% ammonium sulphate the total protein concentration was now approximately 10mg/litre of starting culture.

3.3.6. Partial purification of ICP34.5 using anion-exchange chromatography.

Many attempts were made to find a column and conditions of pH at which ICP34.5 bound. In almost every case it was found that either the protein did not bind, or it bound but was eluted over almost the entire gradient, making purification almost impossible (table 3.6.). One such case is demonstrated in fig.3.30.. Here an XK 16 column was packed with 10ml S-sepharose (Pharmacia) and equilibrated with 100ml buffer A, (in this case 50mM NaCl,

3.27. Ammonium sulphate fractionation of ICP34.5 from crude bacterial extracts.

Saturated ammonium sulphate was added to the crude extract and following centrifugation, the pellets (P) and supernatants (S) from the fractions indicated were loaded on the gel. The expected position of the band representing ICP34.5 is indicated (■). Both pellet and supernatant fractions correspond to an equal volume of crude extract and were equalized to 60% $(NH_4)_2SO_4$ prior to loading onto the gel. Molecular weight markers are on the left-hand side.

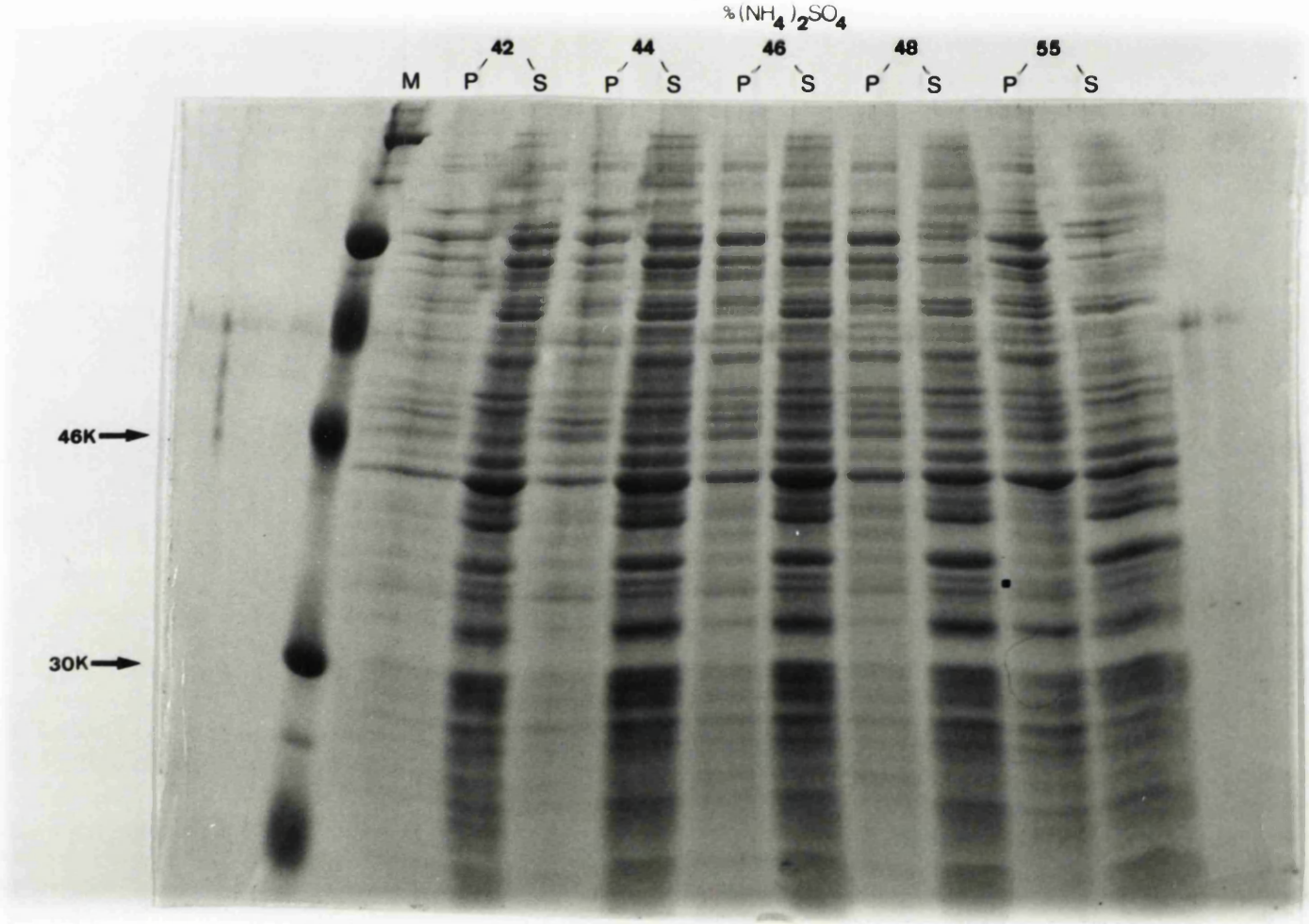

% $(NH_4)_2SO_4$
42
44
46
48
55
M
P
S
P
S
P
S
P
S
P
S
46K
30K

Figure 3.28. Purification of ICP34.5 by ammonium sulphate fractionation.

Saturated ammonium sulphate (10%, 20%, 30%, 40% and 50%) was added to crude extract and after centrifugation, equal volumes of supernatant and pellet fractions were loaded onto a gel. Following electrophoresis, the proteins were transferred to nitrocellulose and Western blotting was carried out using peptide antiserum 78, at a 1/50 dilution. The relative quantity of ICP34.5 in the pellet and supernatant fractions at each salt concentration was compared.

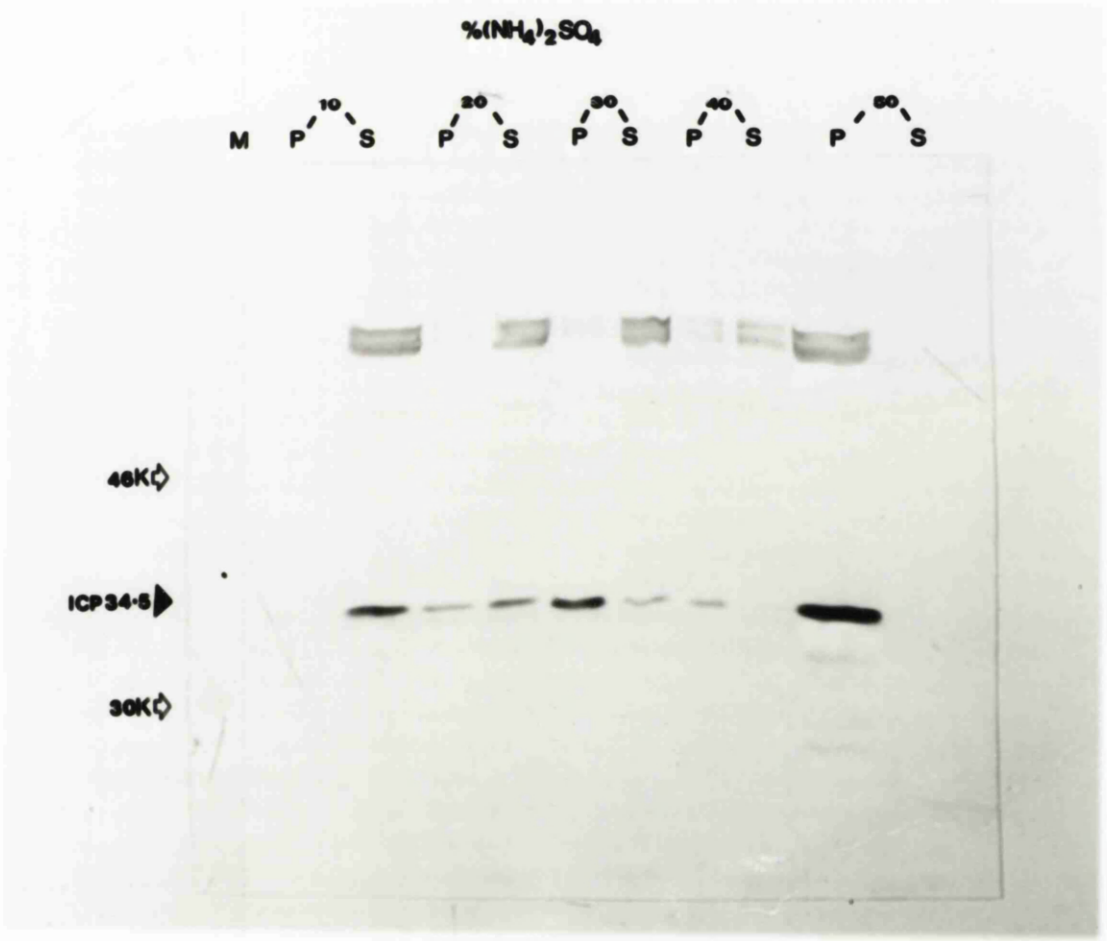

%(NH4)2SO4
M
P
10
S
P
20
S
P
30
S
P
40
S
P
50
S
46KΦ
ICP34·5
30KΦ

Figure 3.29. Western blotting of ammonium sulphate fractions to confirm the presence of ICP34.5

Saturated ammonium sulphate was added to crude extract. After centrifugation, the pellets from the fractions indicated were loaded onto a gel. Following electrophoresis, the proteins were transferred to nitrocellulose and Western blotted using peptide antiserum 78, at a 1/50 dilution, without (A) or with (B) pre-incubation with the relevant peptide. The large arrow indicates the position of ICP34.5. In (A), an air-bubble between the 50% and 60% ammonium sulphate fractions has blocked the efficient transfer of proteins onto the nitrocellulose.

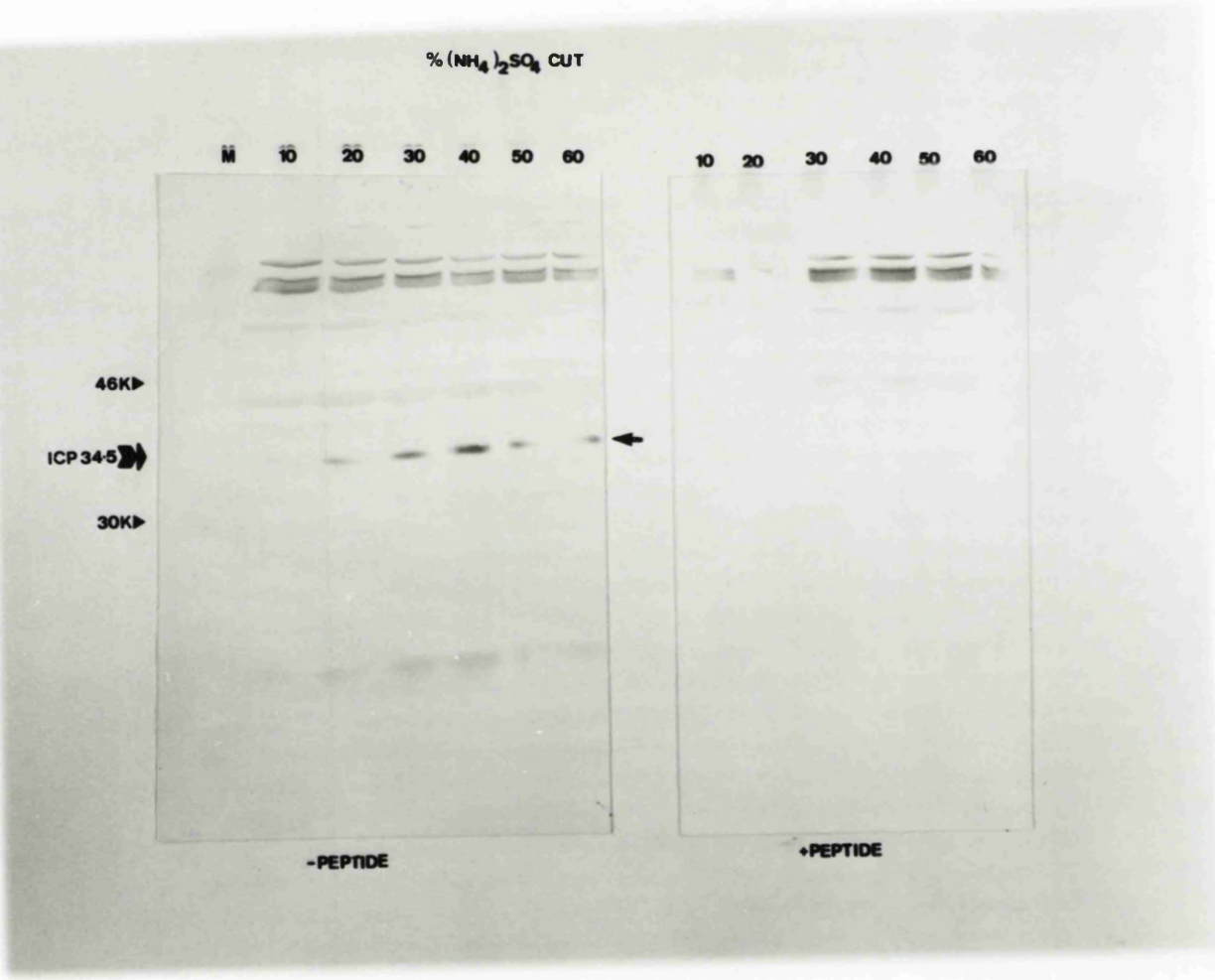

% $(NH_4)_2SO_4$ CUT
M
10
20
30
40
50
60
10
20
30
40
50
60
46K
ICP 34·5
30K
-PEPTIDE
+PEPTIDE

Table 3.6. Columns and conditions tested for purification of *E.coli*- expressed ICP34.5

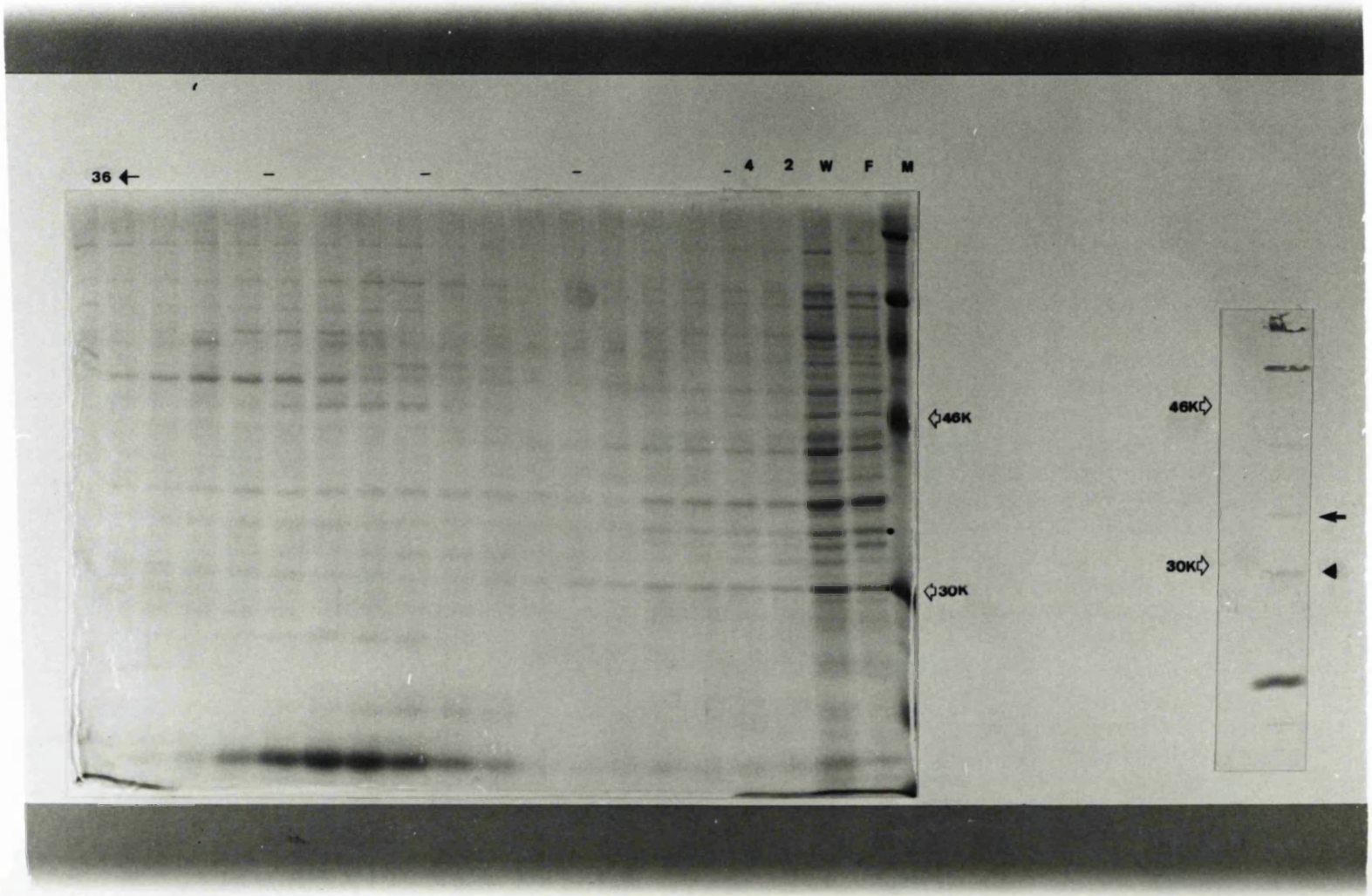
Column	pH/buffer	result
1) Mono S	pH 6.5/ MES	no binding
	pH 5.5/ MES	no binding
	pH 7.2/ Tris-HCl	some binding (aggregation ?)
2) Mono Q	pH 8.0/ Tris-HCl	no binding
	pH 7.6/ HEPES	no binding
	pH 6.8/ HEPES	no binding
	pH 6.0/ MES	no binding
3) Phenyl sepharose	pH 7.6/ Tris-HCl	low yields (~1mg total protein/ liter crude extract)
4) S-sepharose	pH 7.6/ Tris-HCl	Some binding (aggregation ?)
5) DNA-cellulose*	pH 7.6/ HEPES	no binding.

*Dr.F.Amina, personal communication.

In 1-4 the source of ICP34.5 was ammonium sulphate fractionated crude bacterial extracts whereas in number 5, the source of ICP34.5 was from HSV-1 strain F infected cell extracts.

3.30. Purification of ICP34.5 using S-sepharose.

Gradient elution of salt-fractionated *E.coli* expressed ICP34.5 was carried out using a 10ml S-sepharose column. (A) Commassie-blue stained SDS-PAGE. Lane numbers correspond to the fraction numbers. Only every second fraction was run. Molecular weight markers are on the right-hand side. F and W correspond to the flowthrough and wash fractions respectively. The positions of ICP34.5 and the lower molecular weight ICP34.5 associated band are indicated (●). (B) Confirmation of the band corresponding to *E.coli* expressed ICP34.5 was provided by Western blotting the flowthrough fraction using peptide antiserum 78 at a 1/50 dilution. ICP34.5 is indicated by an arrow. The position of the lower 30kD associated band is also indicated (▶).

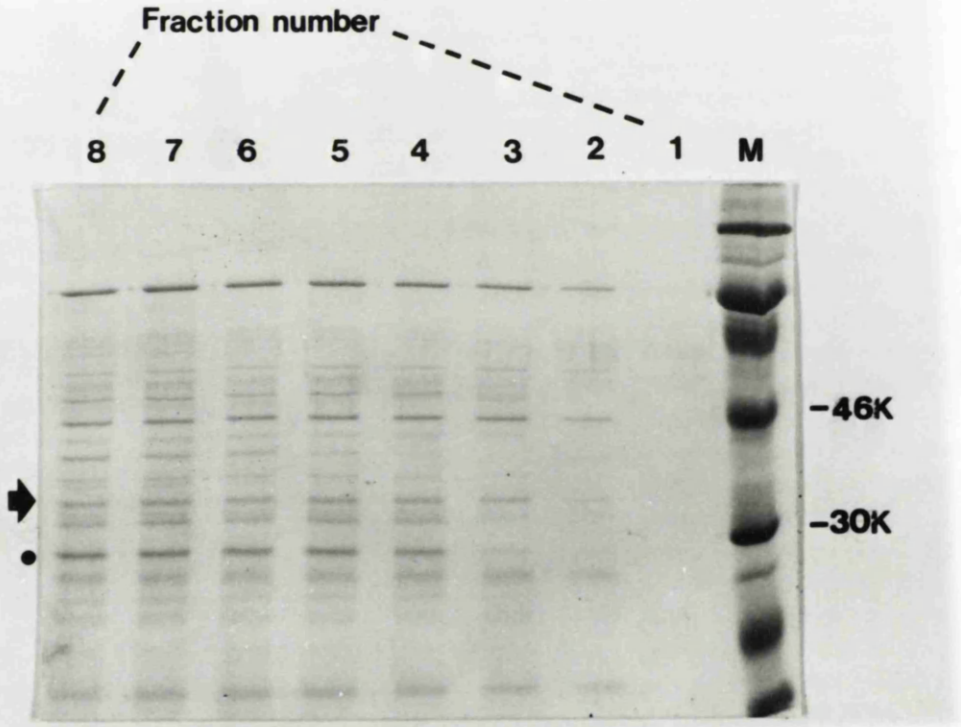

36
4
2
W
F
M
46K
30K
46K
30K

50nM M.E.S, pH6.5) at 4°C. Two litre equivalent of crude extract, which had been partially purified by taking a 50% $(NH_4)_2SO_4$ cut was applied to the column and after washing with 1 column volume of buffer A, a 100ml gradient was applied to the column ranging from 50mM NaCl , 50mM (2-[N-Morpholino]ethanesulfonic acid)(M.E.S.), pH6.5 to 1M NaCl, 50mM M.E.S., pH 6.5 . Two ml fractions were collected and analysed by SDS-PAGE. At first it appeared that the protein had not bound to the column at all, but closer examination of the wash and fractions from the gradient revealed that the protein had bound and was being gradually washed off rather than specifically eluted. It is possible that the *E.coli* -expressed ICP34.5 aggregates either with itself, or with other proteins in the crude extract thus preventing specific binding of the protein to the column matrix and causing the protein either to flow through the column or to be eluted with other proteins in the sample. Some proteins which are at a high concentration in solution are known to form aggregates, and this may be the situation in the crude extract. However in a concentrated solution they tend to be more stable and thus a compromise must be reached. In the case of ICP34.5, reducing the concentration of the solution by increasing the volume of the sample with buffer did not appear to improve elution.

A more satisfactory approach to purify ICP34.5 was attained by anion exchange FPLC. In thiscase, the majority of the *E.coli* proteins in the sample bound but the expressed protein didnot. As the protein pellets from the ammonium sulphate fractionation were generally resuspended in 50mM NaCl, 50mM Tris-Cl pH 7.6 it was decided that this would be the easest buffer to try first. A Mono Q 5/5 HR column was equilibrated first with buffer B (1M NaCl, 50mM Tris-Cl,pH7.6) and then buffer A (50mM NaCl, 50mM Tris-Cl ,pH7.6) before application of the sample. The binding capacity of this column is approximately 25ng protein, which in this case is equivalent to 2.5 litres of ammonium sulphate fractionated material. Fractions of 1ml were collected and analysed by SDS-PAGE. An example of consecutive flow-through fractions is shown in fig.3.31.. Although there are still some contaminating bacterial bands, purification of ICP34.5 was greatly enhanced (see fig. 3.27. for an idea of the increase in purity). Since the protein concentration was

3.31. Partial-purification of ICP34.5 using Anion-exchange chromatography.

Salt-fractionated *E.coli* - expressed ICP34.5 was further purified on a Mono Q HR 5/5 column. A total of 25mg (40ml volume) was loaded on the column. Flowthrough fractions were collected and bound proteins were washed off using a buffer containing 1M salt. Lane numbers of the Commassie blue stained gel correspond to consecutive flowthrough fractions. Molecular weight markers are shown on the right-hand side. The arrow indicates the 37kd ICP34.5 polypeptide and● represents the associated 30kd band.


Fraction number
8
7
6
5
4
3
2
1
M
-46K
-30K

only about 1mg/litre starting material after anion exchange FPLC, further purification of the protein was not attempted due to the risk of losing all material. It was considered that the protein would be sufficiently pure to be used for the production of a polyclonal antiserum.

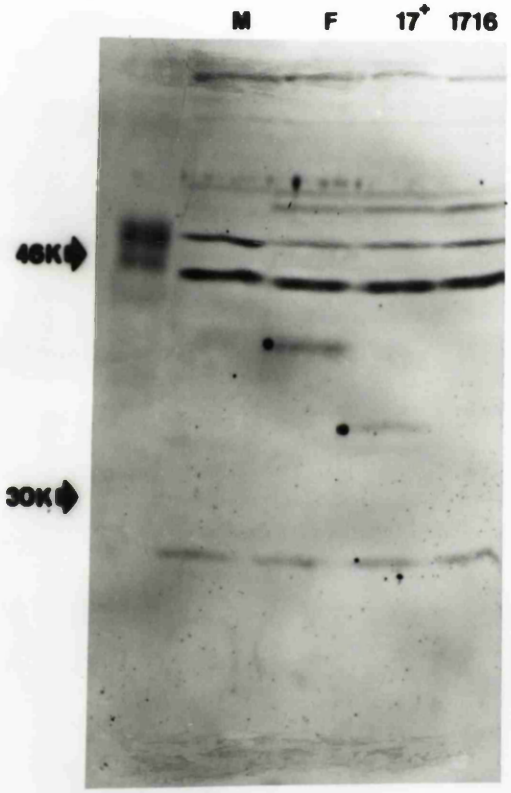
3.3.7. Polyclonal antisera production and detection of ICP34.5 in HSV-1 infected cells.

Two rabbit antisera to ICP34.5 were produced using concentrated samples of partially purified ICP34.5. Two mg of protein (containing approximately 100ug *E.coli* -expressed ICP34.5) were used for each immunization and both rabbits given 4 boosts before being bled out. Various dilutions of the antisera were used to try and detect ICP34.5 in both HSV-1 and HSV-2 infected cell extracts by Western blotting. Antibody incubation was carried out at temperatures of 4°C, RT and 37°C. Incubation of the antiserum overnight, at RT, at a 1/20 dilution was chosen as the preferred conditions. Only 1 of the 2 antisera recognised ICP34.5 in HSV-1 infected cell extracts. Neither antiserum recognised an ICP34.5 homologue in HSV-2 strain HG52, even at a dilution as low as 1/10 (data not shown).

It has previously been shown that HSV-1 strain 17^+ produces a lower M_r ICP34.5 than HSV-1 strain F (MacKay *et al.*, 1993). This difference is due at least partly to an amino acid sequence, PAT, repeated 10 times in the HSV-1 strain F protein, but only 5 times in the HSV-1 strain 17^+ protein. Peptide antiserum, 78, was ~30 fold less sensitive at detecting the HSV-1 strain 17^+ protein than the HSV-1 strain F protein (MacKay *et al.*, 1993) and this could be due to one (or both) of the following reasons: either in HSV-1 strain 17^+ infected cells compared to HSV-1 strain F infected cells there are lower levels of ICP34.5 synthesized, or because the peptide antiserum was raised against 10 copies of the PAT repeat, the difference could simply be due to antibody affinity differences.

Using the rabbit polyclonal antiserum, HSV-1 strain 17^+ ICP34.5 was detected at comparable levels to the HSV-1 strain F polypeptide (fig.3.32.) indicating that the

Figure 3.32. Detection of ICP34.5 in HSV-1 infected cells using the protein antisera. Specific recognition of bands corresponding to ICP34.5 in HSV-1 strain F and HSV-1 strain 17^+ infected cells using the protein antiserum at a 1/20 dilution. Bands were visualised using ECL (Amersham). Lanes are labelled at the top of each track. Molecular weight markers are on the left-hand side.


M
F
17+
1716
46K
30K

previous differences in detection had been due to antibody affinity differences and that the protein accumulated to equivalent levels in cells infected with either strain. In HSV-1 strain 17^+ infected cell extracts, 2 bands are detected which are absent from cells infected with the RL1 deletion variant 1716, one corresponding to ICP34.5 which is indicated, and another fainter one running just below ICP34.5. It would be reasonable to assume that the HSV-1 strain 17^+ stock is heterogeneous with respect to the number of copies of the DNA sequence coding for the PAT repeat and this lower M_r band represents an ICP34.5 polypeptide with less than the believed 5 copies of PAT. If as we suspect, the previous differences in detection of the strain 17^+ polypeptide compared to the strain F polypeptide were due to antibody affinity differences, this would explain why the lower molecular weight band has not previously been observed using the peptide antiserum. Alternatively, this lower molecular weight band may simply be a breakdown product of the larger polypeptide. If ICP34.5 is degraded or processed in infected cells, the previous difficulties in detection of the protein would be explicable.

Unfortunately the polyclonal antiserum produced did not have as high an affinity as had been anticipated and was no more sensitive at detecting HSV-1 strain F ICP34.5 than the available peptide antiserum. We were unable to detect ICP34.5 from HSV-1 strain F, HSV-1 strain 17^+ and HSV-2 strain HG52 by immunoprecipitation (data not shown).

3.3.8. Detection of ICP34.5 by immunofluorescence.

During the course of this study HSV-1 strain F ICP34.5 was localized to the cytoplasm of infected cells by immunofluoresence using the antipeptide serum, 78 (section 3.2.10.). These experiments were repeated using the polyclonal antiserum. Before use the polyclonal antiserum was preincubated with *E.coli* cell extracts to cut down non-specific binding (section 2.2.35.). Slides were prepared as described previously except that the cells were incubated with the first antibody under 3 different conditions: (i) RT, 1hr; (ii) 37°C, 30minutes; (iii) 37°C, 1hr. The best results were obtained when incubation was carried out at 37°C for 1hr.

As observed previously, no distinct localized staining could be detected in mock (fig.3.33a.) or RL1 deletion mutant (in this case 1771) (fig.3.33b.) infected cells whereas dense punctate patches of staining were observed in the cytoplasm of strain 17^+ infected cells (fig.3.33c.) Thus the distribution of ICP34.5 following both HSV-1 strain 17^+ and HSV-1 strain F infection appears identical.

Figure 3.33. Localization of ICP34.5 to the cytoplasm of HSV-1 strain 17^+ infected cells using the rabbit protein antiserum.

Immunofluoresence of mock (top), 1771 (middle) and HSV-1 strain 17^+ (bottom) infected cells incubated with the ICP34.5 rabbit protein antiserum a 1/50 dilution. Anti-rabbit IgG FITC conjugate was used as the second antibody.

Chapter 4- Characterization of a U_S deletion variant

4.1. Introduction

During the analysis of single plaque isolates from a transfection, an isolate was identified which had an apparent deletion in the short unique(U_S) region of the HSV-1 genome. This variant had a small plaque morphology on BHK21/C13 cells and in both one-cycle and multi-cycle growth analysis, it had an extended lag phase, although it reached final titres equivalent to the wild-type virus HSV-1 strain 17^+. This suggested that the variant, designated 1772, had a defect in either adsorption, penetration or cell-to-cell spread following infection of BHK21/C13 cells. I decided to determine the precise location of the deletion in 1772 and attempt to further characterize its apparent growth defect.

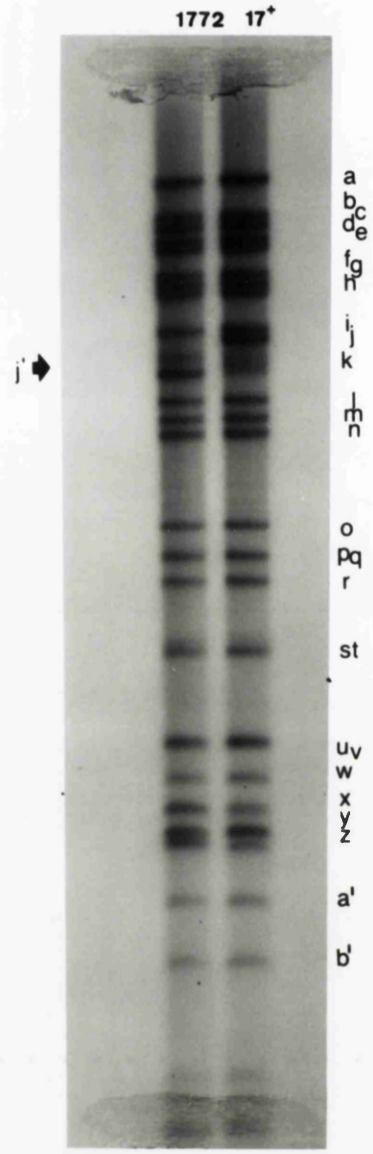
4.2. Isolation of 1772 and localization of the deletion in U_S.

While analysing single plaques, one variant was isolated which had an apparent 500-700bp deletion in the BamHI j restriction enzyme fragment (fig.4.1.) within the U_S region of the HSV-1 genome (see fig.3.6 for HSV-1 strain 17^+ BamHI restriction enzyme map). Confirmation that the deletion was in BamHI j was provided by Southern blotting (section 2.2.21.).

One ug of both wild-type HSV-1 strain 17^+ DNA and 1772 DNA was digested with BamHI and the resulting fragments separated on 0.8% agarose gels. Four duplicate sheets of nylon membrane were prepared as described in section 2.2.21. and the DNA probed with 4 separate randomly primed subfragments from the wild-type BamHI j restriction enzyme fragment. These probes are listed below.

Figure 4.1. BamHI restriction enzyme profile of 1772.

Autoradiograph of a BamHI digest of viral DNA. The wild-type BamHI restriction enzyme fragments are indicated on the right-hand side. The novel 1772 BamHI j fragment is marked by an arrow and is designated by the letter of the band from which it was derived plus a prime symbol (‘). Lanes are labelled at the top of each track.


1772
17+
j'
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
u
v
w
x
y
z
a'
b'

Probe	Restriction fragment	Nucleotide positions*	Size
1	BamHI - PvuII	136288-138072	1784bp
2	PvuII - BamHI	138574-142746	4172bp
3	BstXI - BstXI	139371-139844	473bp
4	BstXI - BamHI	139844-142746	2902bp

* According to McGeoch et.al.(1988a).

It was hoped that Southern blotting using these 4 separate probes would help localise the deletion in 1772. However this was not the case. Similar results were obtained with all 4 probes (see fig 4.2. for examples). In each case, the probe hybridized to a 6.4kb band the expected size of BamHI j in the HSV-1 strain 17^+ digest, but to a 5.7-5.9kb band in the 1772 digest. This result did however, confirm that the deletion was in BamHI j.

Proteins encoded by this region of the HSV-1 genome are a protein kinase, gG, gD, US5, gI and gE (US3-7) (fig. 4.3.). Because 1772 had a small plaque morphology on BHK21/C13 cells it was likely that one of the "non-essential" glycoproteins (Longnecker and Roizman, 1986, 1987; Longnecker *et al.*, 1987) was either totally or partially deleted and we decided to sequence the end-points of the deletion to identify which gene(s) the deletion affected.

4.3. Sequence analysis of 1772

For sequence analysis of 1772, the BamHI j restriction enzyme fragment from this variant was cloned into the BamHI restriction enzyme site of pGEM 3zf(-). Restriction enzyme analysis was carried out to localize a fragment, of suitable size for sequencing (~1kb), which contained the 1772 deletion.

Digestion of wild-type HSV-1 strain 17^+ BamHI j, cloned into pGEM 3zf(-), with BstXI and SacI (recognition sites for these enzymes are not found in pGEM3zf(-)) gives 6 fragments; 1 ~4.7kb in size which contains the plasmid backbone and 1.6kb of HSV-1 sequence, and 5 other HSV-1 fragments of 1503, 573, 311, 633 and 1883bp.

Figure 4.2. Detection of BamHI j by Southern blotting.

An autoradiograph of a Southern blot of BamHI digested HSV-1 strain 17^+ and 1772 DNA probed with 2 subfragments from BamHI j (see page 108). The deleted BamHI j band in 1771 is marked j'. M is a molecular weight marker tract. The 4172bp probe contains contaminating pAT153 sequences which hybridize to the 1.6kb fragment in the marker tract.

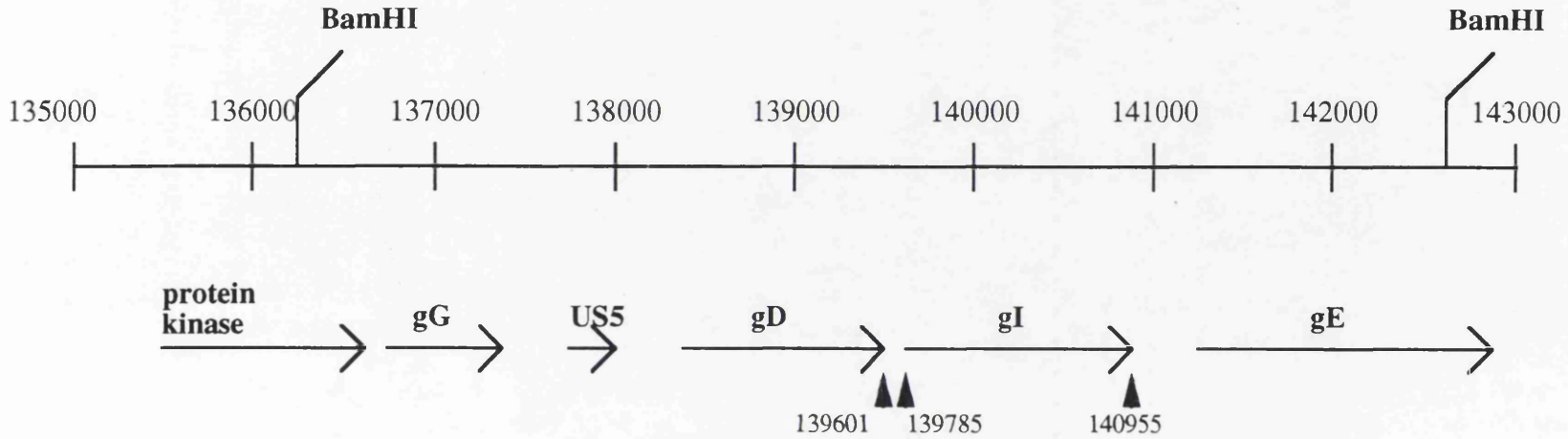
4172 bp probe

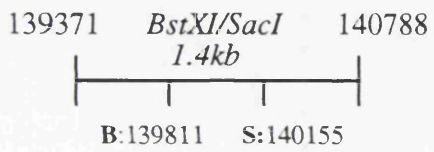
2902 bp probe

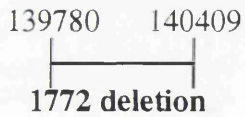
Figure 4.3. Genetic content of the BamHI j restriction enzyme fragment.

Diagramatic representation of the genes encoded in the BamHI j restriction enzyme fragment. Nucleotide positions (McGeoch *et al.*, 1988a) are shown on the top line. The position of the termination-codon of gD (np 139601), the initiation-codon of gI (np 139785) and the termination-codon of gI (np 140955) are marked, and their positions indicated with arrowheads.

The deletion in 1772 was mapped to a 1.4kb BstXI/SacI sub-fragment of BamHI j from which a BstXI (B) and a SacI (S) restriction enzyme site had been deleted. Sequence analysis of this fragment revealed that 1772 has a 630bp deletion which removes the initiating methionine of gI and extends to the 3' part of the gene.


BamHI
BamHI
135000
136000
137000
138000
139000
140000
141000
142000
143000
protein kinase
gG
US5
gD
gI
gE
139601
139785
140955


139371
BstXI/SacI
1.4kb
140788
B:139811
S:140155


139780
140409
1772 deletion

Digestion of 1772 BamHI j cloned into pGEM 3zf(-) gives only 4 fragments; the ~4.7kb fragment containing the plasmid backbone, the 1883bp and the 1503bp fragments. The 573, 311 and 633bp fragments (which are adjacent to each other on the HSV-1 genome) are missing and are replaced by a single fragment of ~800bp. This indicates that the deletion in 1772 had removed 2 restriction enzyme sites- a BstXI site at np 139811 and a SacI site at np 140155-and the deletion is thus contained within a sub-fragment of BamHI j (np 139371- np 140788) which is 1.4kb in the wild-type virus HSV-1 strain 17^+, but only ~800bp in 1772.

This fragment was purified, blunt-ended and cloned into M13. It was sequenced in both directions as described in section 2.2.25. using a M13 universal primer (fig.4.4). This revealed that 1772 had a 630bp deletion removing np 139780-np 140409. The deletion was in the region of U_S encoding gI and removed the initiating ATG of the gene and 625bp of the 5' end; 546bp of the 3' end of the gene were left intact. The sequence of 1772 showed 100% homology (~200 bases were read) with the published sequence of this region on either side of the deletion (fig 4.5.), showing that there was no rearrangement at the deletion endpoints.

4.4. Immunoprecipitation of gI using MAb 3104.

Cells infected with HSV-1 strain 17^+ or 1772 were labelled 3-7hrs post-infection with ^{35}S methionine, harvested into 1ml extraction buffer and immunoprecipitated using MAb 3104, specific for gI (section 2.2.39.). In extracts from HSV-1 strain 17^+ infected cells MAb 3104 precipitates both the immature form of gI (pgI) and mature forms of gI (fig.4.6.). Due to their association in infected cells with gI, gE in both the immature (pgE) and mature (gE) forms are coprecipitated by the MAb 3104-gI complex. The amount of coprecipitation of gE with the MAb 3104-gI complex is known to vary from experiment to experiment , and can be disrupted by heat and SDS (Johnson *et al.*, 1988).

Neither gI or gE were precipitated by MAb 3104 in mock or 1772 infected cells, confirming the sequencing data which had revealed that in 1772, gI is deleted. The

Figure 4.4. Sequence analysis of 1772.

An autoradiograph of the DNA sequence of the HSV-1 strain 17^+ variant 1772, showing the position of the deletion which removes np 139780-np 140409. Sequences were obtained by the dideoxynucleotide sequencing method using T7 DNA polymerase.

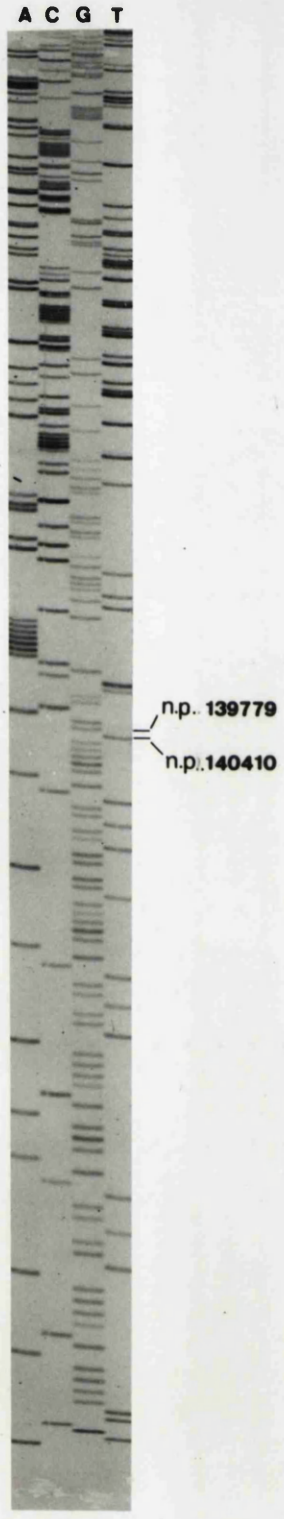

A C G T
n.p. 139779
n.p. 140410

Figure 4.5. Computer analysis of the 1772 deletion.

Computer analysis of the 1772 deletion was carried out on a MicroVAX computer using the University of Wisconsin Computer Genetics Group software package. The sequenced fragment of 1772 shows 100% homology with the published sequence of HSV-1 strain 17^+ on either side of the deletion. The sequence of the BstXI/SacI BamHI j sub-fragment from 1772 is on the top line, the wild-type HSV-1 strain 17^+ sequence is on the bottom line. Homologous sequences are indicated by vertical bars. The nucleotide numbers of the last undeleted base on either side of the deletion are marked.

```
SCORES      Init1: 304 Initn: 304 Opt: 304
            100.0% identity in 76 bp overlap

                                                 140410
                                                    |          10          20          30
726a                                                ACCCCCTCGACCACCACCTCCACCCCCTCG
                                                    ||||||||||||||||||||||||||||
Hsv1.S GTATACCAACCCGCCCCTAACCAGGCCTCCACCCCCTCGACCACCACCTCCACCCCCTCG
    14038

               40          50          60          70
726a   ACCACCATCCCCGCTCCCTCGACCACCATCCCCGCTCCCCAAGCAT
       ||||||||||||||||||||||||||||||||||||||||||||
Hsv1.S ACCACCATCCCCGCTCCCTCGACCACCATCCCCGCTCCCCAAGCATCGACCACGCCCTTC
    14044

Hsv1.S CCCACGGGAGATCCAAAACCACAACCTCCCGGGGTCAACCACGAACCCCCATCTAATGCC
    14050


726a /rev
_Dub0:[Hsv.Dna]Hsv1.Seq



  SCORES      Init1: 520 Initn: 684 Opt: 520
              100.0% identity in 130 bp overlap

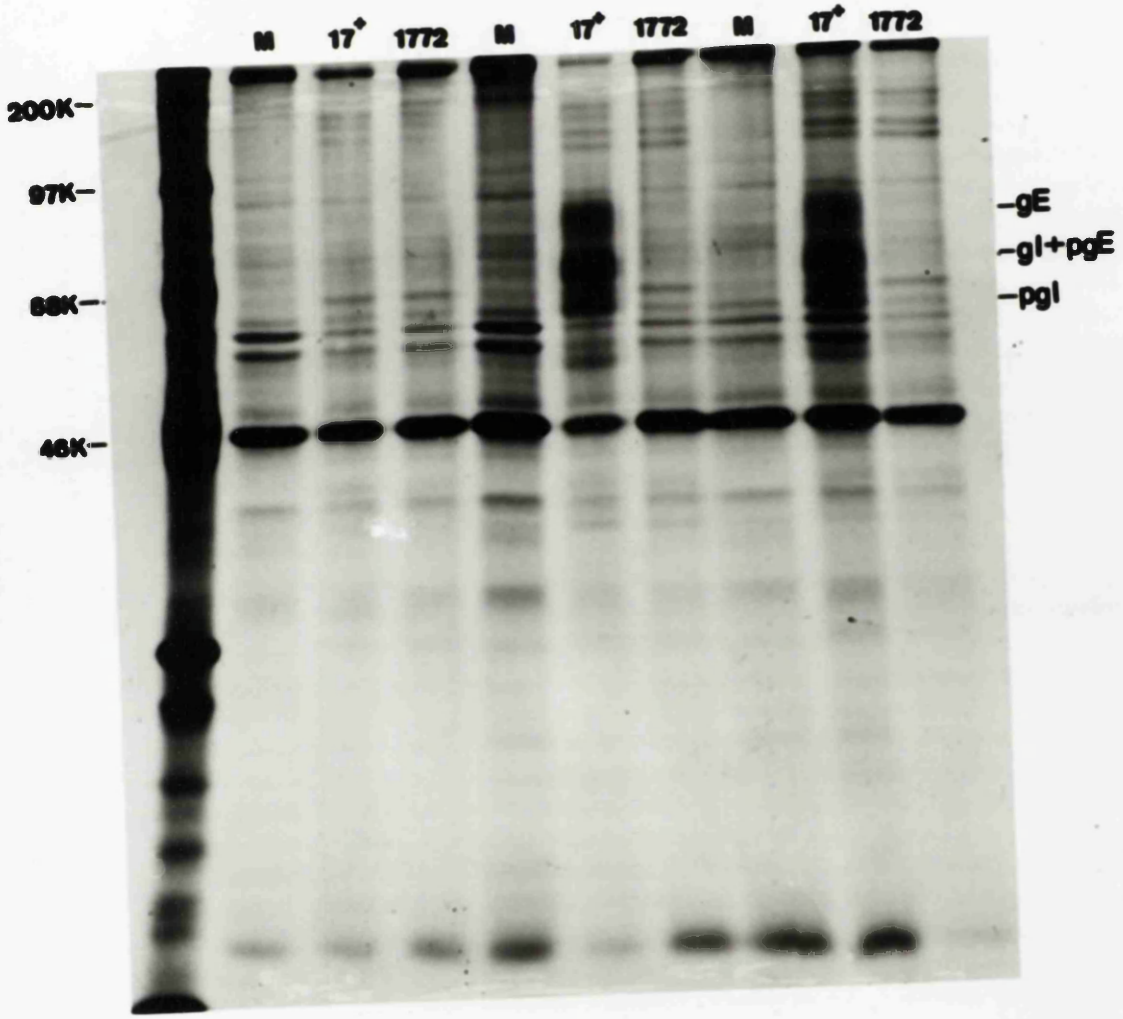
                                                          10          20          30
  726l                                                    TTGGGATGGGACCTTAACTCCATATAAAGC
                                                          ||||||||||||||||||||||||||||||
  Hsv1.S AATGGGTGCGGGGGGGTCAGGTCTGCGGGGTTGGGATGGGACCTTAACTCCATATAAAGC
         13962

                  40          50          60          70          80          90
  726l    GAGTCTGGAAGGGGGGAAAGGTGGACAGTCGATAAGTCGGTAGCGGGGGACGCGCACCTG
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
  Hsv1.S GAGTCTGGAAGGGGGGAAAGGTGGACAGTCGATAAGTCGGTAGCGGGGGACGCGCACCTG
         13968

                100         110         120         130         140         150
  726l    TTCCGCCTGTCGCACCCACAGCTTTTTTTGCGAACCGTCCACCCCCTCGACCACCACCTC
          ||||||||||||||||||||||||||||||||||||||
  Hsv1.S TTCCGCCTGTCGCACCCACAGCTTTTTTTGCGAACCGTCCCGTTCCGGGATGCCGTGCCG
         13974                                    |
                                                  139779
```

Figure 4.6. Immunoprecipitation of gI using MAb 3104.

Immunoprecipitation of gI and gE and their precursors, pgI and pgE respectively. BHK21/C13 cells were labelled with ^{35}S methionine from 3-7hrs post infection. Extracts were precipitated with either ascites control or MAb 3104 specific for gI . The positions of gI , gE, pgI and pgE are indicated. The molecular mass markers are indicated on the left-hand side. Tracks are labelled at the top of the gel.


M
17+
1772
M
17+
1772
M
17+
1772
200K
97K
68K
46K
gE
gI+pgE
pgI

specificity was indicated by the failure to precipitate gI or gE polypeptides with control ascites.

4.5. *In vitro* growth characteristics of 1772

The *in vitro* growth characteristics of 1772 were determined on BHK21/C13 cells and compared to the wild-type virus HSV-1 strain 17^+. In multi-cycle growth experiments 1772 had an extended lag phase in comparison to the wild-type virus., although this lag was not as obvious in one-cycle growth experiments. Both HSV-1 strain 17+ and 1772 had comparable final titres (fig 4.7(a).&4.7 (b).). This indicated that 1772 was delayed in entry into BHK21/C13 cells and suggested a defect in adsorption, penetration or cell fusion. The stocks of HSV-1 strain 17^+ and 1772 had similar particle:p.f.u. ratios; 14:1 and 5:1 respectively, indicating the extended lag phase was not due to interference by non-infectious particles.

4.6. Kinetics of polypeptide synthesis by 1772

To examine whether 1772 was delayed in the initiation of protein synthesis following infection of BHK21/C13 cells, we looked at the kinetics of synthesis of each class of polypeptide following 1772 infection. Cells were infected with wild-type HSV-1 strain 17^+ and 1772 at a m.o.i. of 5 p.f.u./cell and harvested at various times post-infection. Proteins were separated by SDS-PAGE and analysed by Western blot as described in section 2.2.37.. Synthesis of all classes of HSV-1 polypeptides was examined; Vmw110 as a representative immediate-early polypeptide; 65KDBP, a representative early polypeptide and 21K a member of the true-late class of HSV-1 polypeptides.

Initial experiments indicated that 1772 was delayed in the initiation of synthesis of all classes of HSV-1 polypeptides. This was consistent with a delay in entry into BHK21/C13 cells. However, when different extracts were prepared and the experiments repeated, no difference was observed between the wild-type virus and 1772. Subsequent experiments

Figure 4.7 (a). One-cycle growth curve of 1772.

One-cycle growth curve of HSV-1 strain 17^+ and 1772. BHK21/C13 cells were infected at a m.o.i. of 10 p.f.u./cell, and following absorption at 37°C for 1hr, the monolayers were washed twice with PBS/calf, and incubation continued at 37°C. Plates were harvested at 0, 2, 4, 6, 8, 12, and 24 hrs post-infection and titrated on BHK21/C13 cells.

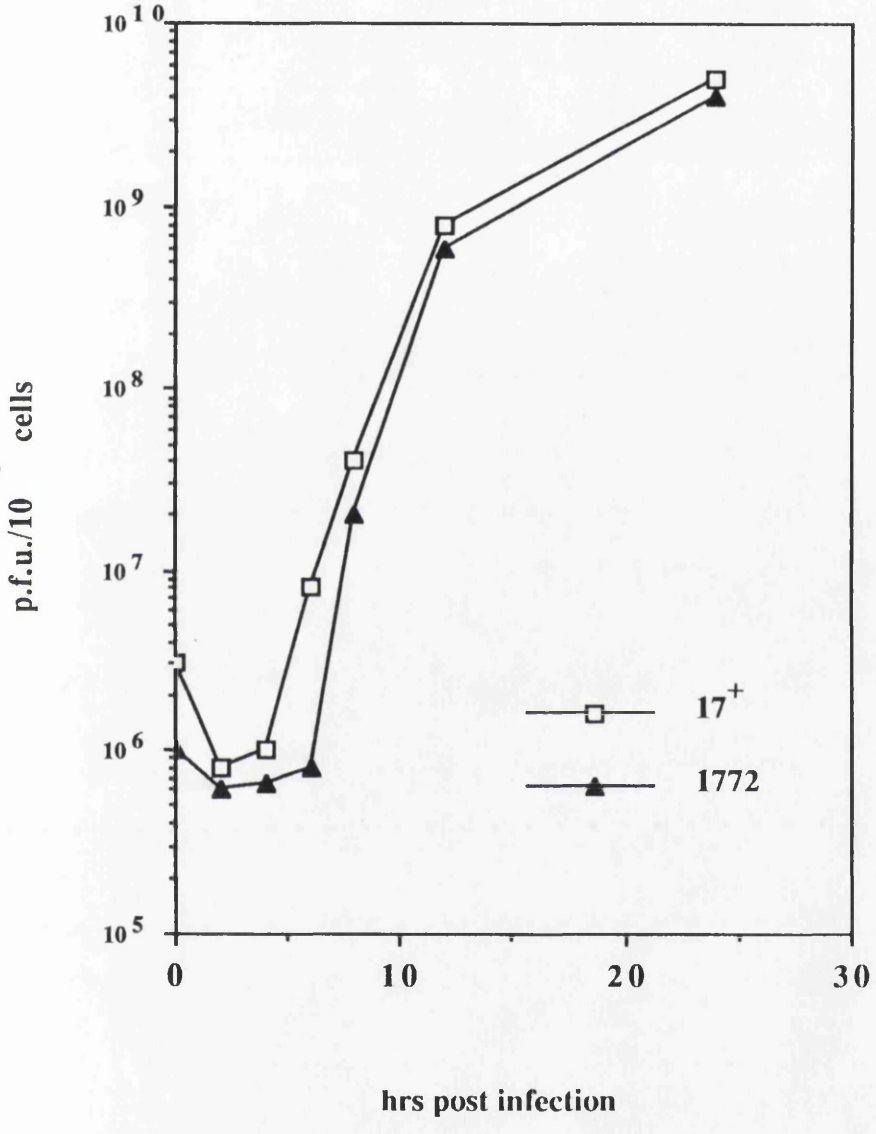
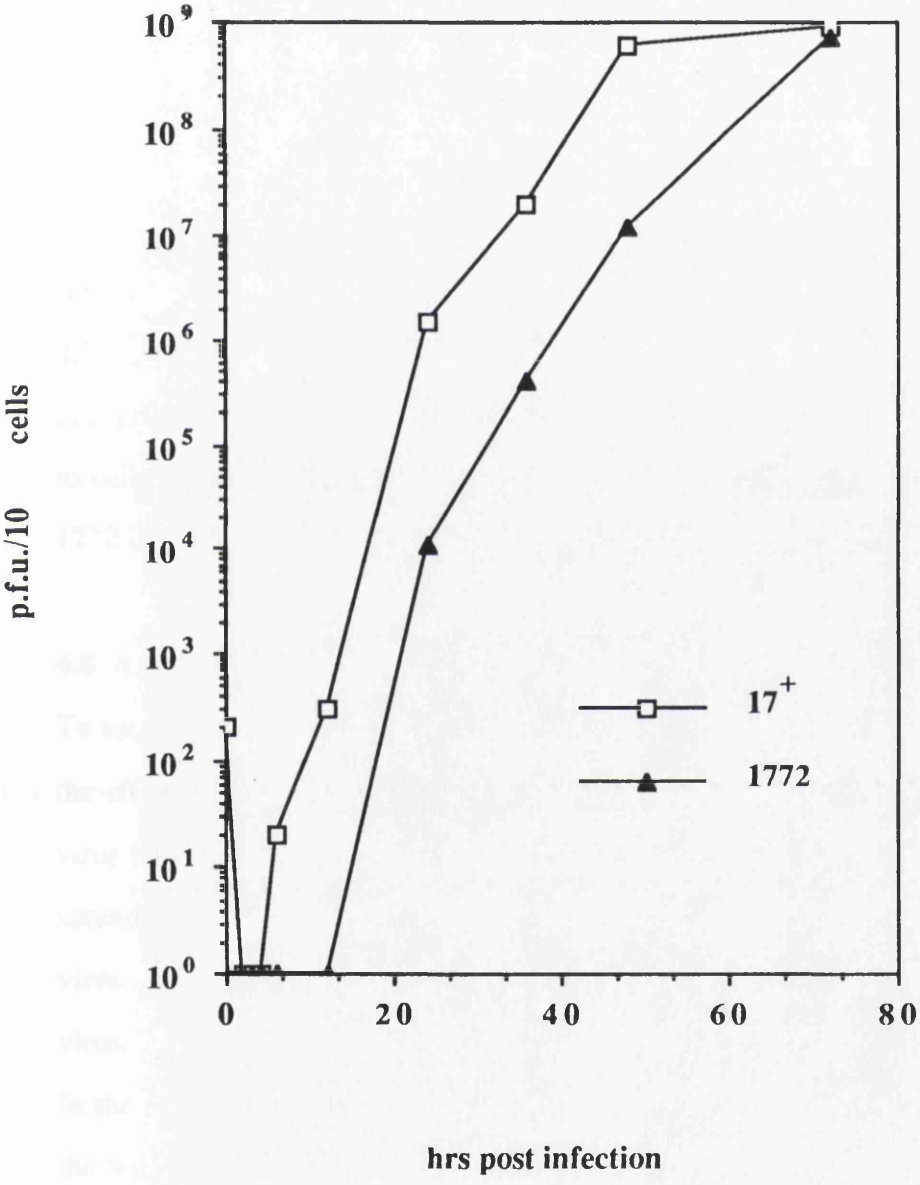

p.f.u./10 6 cells
10 10
10 9
10 8
10 7
10 6
10 5
0
10
20
30
17+
1772
hrs post infection

Figure 4.7 (b). Multicycle growth curve of 1772.

A multicycle growth curve of HSV-1 strain 17^+ and 1772 was carried out on BHK21/C13 cells. Cells were infected at a m.o.i. of 0.001 p.f.u./cell, and following adsorption at 37°C for 1hr, the monolayers were washed twice with PBS/calf and incubation continued at 37°C. Plates were harvested at 0, 2, 4, 6, 8, 12, 24, 36,48 and 72 hrs post-infection and titrated on BHK21/C13 cells.


p.f.u./10^6 cells
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
20
40
60
80
17+
1772
hrs post infection

have yielded variable results, therefore we are unable to conclude whether or not 1772 is delayed in the initiation of protein synthesis following infection of BHK21/C13 cells.

4.7. Adsorption and penetration of 1772 onto cells.

HSV-1 glycoproteins play a crucial role in the initial stages of virus adsorption and penetration into tissue culture cells. Although gI has not previously been reported to play a role in virus adsorption or penetration in tissue culture it is possible that in some cell types gI may play a role in these initial stages of virus infection.

The ability of 1772 to adsorb to and penetrate BHK21/C13 cells was compared to the wild-type virus HSV-1 strain 17^+ as described in sections 2.2.4. and 2.2.5. In both cases no difference in adsorption / penetration kinetics was observed between the wild-type virus and 1772 (fig 4.8. & fig 4.9.). Attempts to examine the percentage of input virus adsorbed to cells were unsuccessful due to poor labelling of virion preparations. It is possible that 1772 is defective in cell-to-cell spread and this was later analysed.

4.8. Analysis of cell-to-cell spread of 1772

To ascertain whether cell-to-cell spread is impaired during 1772 infection, we determined the effect of the presence of neutralizing antibodies (human serum) in the growth media on virus titre. The rationale for this experiment was that if gI plays a role in direct cell-to-cell spread, the presence of neutralizing antibodies should cause a reduction in virus titre as virus cannot spread *via* the alternative pathway which is through absorption of released virus. The results are summarized in figure 4.10.

In the absence of neutralizing antibody in the overlay, 1772 reached comparable titres to the wild-type virus HSV-1 strain 17^+ again with a delay in the lag phase. However, in the presence of human serum, the titre of 1772 was reduced by ~30 fold compared to the wild-type virus after 96hrs growth on BHK21/C13 cells and c.p.e. was much less extensive indicating poorer spread of the virus. These results indicate that gI does play a direct role

Figure 4.8. Adsorption of 1772 onto cells.

Pre-cooled BHK21/C13 cells on 50mm plates were infected with 400 p.f.u./plate HSV-1 strain 17^+ or 1772 at 4^oC. 0, 15, 30, 45, 60, 80, 100 , 120 and 240 minutes post-infection unadsorbed virus was removed by washing twice with PBS/calf and the plates overlaid with 4ml EMC10%. Following incubation at 37^oC for 2 days, plates were stained and the number of plaques on each counted. Bound virus was calculated as a percentage of final virus bound at T=240. Each timepoint represents the mean of 3 individual plates.

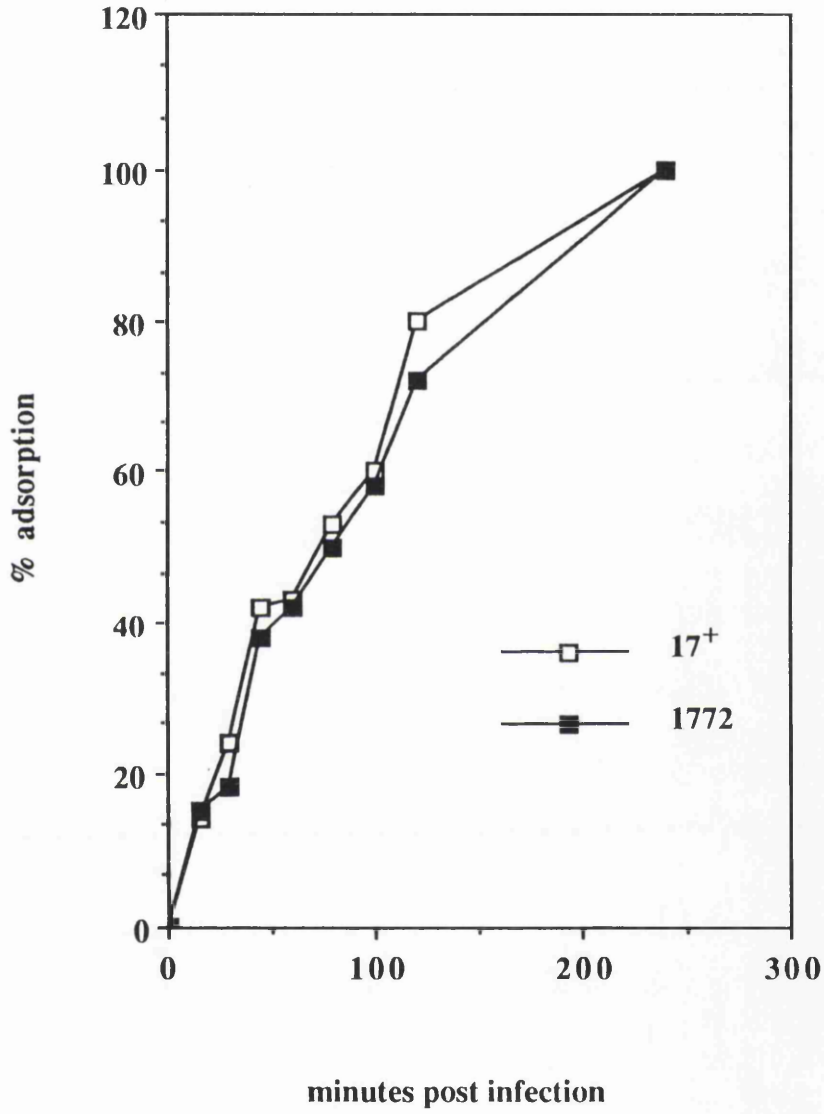

120
100
80
60
40
20
0
% adsorption
0
100
200
300
minutes post infection
17+
1772

Figure 4.9. Penetration of 1772 into cells.

Pre-cooled BHK21/C13 cells were infected with 400 p.f.u. HSV-1 strain 17^+ or 1772. Following absorption at 4°C for 1hr, plates were washed twice to remove any unabsorbed virus, overlaid with 4mls ETC10 and incubated at 37°C to enable virus penetration. 0, 5, 10, 20, 30, 45 and 60 minutes post-incubation at 37°C, media was removed, 1ml citrate buffer added to inactivate unpenetrated virus, and incubation continued at RT for a further 5 minutes. Plates were washed twice with PBS/calf to remove the citrate buffer, overlaid with 4mls EMC10% and incubated at 37°C for 2 days. Plates were stained and the number of plaques on each counted. Virus penetration at each timepoint was calculated as a percentage of the virus bound at T= 60. Each timepoint represents the mean of 3 individual plates.

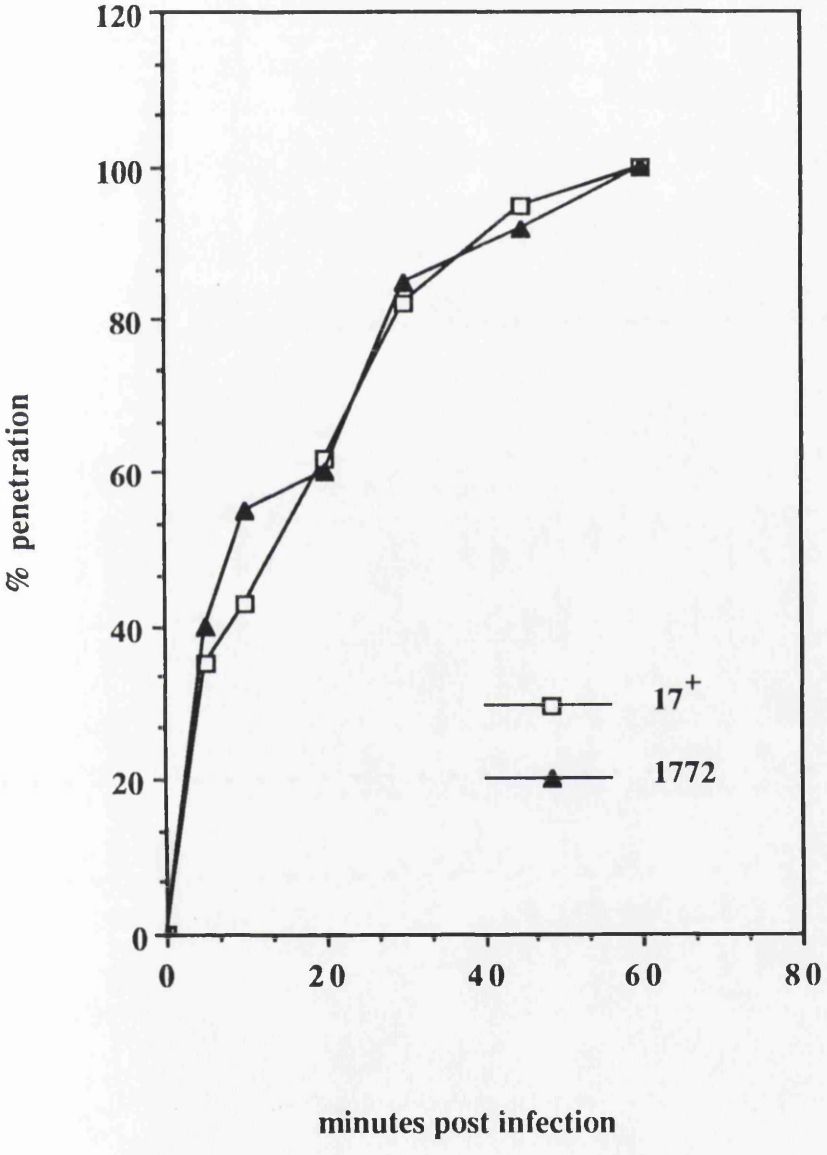
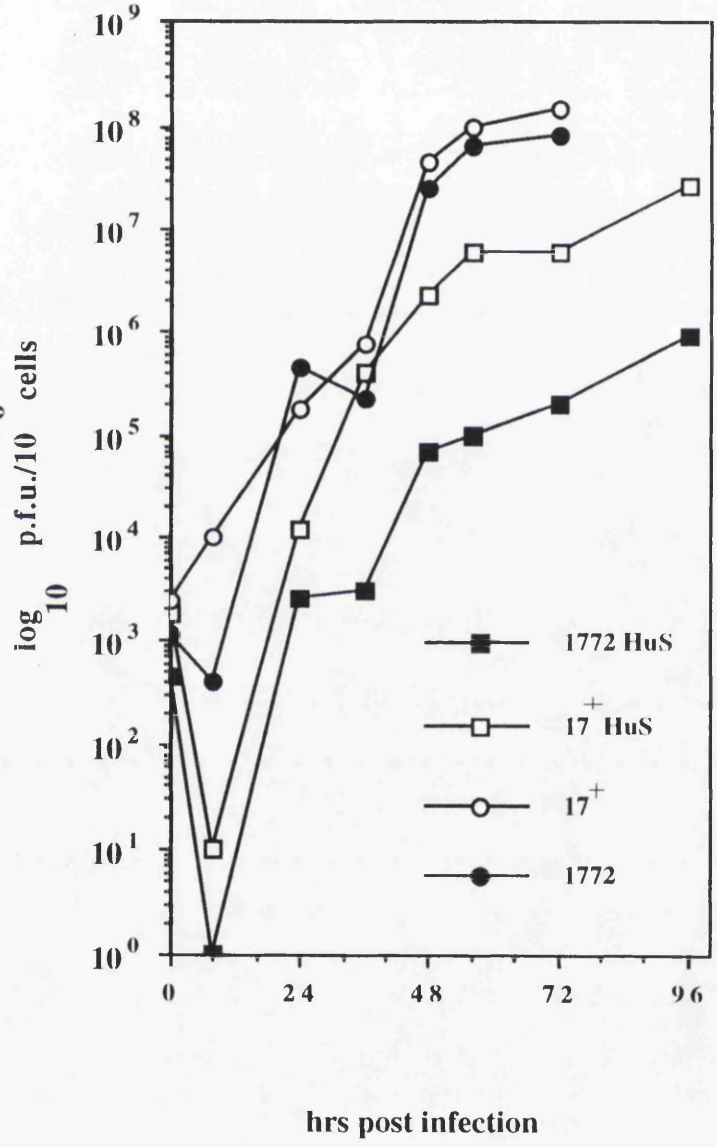

120
100
80
60
40
20
0
0
20
40
60
80
% penetration
minutes post infection
17+
1772

Figure 4.10. Analysis of cell-to-cell spread in 1772.

Confluent monolayers of BHK21/C13 cells were infected with 0.001 p.f.u HSV-1 strain 17^+ or 1772. After absorption at 37°C for 1hr, plates were washed twice with PBS/calf, overlaid with ETC10 +/- neutralizing antibodies (human serum) and incubation continued at 37°C. Plates were harvested at 0, 8, 24, 36, 48, 56, 72 and 96 hrs post-infection and titrated on BHK21/C13 cells.


10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
24
48
72
96
$\log_{10}$ p.f.u./10^6 cells
1772 HuS
17$^+$ HuS
17$^+$
1772
hrs post infection

in cell-to-cell transmission of HSV-1 strain 17^+. As expected the rate of HSV-1 growth was inhibited by the presence of human serum.

4.9. Neurovirulence of 1772

Previous studies (Meigner *et al.*, 1988; Nishiyama *et al.*, 1993) have examined the neurovirulence of all genes in the U_S region of the HSV-1 genome, with the exception of US7. In PRV gp63, the HSV-1 gI homologue, is required in conjunction with the HSV-1 gE homologue (gI) for viral neurotropism. Viral mutants with deletions in either (or both) of these genes are unable to infect specific classes of neurons in the rat retina.

The neurovirulence of 1772 was determined by estimation of its LD_{50} value following intracerebral inoculation of 3-week old BALB/c mice compared to the wild-type virus HSV-1 strain 17^+. As we could not anticipate the virulence of 1772 it was injected at a range of doses (10^2-10^6 p.f.u./mouse). The wild-type virus was injected at doses of 10^1 and 10^2. The results are shown in table 4.1..

As expected the wild-type virus was highly virulent, with only 1 survivor at a dose of 10^1, giving a LD_{50} of 7 p.f.u./mouse. All deaths occurred by day 6 post-inoculation.

In contrast 1772's neurovirulence was attenuated . When injected at doses of 10^5 or 10^6 all mice died, but not until an average of 4 days post-inoculation. Two mice died at doses of 10^4 and 10^3 post-inoculation, but again there was a delay in the time of death (6-7 days post-inoculation). No mice died at a dose of 10^2 p.f.u./mouse, giving an LD_{50} of 3.6 $x10^3$ p.f.u./mouse. These results indicate that gI may play an important role in infection of some cell types *in vivo* as is observed with its PRV homologue. Unfortunately time constraint did not allow us to look at 1772 virulence *via* a peripheral route of inoculation.

Table 4.1. Neurovirulence of 1772 following intracerebral inoculation of 3-week old BALB/c mice.

virus \ dose	10^1	10^2	10^3	10^4	10^5	10^6	LD50 p.f.u./ mouse
17 +	3/4	4/4	ND	ND	ND	ND	7
1772	ND	0/4	2/4	2/4	4/4	4/4	3.6×10^3

*no. of deaths/ no. injected
ND= not done.

Chapter 5 - Discussion

5.1.1. Objectives

The work presented in this thesis had three main objectives: (i) to verify that the region between Vmw110 and the 'a' sequence contains an ORF coding for ICP34.5 in HSV-1 strain 17^+, (ii) to confirm, in HSV-1 strain 17^+, that this protein plays a crucial role in neurovirulence following intracerebral inoculation of mice , and (iii) to further characterize ICP34.5.

5.1.2. Reinvestigation of the HSV-1 strain 17^+ RL1 sequence.

The existence of a neurovirulence determinant in IR_L and the adjacent region of U_L was first demonstrated by Thompson *et al.* (1983). These workers had previously found that the HSV1/2 intertypic recombinant RE6 (Marsden *et al.*, 1978) was completely non-neurovirulent in mice following intracerebral inoculation of $3.2x10^7$ p.f.u.. Since 10 p.f.u. of either HSV-1 strain 17^+ or HSV-2 strain HG52 (the "parental" strains of this recombinant) were lethal for mice on intracerebral inoculation, RE6 was at least 10 million-fold less neurovirulent than the wild-type strains from which it was derived (Thompson and Stevens, 1983b). By transfecting intact RE6 DNA with cloned fragments of DNA from HSV-1 strain 17^+ onto tissue culture cells , and selecting for neurovirulent recombinants *in vivo*, it was revealed that HSV-1 information residing between 0.71 and 0.83m.u. encoded a gene function(s) which was a determinant of neurovirulence in HSV-1 strain 17^+ (Thompson *et al.*, 1983). Further *in vitro* recombination / *in vivo* selection experiments demonstrated that recombination with a cosmid clone of the HSV-1 strain 17^+ HindIII c fragment (0.64-0.87 m.u.) could restore a neurovirulent phenotype to RE6. Recombinants generated with this fragment had LD_{50} values 5 orders of magnitude lower than RE6, with a $LD_{50}=1.1x10^3$ (Thompson *et al.*, 1985). In this study however, other recombinants were generated which had LD_{50} values intermediate between RE6 and wild-type virus (egs. $1x10^4$ and $2.2x10^5$). Sequence analysis was not carried out across the 'restored' sequences in any rescuant, complicating interpretation of these results. Subsequently, it was found that

sequences residing between 0.698 and 0.721 m.u. could confer partial neurovirulence on RE6. Recombinants generated with cloned DNA spanning this region were only 50-fold more neurovirulent that RE6 (Thompson and Wagner, 1988), but again sequence analysis was not carried out on the 'restored' fragments. These anomalies were not discussed in later papers, and the authors went on to demonstrate that a 1.6kb cloned fragment (SstI/ XhoII, 0.82-0.832m.u.) conferred a highly neurovirulent phenotype to RE6 (Thompson *et al.*, 1989).

In our laboratory, this region of the HSV-2 strain HG52 genome had previously been found to encode neurovirulence related sequences (Taha *et al.*, 1989a, 1989b), which were not required for virus growth *in vitro*. A variant, JH2604, with a 1.5kb deletion within each copy of R_L, between 0 to 0.02 and 0.81 to 0.83 map units was isolated (Harland and Brown, 1985). This deletion removed 1 complete copy of the 17bp DR1 element of the 'a' sequence and terminated 522bp upstream of the 5' end of RL2 (Taha *et al.*, 1989b). Following intracerebral inoculation of mice, JH2604 was completely non-neurovirulent and failed to replicate in mouse brain (Taha *et al.*, 1989a). These findings pointed to sequences, located between the 'a' sequence and Vmw110, coding for a protein involved in neurovirulence in both HSV-2 strain HG52 and HSV-1 strain 17^+.

In HSV-1 strain F, one gene product had been localised within the smallest fragment capable of restoring a highly neurovirulent phenotype to both JH2604 and RE6 (Ackermann *et al.*, 1986; Chou and Roizman, 1986). By means of an antibody produced against 10 copies of the trimer Pro-Ala-Thr (PAT), encoded by the HSV-1 strain F RL1 ORF, which predicted a protein of 358 amino acids in size, a 43.5K protein was specifically detected in HSV-1 strain F infected cell extracts. This protein was designated ICP34.5 (Ackermann *et al.*, 1986) and was shown to be a determinant of HSV-1 strain F neurovirulence (Chou *et al.*, 1990).

In HSV-1 strain 17^+, the ORF ascribed to ICP34.5 was "thoroughly disrupted" (McGeoch *et al.*, 1988a; Perry and McGeoch, 1988) and the existence of a gene in this locality could not be positively established. In 1990, when Chou and Roizman republished the sequence of the gene encoding ICP34.5 in HSV-1 strain F they made 25 alterations to the sequence published in 1986 and reported that the gene contained only 263 codons. The HSV-1 strain

F revised sequence was now almost identical to the published HSV-1 strain 17^+ sequence (Perry and McGeoch, 1988), with the only major difference being a 2bp insert in the HSV-1 strain 17^+ sequence which rendered 60% of the HSV-1 strain 17^+ sequence (including the PAT repeat) out of frame. The ORF identified by Chou and Roizman (1990) was conserved in two other "limited passage" strains but not in HSV-1 strain 17^+. This was attributed to the alleged multipassage history of HSV-1 strain 17^+ which it was claimed, led to the accumulation of mutations. However if ICP34.5 was not produced by HSV-1 strain 17^+ it should at least be modified in neurovirulence if not totally non-neurovirulent compared to other wild-type viruses. This is obviously not the case (see table 3.1.). In fact HSV-1 strain 17^+ is more neurovirulent than HSV-1 strain F (see table 3.3.).

In our laboratory, the isolation and characterization of a deletion variant, 1716 (MacLean, A., *et al.*, 1991a) demonstrated that the region spanning the deleted sequences in JH2604 was also required for neurovirulence in HSV-1 strain 17^+. Sequence analysis of 1716 revealed that it had a 759bp deletion which removed 1 complete 18bp DR1 element from the 'a' sequence and terminated 1105bp upstream of the 5' end of Vmw110. 1716 was found to be totally non-neurovirulent following intracerebral inoculation of BALB/c mice with a LD_{50} of $7x10^6$ p.f.u./mouse compared to <10 p.f.u./mouse for the parental wild-type strain 17^+. Analysis of the *in vivo* growth kinetics of 1716 demonstrated that the non-neurovirulent phenotype was due to an inability to replicate in mouse brain. Rescuants of 1716 had LD_{50} values identical to those of wild-type HSV-1 strain 17^+.

The initial stage of this project involved reanalysis of the HSV-1 strain 17^+ sequence at the terminus of the long repeat, in the region where the putative frame-shift occurred. For this purpose a 1.46kb subfragment of the BamHI k fragment (np 125074-np 126530) was cloned into M13 and sequenced. At the same time, an independently produced XhoI c clone comprising the region spanning the disputed bases was sequenced by Mr A.Dolan in Dr D.J. McGeoch's laboratory. In both cases, the number of bases was identical to that in the published sequence of HSV-1 strain F but not to that in the strain 17^+ published sequence. This showed that the original sequence obtained for this locus of HSV-1 strain 17^+ was

inaccurate and probably came from an atypical plasmid clone. The agreement between the sequences of the XhoI c and BamHI k subfragment clones, and the ability of the sequenced BamHI k subfragment to restore neurovirulence (MacLean, A., *et al.*, 1991a; this thesis) argue strongly that the shorter version of the sequence (residues 823 and 824 removed) must represent HSV-1 strain 17^+ genomic DNA correctly. Correction of the strain 17^+ sequence to that of the newly sequenced clones (Dolan *et al.*, 1992) opens the reading frame proposed by Chou and Roizman (1990) to encode a protein of 248 amino acids with an apparent M_r of 26184. This ORF has been designated RL1 (McGeoch *et al.*, 1991).

The difficulties which arose over the sequencing of this genomic region and which originally cast doubts on the ability of a "non-coding" sequence in strain 17^+ to confer neurovirulence emphasizes the need to rapidly redetermine the sequence of any intrastrain differences which may arise in the future and reinforces the value in determining the equivalent HSV-2 sequence. Colinear regions of homology between HSV-1 and HSV-2 may reveal genes which play an important role in the viral life-cycle and which would be otherwise unidentified. This is illustrated by recent determination of the HSV-2 strain HG52 sequence in R_L and the adjoining parts of U_L (McGeoch *et al.*, 1991), and in which comparison of the HSV-2 UL56 gene sequence with its HSV-1 counterpart revealed an apparent frame-shift adjacent to the 3' end of the HSV-1 ORF. Re-analysis of both sequences confirmed that the HSV-2 sequence was correct, but that in determining the HSV-1 sequence a compression had been incorrectly resolved (McGeoch *et al.*, 1988a; Perry and McGeoch, 1988). Thus UL56 of both HSV-1 and HSV-2 possesses an uncharged and highly hydrophobic section of 18 amino acids which could constitute a membrane-spanning domain, suggesting that UL56 is a membrane associated protein.

Having demonstrated that HSV-1 strain 17^+ contains a gene, RL1, which encodes an ICP34.5 homologue, we wished (i) to confirm that this ORF was translated and (ii) to confirm the importance of this gene in neurovirulence. Several approaches were taken: (i) insertion of a stop-codon in only 1 frame into the predicted RL1 ORF to demonstrate that this led to a non-neurovirulent phenotype and (ii) expression of ICP34.5 under the control of

a strong *E.coli* promoter and detection of the protein using pre-existing peptide antisera. Purification of the *E.coli* -expressed protein also allowed generation of antisera which subsequently recognised ICP34.5 in HSV-1 strain 17^+ infected cell extracts.

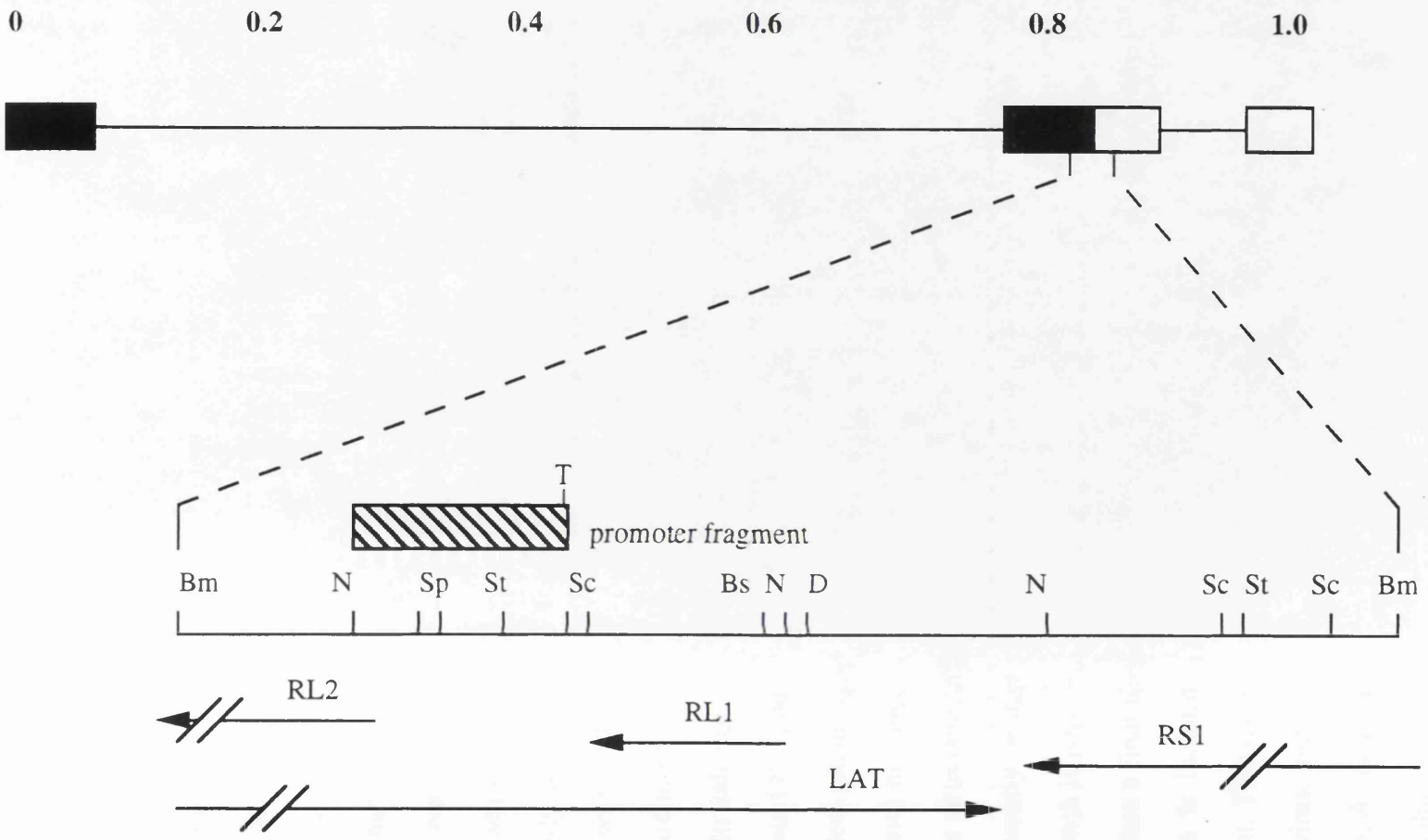
5.1.3. Construction and characterization of a HSV-1 strain 17^+ RL1 variant.

No HSV-1 strain 17^+ mutants with lesions solely in the RL1 ORF have previously been described, although Chou *et al.* (1990) have partially confirmed the coding assignment of the homologous protein in HSV-1 strain F, by inserting a six-frame stop-codon ~90bp from the initiating ATG. However, this mutation would also affect overlapping reading frames. Recently the identification of a novel promoter downstream of RL1 (figure 5.1), from which two transcripts of 0.9kb and 4kb, antisense to RL1 have been detected by Northern blotting (Bohenzky *et al.*, 1993), has potentially complicated the analysis of existing RL1 mutants. The 3' ends of these transcripts have not been mapped, but they can be detected in cells infected with HSV-1 mutants lacking the LAT promoter and are therefore not processed forms of LAT. Verification that lack of synthesis of ICP34.5 alone, was responsible for a non-neurovirulent phenotype was achieved through the construction and characterization of a HSV-1 strain 17^+ RL1 variant, 1771, with a stop-codon in only the ICP34.5 reading frame, 9 base pairs downstream from the initiating ATG.

It was initially decided to insert an in-frame stop-codon close to the initiating ATG of RL1 by site-directed mutagenesis using as a starting template, the M13 clone which had been used to resequence the gene. However, it soon became clear that this was not the best approach to use. Although oligonucleotides 18-42 bases long were used under high stringency conditions, they consistently either hybridized non-specifically to the M13 template or failed to hybridize; this could be attributed to the high G+C content of R_L (71.6% G+C; Perry and McGeoch, 1988), and the high G+C content of the oligonucleotides used. The high G+C content of the oligonucleotides may also have led to the formation of secondary structures which prevented correct annealing. Similar results have been found with HSV-2 RL1 (Miss S. Bdour, personal communication); using the same starting template, site-directed mutagenesis has been successful in some regions of RL1, but not in

Figure 5.1. Position of the novel promoter indentified by Bohenzksky *et al.* (1993).

Diagramatic representation of the HSV-1 genome with an expanded region flanking the L-S junction composed of the BamHI restriction enzyme fragment k (np 123459-np 129403). The promoter fragment is indicated by a hatched box. The position of the putative TATA box (which is located 54bp downstream of the SacI site) is indicated (T). The solid arrows denote the mRNAs for RL2, RS1, RL2, and the primary transcript of LAT. The symbols for restriction enzyme sites are as follows: Bm, BamHI; Bs, BstEII; D, DraI; N, NcoI; Sc, SacI; Sp, SphI; St, StuI.


0
0.2
0.4
0.6
0.8
1.0
T
promoter fragment
Bm
N
Sp
St
Sc
Bs
N
D
N
Sc
St
Sc
Bm
RL2
RL1
RS1
LAT

others, presumably due to variations in the G+C content of the regions undergoing mutation. Interestingly, Mr A.Dolan (personal communication) has been unable to successfully amplify by PCR, the region spanning RL1 despite success in other regions of the genome; this again, is probably due to the high G+C content of R_L. As an alternative approach, an in-frame stop-codon was introduced into the RL1 ORF using the plasmid pEA.10 whose construction was described in an earlier section. The only disadvantage with this method was the large size of the oligonucleotide linkers used (91mer and 92mer) and the possibility that aberrant bases could be introduced into the RL1 sequence. This was however eliminated, by sequencing the entire 92bp insert, which confirmed that it was identical to the wild-type RL1 sequence, except for the 6bp insert.

1771 was totally non-neurovirulent following intracerebral inoculation of 3-week old BALB/c mice with a $LD_{50}>10^6$ p.f.u./mouse compared to <10p.f.u./mouse for the wild-type HSV-1 strain 17^+. The LD_{50} values of 1771 and 1716 are 7-fold lower than the published LD_{50} value of 1716 (MacLean, A., *et al.*, 1991a), but it should be pointed out that in this thesis 1771 and 1716 were only injected at doses up to 10^6 p.f.u./mouse. The non-neurovirulent phenotype of 1771 was shown to be due to an inability to replicate in mouse brain, with no detectable virus present at 3 days post-inoculation. It would seem unlikely that the virus detected at days 1 and 2 post-inoculation is newly replicated virus, but this cannot presently be discounted. Like 1716, (MacLean, *A.*, *et al.*, 1991a) 1771 is not impaired in growth *in vitro* in BHK21/C13 cells. In confluent mouse embryo fibroblast 3T6 cells 1771 reaches a final titre ~4 logs lower than the wild-type HSV-1 strain 17^+ and its growth pattern is indistinguishable from that of 1716 (Dr. S.M. Brown, personal communication). This tissue culture system apparently reflects the *in vivo* phenotype of RL1 negative virus, and correlates with *in vivo* growth data available for both 1771 and 1716 (MacLean, A., *et al.*, 1992; section 3.1.7). The replication defect observed in variants which fail to produce ICP34.5, is tissue and/or cell type specific as 1716 replicates in mouse peripheral tissue (Robertson *et al.*, 1992) As both 1716 and 1771 replicate in undifferentiated F9 cells (Dr S.M. Brown, personal communication) (a mouse embryo

testicular carcinoma cell line) this would indicate that the replication defect is not host specific.

The mechanism by which ICP34.5 enables growth in the nervous system is unclear. It is unlikely that ICP34.5 is involved in attachment, penetration or uncoating as both HSV-1 and HSV-2 variants which fail to synthesize ICP34.5 can establish and reactivate from latent infection in mice (Thompson *et al.*, 1989; MacLean *et al.*, 1991b; Robertson *et al.*, 1992), demonstrating that virus can at least enter neurons. In addition, as far as we can determine ICP34.5 is not present in virions or associated with membranes (MacKay *et al.*, 1993). One possible explanation for the apparent growth difference of ICP34.5 negative variant between cells of neuronal and other cells of nonneuronal origin could be that in cells eg. BHK21/C13, MDK or Vero cells, and peripheral tissues *in vivo* where ICP34.5 deletion variants are not impaired in growth, a cellular homologue can compensate for the lack of production of ICP34.5; this homologue may not however, be present in cells of neuronal origin or certain other defined cell types.

The precise role which ICP34.5 plays in the virus life-cycle is unclear, but a recent study in one neuroblastoma cell line (Chou and Roizman, 1992) claimed that in the absence of ICP34.5, apoptosis occurred following infection with HSV-1. Infection of this one neuroblastoma cell line with mutants incapable of expressing ICP34.5 results in shutoff of cellular protein synthesis and no virus replication. Infection of these cells with wild-type virus results in sustained protein synthesis and production of infectious progeny. No such defect was seen in the Vero cells used as controls. However the mutant 1716 fails to induce cell-death or apoptosis in tissue culture (Dr S.M. Brown, personal communication).

Two homologues of ICP34.5 which originate from 2 quite unrelated sources have recently been described (Barnett and McGeoch, 1991; Sussman *et al.*, 1992) - MyD116, a murine myeloid differentiation primary response gene, and LMW23-NL, an ORF in the African Swine Fever Virus genome. Although the function of LMW23-NL is unknown, its similarity with genes involved in myeloid cell differentiation and viral host range suggest a possible role for it in African Swine Fever host range.

Although they differ in size, ICP34.5, MyD116 and LMN23-NL contain a centrally located acidic region with a highly conserved, hydrophilic 56-amino acid domain located at the carboxy terminus. LMN23-NL and ICP34.5 both contain a highly basic amino terminus composed of 8 to 10 lysine and arginine residues. In the conserved domain, LMW23-NL and MyD116, show 78% overall conservation, whereas LMW23-NL and ICP34.5 have 55% overall conservation. These sequence determinations suggest that in HSV-1 and HSV-2 the carboxyterminus of the protein, is the region most highly conserved in all these proteins, constitutes an important functional domain. Furthermore, mutations affecting the conserved domain of HSV-2 strain HG52 RL1 completely abolish neurovirulence (Miss S. Bdour, personal communication).

In summary having confirmed that the HSV-1 strain 17^+ genome encodes a HSV-1 strain F ICP34.5 homologue we have constructed a HSV-1 strain 17^+ RL1 deletion variant 1771 with a stop-codon in only the ICP34.5 reading frame. This mutant has been used to demonstrate conclusively that deletion of ICP34.5 alone is sufficient to abolish neurovirulence following intracerebral inoculation of mice with HSV-1 strain 17^+.

5.1.4. Construction and characterization of an HSV-1 strain F ICP34.5 deletion variant.

In a previous attempt to detect ICP34.5 from HSV-1 strain 17^+ infected cells, synthetic oligopeptides representing different regions of the predicted open reading frame were used to generate antisera (figure 3.13.). Until recently (MacKay *et al.*, 1993), none of these peptide antisera were successful in identifying ICP34.5 in HSV-1 strain 17^+ infected cells, although 1 which was directed against 10 copies of the PAT repeat strongly recognised ICP34.5 in HSV-1 strain F infected cell lysates. Another antiserum directed against 5 copies of the PAT repeat weakly recognised HSV-1 strain F ICP34.5 in infected cell lysates.

The strain F deletion variant, F11, was constructed to confirm the specifity of the antiserum against the PAT tenmer, to use as a negative control in ICP34.5 localization studies and to show that loss of ICP34.5 expression specifically correlated with a loss of neurovirulence.

The simplest method for construction of this variant was to linearise a plasmid, containing a HSV-1 strain 17^+ fragment spanning the 1716 deletion, and to cotransfect this onto BHK21/C13 cells along with intact HSV-1 strain F DNA. An isolate with a genome profile identical to 1716 was then selected for further analysis.

1716 and F11 are totally non-neurovirulent following intracerebral inoculation, with LD_{50} values of $>10^7$ p.f.u./mouse. By comparison HSV-1 strain 17^+ and HSV-1 strain F had LD_{50} values of $<10^2$ p.f.u./mouse. It has previously been shown (MacLean, A., *et al.*, 1991a) that the non-neurovirulent phenotype of 1716 is caused by its inability to replicate in mouse brain. As F11 contains exactly the same RL1 deletion in its genome, it would be reasonable to assume that it also, is unable to replicate when injected intracerebrally into mouse brain.

The main aim of this study was to demonstrate that a lack of synthesis of ICP34.5 correlated with a loss of neurovirulence. By Western blot analysis of infected cell extracts, it was demonstrated that F11 did not produce ICP34.5. In conjunction with neurovirulence studies of F11 we confirmed that lack of synthesis of ICP34.5 specifically correlated with the acquisition of an non-neurovirulent phenotype.

Ackermann *et al.* (1986) have reported the accumulation of HSV-1 strain F ICP34.5 largely in the cytoplasm of Hep-2 cells, and these results have been confirmed for HSV-1 strain 17^+ using cell fractionation studies (MacKay *et al.*, 1993). BHK21/C13 infected cell extracts were separated into cytosolic and membrane fractions with all detectable ICP34.5 being found in the cytosolic fraction. Similarly when infected cells were separated into nuclear and cytoplasmic fractions, less than 1/320 of ICP34.5 was present in the nucleus with the remainder in the cytoplasm. This result was confirmed by immunofluorescence using the wild-type HSV-1 strain F and F11 as a negative control. ICP34.5 was localised in discrete punctate patches within the cytoplasm, suggesting possible localization of the protein in specific cytoplasmic organelles eg. golgi apparatus or ribosomes.

The major difference between 1716 and F11 is the ability of these 2 viruses to reactivate from latency. In a previous study (Robertson *et al.*, 1992) the RL1 deletion variant 1716 was found to be impaired in reactivation from latency compared to the wild-type virus - a

result which was confirmed in this study. Reactivation of both 1716 and F11 from latency is dose-dependent. HSV-1 strain F appeared to establish and reactivate from latency in significantly fewer ganglia than HSV-1 strain 17^+ (15% of ganglia infected with HSV-1 strain F reactivating compared to 55% of ganglia infected with HSV-1 strain 17^+), a consistent finding in our laboratory (Dr. L.Robertson, Dr. A. MacLean, personal communication). Taking this into account, F11 did not show any difference in reactivation kinetics compared to HSV-1 strain F. F11 in not impaired in reactivation from latency compared to HSV-1 strain F, although it is impaired in reactivation from latency compared to HSV-1 strain 17^+.

The LD_{50} of HSV-1 strain F is consistently 1 log higher than that of HSV-1 strain 17^+ following intracerebral inoculation of mice, which implies poorer replication of the virus *in vivo*. If HSV-1 strain F also replicates less efficiently than HSV-1 strain 17^+ in the DRG following inoculation at the periphery, less virus will be available for establishment of a latent infection, leading to poorer reactivation kinetics for HSV-1 strain F compared to HSV-1 strain 17^+. This could explain the lack of difference in reactivation frequency between F and F11. This hypothesis could be tested by comparing the replication kinetics of both HSV-1 strain F and HSV-1 strain 17^+ in DRG, and their LD_{50}s, following footpad inoculation

MacLean *et al.* (1991b) have previously demonstrated that the HSV-2 strain HG52 mutant, 2604, which has a similar deletion in its genome to F11 and 1716 is not impaired in reactivating from latency compared to the wild-type parental virus. Following inoculation with JH2604 at a dose of 10^5 p.f.u., virus reactivated from 74% of ganglia, while at the same dose of HSV-2 strain HG52, virus reactivated from 81% of ganglia. It would thus appear that the factors which determine establishment and/or reactivation from latency differ depending on the viral background. These factors also seem to be separate from those affecting neurovirulence.

It is possible that the functional domain of LAT and hence the effect which the deletions in JH2604 and 1716 have on LAT, varies between HSV-2 strain HG52 and HSV-1 strain 17^+. Recent comparison of HSV-2 strain HG52 R_L with its HSV-1 strain 17^+ counterpart (McGeoch *et al.*, 1991) has demonstrated that the sequence of the LAT transcript is

dissimilar, whereas the probable LAT promoter region of HSV-2 strain HG52 shows similarities to that of HSV-1 strain 17^+. Hence the regions of the LAT transcript deleted in JH2604 and 1716 may differ. If a functional domain of LAT is deleted in 1716 but not JH2604 this might account for the reactivation kinetics observed with the 2 viruses. Analysis of the latency characteristics of 1771 with only 1 inserted stop-codon and hence minimal effect on the antisense LAT should answer this question.

During the course of this study, 2 other recombinant wild-type viruses, 1716a and F11a were constructed. 1716a contains the HSV-1 strain F RL1 promoter and 5' coding sequences in a strain 17^+ background, whereas F11a contains the HSV-1 strain 17^+ RL1 promoter and 5' coding sequences in a strain F background. If ICP34.5 is underproduced in HSV-1 strain 17^+, and this is a function of the viral background, then 1716a would synthesize lower amounts of the protein than F11a. The results obtained are ambiguous. Both F11a and 1716a produce a protein with a similar M_r to that of HSV-1 strain F (as confirmed by Western blotting) suggesting that other viral gene(s) are also involved in ICP34.5 production and processing. The idea that ICP34.5 is underproduced in HSV-1 strain 17^+, rather than being less strongly recognised by the peptide antiserum might be supported by the fact that 1716a and F11R were marker rescued using the same fragment of DNA, but in 1716a, with a strain 17^+ background, strain F ICP34.5 is apparently underproduced. However this does not correlate with later evidence obtained using the polyclonal antiserum, which clearly indicates that ICP34.5 is produced in similar quantities in HSV-1 strain F and HSV-1 strain 17^+. Natural variation in the number of copies the DNA sequence encoding the PAT repeat within the 1716a stock, would lead to the presence of isolates with fewer copies of the PAT repeat. These might not be detected due to poor antibody affinity of the peptide antiserum. Two other HSV-1 strains, MGH-10 and CVG-2 sequenced by Chou and Roizman (1990) had 6 copies of the PAT repeat, so it would not be unreasonable to assume that natural variation in the number of PAT repeats could occur. Alternatively, infection with 1716a may be poorer and hence the apparent underproduction of protein.

Confirmation that the wild-type phenotype had been restored to F11a and 1716a was provided by determination of their LD_{50} values in BALB/c mice following intracerebral inoculation. In both cases the neurovirulent phenotype was found to be restored to the recombinant virus to a level comparable with HSV-1 strain F.

It is interesting to note that HSV-1 strain F, which was isolated from a recurrent facial lesion, is of moderate virulence, with a 50% lethal dose of approximately 100 p.f.u. by intracerebral inoculation of mice. In contrast, MGH-10, derived from a case of human encephalitis, has a 50% lethal dose of 1 to 5 p.f.u. by the same route in identical mouse strains (Meigner *et al.*, 1988). Similarly, in our system the LD_{50} of HSV-1 strain F is consistently around 1 log higher than that of HSV-1 strain 17^+. It is possible that these observed differences in HSV-1 neurovirulence could be ascribed to differences in the nucleotide sequence, in particular the PAT copy number of ICP34.5 with strains having fewer copies of the PAT repeat being more neurovirulent. This hypothesis has not been tested. Alternatively, it could be due to alterations elsewhere in the genome.

5.1.5. Over-expression of RL1 in a bacterial system, and partial purification of ICP34.5.

We chose to express ICP34.5 in *E.coli* for several reasons: (i) ICP34.5 had not previously been detected in HSV-1 strain 17^+ infected cells, probably due to a low level of synthesis. By placing the gene under the control of a strong promoter we hoped to be able to detect a protein product using pre-existing peptide antisera, (ii) At the initiation of this project we were concerned that we would be unable to detect ICP34.5 in HSV-1 strain 17^+ infected cells using peptide antisera. By expressing the protein in a bacterial expression system, we hoped to raise polyclonal anti-ICP34.5 sera which would specifically recognise ICP34.5 in HSV-1 strain 17^+ infected cells and might also detect the semi-conserved polypeptide in HSV-2 strain HG52 infected cells, (iii) MacKay *et al.* (1993) have recently optimized our assay conditions such that a peptide antiserum raised against 10 copies of the PAT repeat specifically recognises ICP34.5 in HSV-1 strain F and HSV-1 strain 17^+ infected cells. The level of detection of ICP34.5 in HSV-1 strain 17^+ was 30-fold lower than in HSV-1 strain

F; a polyclonal antiserum would enable determination of whether the differences in the levels of detection between the HSV-1 strain 17^+ and HSV-1 strain F polypeptides were real, or merely due to antibody affinity differences, and (iv) purification of the *E.coli* -expressed ICP34.5 might have enabled us to undertake functional studies.

The *E.coli* pET system (Studier *et al.*, 1990) has previously been used for high level expression of HSV-1 R1 (Furlong *et al.*, 1991) and R2 (Lankinen *et al.*, 1991) and was chosen for expression of the entire ORF of ICP34.5. However, ICP34.5 was not expressed at a particularly high level in this system (~1% R1 (Furlong *et al.*, 1991)). Several attempts were made to increase the level of production of ICP34.5 in this system. Variation in growth temperatures had no effect on the quantity of ICP34.5 produced, neither did addition of ampicillin, at various stages of growth, to ensure maintenance of pET34.5 within the *E.coli* cells. When single plaques from a plate were used to inoculate 500ml cultures directly, the quantity of ICP34.5 decreased by ~50%. In no case was an obvious increase in the level of ICP34.5 production detected and there did not appear to be any significant fluctuations in the quantity of ICP34.5 produced in individual experiments.

When cultures were maintained at 37°C following induction, ICP34.5 was produced in an insoluble form. Subsequent experiments revealed that lowering the growth temperature of the cultures to 28°C following induction was sufficient to enable production of soluble *E.coli* -expressed ICP34.5. Because ICP34.5 was not expressed to high levels and thus was not a major constituent of the bacterial lysate, and previous studies using the peptide antisera indicated that it might not be highly immunogenic, we felt it necessary to remove as many contaminating bands from the crude extract before using it for the production of polyclonal antisera. A two stage programme was developed for the partial purification ICP34.5. The first stage in purification was ammonium sulphate fractionation. By 50% ammonium sulphate, almost all ICP34.5 had been precipitated from crude bacterial extract, therefore this percentage ammonium sulphate was routinely used for purification. As a second step, several columns were tested for ICP34.5 binding at various pH ranges and salt concentrations, but in almost every case the protein failed to bind. When it did bind, the protein tended to elute over almost the entire salt gradient suggesting that the *E.coli*

expressed ICP34.5 was aggregating with other proteins in the sample. Although the theoretical isoelectrical point of ICP34.5 is 4.3, the protein failed to bind to a mono P column in the pH range 4-6 (Dr. M. Denheen, personal communication). Again this could be due to aggregation of the protein in solution preventing binding to the column or alternatively the protein may be folded in such a manner that charged amino acids are screened.

Using the MonoQ as the second step in purification was a compromise; although ICP34.5 did not bind to this column, most bacterial bands did and thus the flowthrough gave larger and purer yields of protein than any other column tested. Despite this, only small amounts of protein remained following purification using the MonoQ column. We did not consider it necessary therefore to attempt further purification of the protein prior to use as an immunogen.

Two New Zealand White rabbits were immunized intramuscularly, first with 2mg total protein (approximately 100ug ICP34.5) in Freund's complete adjuvant followed by 4 boosts, each at a 14 day interval, using the same amount of antigen but in Freund's incomplete adjuvant. Rabbits were bled 10 days after each boost and the antisera tested at different dilutions against HSV-1 strain F and HSV-1 strain 17^+ infected cell extracts for reaction with ICP34.5 specific bands. After 5 injections only 1 rabbit had produced an immune response, with an antibody titre which appeared to have plateaued. This animal was bled out and its serum used as the polyclonal anti-ICP34.5 serum.

The polyclonal antiserum did not have as high an affinity as had been hoped and was actually no more sensitive at detecting the HSV-1 strain F polypeptide than the peptide antiserum which was already available. However, the protein antiserum recognised HSV-1 strain F and HSV-1 strain 17^+ ICP34.5 with equivalent sensitivity, demonstrating that previous differences in detection level (MacKay *et al.*, 1993) were due to antibody affinity differences and that the level of ICP34.5 accumulation in cells infected with each strain is similar. We were unable to detect HSV-2 strain HG52 ICP34.5 using either immunoprecipitation or Western blotting. In immunofluorescence studies, this antiserum was used in conjunction with 1771, to confirm our previous results which had demonstrated the localization of ICP34.5 in discrete punctate patches, specifically in the cytoplasm of infected

cells. This work demonstrates that the distribution of ICP34.5 in infected cells is similar following HSV-1 strain F and HSV-1 strain 17^+ infection.

In summary we have used partially purified *E.coli* -expressed ICP34.5 to produce in rabbits a polyclonal anti-ICP34.5 serum which specifically detects ICP34.5 in HSV-1 strain F and HSV-1 strain 17^+ infected cell lysates. We have demonstrated that the level of ICP34.5 accumulation is similar following HSV-1 strain F and HSV-1 strain 17^+ infection, indicating that previous observed discrepencies in the level of detection between the two strains were due to antibody affinity differences. The polyclonal antiserum was also used to specifically localize ICP34.5 to the cytoplasm of HSV-1 strain 17^+ infected cells.

5.1.6. Characterization of 1772, a deletion variant in U_S.

The U_S region of the HSV-1 genome encodes 13 predicted ORFs (McGeoch *et al.*, 1985; Georgeopoulou *et al.*, 1993). With the exception of gD, these are all dispensible for growth in tissue culture (Post and Roizman, 1981; Longnecker and Roizman, 1986; Umene, 1986; Longnecker *et al.*, 1987; Weber *et al.*, 1987; Georgeopoulou *et al.*, 1993) .The isolation of a spontaneous U_S deletion variant from a transfection experiment was unexpected. In general where spontaneous HSV variants have previously been isolated and described, the deletions have involved one of the repeat regions and adjacent unique sequences (Brown *et al.*, 1984; Harland and Brown, 1985; Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987), suggesting that these act as illegitimate 'hotspots' for recombination.

Following plaque purification and titration on BHK21/C13 cells, it was observed that 1772 produced small discrete plaques, which did not have a normal wild-type morphology. This phenotype was indicative of a defect in cell-to-cell spread, a property which has not previously been ascribed to any gene in U_S. Restriction endonuclease and sequence analysis localized the deletion in 1772 to the BamHI restriction enzyme fragment and subsequently to US7 encoding gI. 1772 has a 630bp deletion which removes the initiating ATG of US7 and extends to the 3' end of the gene. Confirmation that US7 was deleted in 1772 was provided by immunoprecipitation using the previously well characterized MAb 3104 (Johnson *et al.*,

1988). This MAb precipitated proteins with the expected size of gI and pgI and also gE and pgE from HSV-1 strain 17^+ infected cells, but not from mock or 1772 infected cells.

Initial characterization of 1772 involved analysis of the *in vitro* growth characteristics of this variant. In one-cycle, but especially multi-cycle growth analysis, 1772 had an extended lag phase, although it reached final titres comparable to the wild-type virus. Longnecker *et.al.* (1987) demonstrated that deletion of gI did not affect the ability of the virus to replicate in tissue culture, but they did not carry out detailed growth analysis of their US7 deletion variant, hence they did not observe this extended lag phase during the initial stages of growth in tissue culture. If 1772 has a defect in adsorption or penetration into BHK21/C13 cells, this would explain the apparent initial delay in initiation of infection. Entry of herpesviruses into their host cells is believed to occur by fusion of the viral and cellular membranes (reviewed by Spear, 1993). Since 1772 appeared to be defective in the initial stages of viral growth, we thought it was possible that entry of the virus into the cells and initiation of the infectious cycle could be affected by the lack of gI. However, no difference in the time of initiation of immediate-early, early or late protein synthesis in BHK21/C13 cells could be detected, and no difference in the rates of adsorption of wild-type and gI^- virus to BHK21/C13 cells was observed. It is possible that the percentage of 1772 adsorbing to cells in tissue culture is impaired, however experiments to determine if this was the case were unsuccessful due to poor labelling of virion preparations. Development of resistance to low pH ie. virus penetration, between the wild-type and gI^- virus was also similar. Virus preparations of 1772 and HSV-1 strain 17^+ had comparable particle:p.f.u. ratios, ruling out the possibility of interfering virus particles in the 1772 preparation. Thus, if gI plays an auxillary role in the fusion of the viral and cellular membranes during virus entry into cells, the effect is too subtle to be detected by the assays we have used. Through the incorporation of neutralizing antibodies in the overlay it was demonstrated that gI plays a direct role in cell-to-cell spread.

Following intracerebral inoculation of mice, 1772 was intermediate in neurovirulence with a LD_{50}= $3.6x10^3$ p.f.u./mouse compared to 7 p.f.u./mouse for the wild-type virus HSV-1 strain 17^+. Although the actual mean time to death was not calculated, this variant was

impaired in growth *in vivo*. If injected with HSV-1 strain 17^+ at doses of 10^5 or 10^6, mice would be expected to die within 24-48 hrs. However when injected with equivalent doses of 1772, the mean time to death was approximately 4 days. It could be postulated that *in vivo*, wild-type virus spreads either (or both) by direct cell-to-cell transmission or *via* adsorption to uninfected cells of virus that has been released from infected cells. As 1772 is impaired in direct cell-to-cell spread, virus will still spread *via* the other pathway, and this accounts for the reduced virulence. 1772 should similarly be impaired in virulence following peripheral inoculation. In PRV, both gI and gp63 (the HSV-1 gE and gI homologues respectively) are known to play a role in virulence in both pigs, the natural host of this virus (Kimman *et al.*, 1992) and mice (Peeters *et al.*, 1993). gp63 forms a complex with gI and the phenotypes of gI, gp63 and gI + gp63 mutants are similar (Zuckermann *et al.*, 1988) suggesting that the complex of gp63 and gI is the functional unit. Evidence that gI (and thus gp63) is involved in cell-to-cell transmission of the virus was recently reported (Zsak *et al.*, 1992). We have demonstrated this for gI and believe this accounts for the observed *in vivo* phenotype of 1772.

The physiological role played by Fc receptors in the replicative cycles of herpesviruses remains unclear. It has been postulated that the Fc receptor protects the virus, or virus infected cell from host immune attack by binding IgG, thus preventing immune recognition , or sterically hindering destruction by IgG or Fc-dependent effector cells (Cost*a et al.*, 1977; Lehner *et al.*, 1975; Westmoreland and Wadkins, 1974). Adler *et al.* (1978) demonstrated that IgG aggregates protect HSV-1 infected cells from complement-mediated cytolysis or destruction by sensitized lymphocytes, while Dowler and Veltri (1984) demonstrated that HSV-2 virions can resist neutralization by HSV-2 specific antisera by binding non-immune IgG.

The Fc portion of IgG is the region of the molecule responsible for mediating important effector functions, such as complement activation and antibody-dependent cellular cytotoxicity (for overview see Roitt et.al., 1985). Therefore, binding of the Fc end of immune IgG could modify the effectiveness of IgG mediated attack. Recently, Frank and Friedman (1989) demonstrated that the HSV-1 Fc receptor can bind the Fc domain of IgG

which has its antigenic target (in this case a viral glycoprotein) bound at the Fab domain, and thus protects the virus from antibody and complement-dependent neutralization.

It has recently been shown that expression of gE alone results in the induction of Fc receptor activity, while expression of gI alone gives no detectable Fc binding; hence HSV-1 has the potential to induce 2 Fc receptors- a high affinity receptor comprising gI and gE which binds monomeric IgG and a lower affinity receptor comprising gE alone, which binds IgG complexes (Bell *et al.*, 1990; Dublin *et al.*, 1990). The presence of gI could enhance binding of IgG by gE in 2 ways; either indirectly by altering the confirmation of gE to increase the affinity of its Fc receptor-binding domain, or directly by interacting with gE to form a new high-affinity Fc-binding domain. If 1772 produces only a low affinity Fc-receptor as would be suggested by the results obtained by Bell *et al*.(1990) and Dublin *et al.* (1990), virus infected cells may be more susceptible to immune cytolysis and this could perhaps account for the reduction in neurovirulence of 1772.

In PRV however, the gI-gp63 complex does not appear to have IgG Fc-receptor activity (Zuckermann *et al.*, 1988). Thus the significance of the gE-gI complex Fc-receptor expression in HSV-1 virulence is unknown. Although an Fc-receptor could decrease the effects of antibody on infected cells, its reported absence from PRV and PRV-infected cells suggests that the Fc-receptor is not of major importance for the expression of virulence of herpesviruses.

5.2. Future prospects

We have confirmed that ICP34.5 plays a crucial role in neurovirulence following intracerebral inoculation of mice, and it may now be possible to define the functional regions of RL1. Further defined lesions could be constructed along the length of RL1 eg. at the PAT repeat, before the 63 amino acid region of conservation between HSV-2 strain HG52 and HSV-1 strain 17^+ and at various positions within the 63 amino acid conserved domain. It should also be possible to construct a RL1 variant in which the 5' end of RL1 is deleted, through the use of oligonucleotide-linkers. Analysis of the neurovirulence phenotype of the

resulting variants may indicate the functional regions of the gene and help to define the precise role which this protein plays *in vivo*.

The latency characteristics of 1716 and F11 are currently being examined using a mouse corneal model to determine if the results obtained correlate with those obtained using the footpad model. Preliminary results have indicated that in the corneal model, 1716 compared to the wild-type virus is impaired in reactivation from latency to an even greater extent than is observed using the footpad model (Dr. J. Spivak, personal communication). The reason for this defect is at present unclear, but the possibility that ICP34.5 is involved in establishment and/or reactivation from latency is currently under investigation. Through characterization of the latency characteristics of 1771, we should be able to determine if the impairment of 1716 in reactivation from latency is due to an effect of the large genomic deletion on the LAT transcriptional unit, or due to the lack of synthesis of ICP34.5.

1716 is impaired in growth in DRG of mice ; if 1771 is similarly impaired in growth at this anatomical site we can definitively conclude that ICP34.5 is required for cell and/ or tissue specific growth. These studies are currently on-going.

Detection of HSV-2 strain HG52 ICP34.5 will require a higher titre and more sensitive antiserum. There are obvious routes to be taken to achieve this. An alternative expression system eg. baculovirus or a GST-fusion might give higher expression of ICP34.5 and higher levels of starting material for purification. Use of an alternative expression system may not however, overcome the problems of protein aggregation. As an alternative approach 5' deletions are currently being made in the existing PET construct in the hope that a smaller truncated version of the protein will be more readily purified. For an antiserum to cross-react with HSV-2 strain HG52 ICP34.5, it will probably be necessary to raise antisera against an expressed protein in which the 63 amino acid region of homology between HSV-1 strain 17^+ and HSV-2 strain HG52 has been conserved. As ICP34.5 is a small protein (248 amino acids in strain 17^+) this limits the alterations which can be made and it may be necessary to express the HG52 polypeptide to successfully raise an antiserum which recognises it in infected cells. It is hoped in the long term that ICP34.5 will be purified to homogeneity, for use in monoclonal antibody production and functional studies. In conjunction with further

defined lesions in the RL1 ORF monoclonal antibodies will be used to determine the functional regions of ICP34.5.

To further characterize the growth defect in 1772, a wild-type rescuant should be constructed, to exclude the possibility that a mutation elsewhere in the viral genome is contributing to the observed phenotype. If, by rescuing the gI deleted sequences, wild-type growth characteristics and wild-type neurovirulence are restored, we can conclude that gI plays a role in cell-to-cell spread.

In PRV glycoproteins gI, gp63 and gIII are the homologues of glycoproteins gE, gI and gC respectively of HSV-1 (Petroviskis *et al.*, 1986; Robbins *et al.*, 1986a). Mutants defective in only gI, gp63 or gIII exhibit a marginal decrease in neurovirulence compared to wild-type virus. In contrast double mutants either gI$^-$ gIII$^-$ or gp63$^-$ gIII$^-$ are non-neurovirulent for mice, chickens and pigs (Mettenleiter *et al.*, 1987,1988). gIII plays a role in virus adsorption to cells in tissue culture (Schreurs *et al.*, 1988), whereas gI (and also gp63) is involved in cell-to-cell transmission of virus (Zsak *et al.*, 1992). Thus the lack of virulence of a gIII$^-$ gI$^-$ (gp63) virus is probably attributable to its inability to spread by either mode.

In HSV-1 gC is required for viral adsorption onto cells (Kuhn *et al.*, 1990; Herold *et al.*, 1991). If we assume that gI is required for cell-to-cell transmission, then a variant lacking both of these glycoproteins would be unable to spread *via* either adsorption of released virus or cell-to-cell transmission. A HSV-1 variant of this nature might be expected to be non-neurovirulent and thus useful for the development of a live attenuated HSV vaccine. Inoculated virus would be localized at the site of infection which would enable the production of a host-immune response, but subsequent spread of the virus *via* post-synaptic transport, and thus involvement of the nervous system would be unlikely.

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HSV-1 strain 17+  TTTAAAGCCGCGGCGGCGGGCAGCCCGGGCCCCCCGCG---GCCGAGACTAGCGAGTTAGACAGGCAAGCAC-TACTCGC
HSV-1 strain F          T         --                                  G
HG52 strain HG52  C  -    G GCC  CAACA G        TG G A  CCA        GG T     C CT          GG   G


HSV-1 strain 17+   CTCTGCACGCACATGCTTGCCTGTCAAACTCTACCACCCCGGCACGCTCTCTGTCT----------------------
HSV-1 strain F
HSV-2 strain HG52  GGT A    TG     G    GAGTG      C  G     A G     CGGCT  GGGCCTACGCCGAGCCCAGCCG


HSV-1 strain 17+   ----CCATG
HSV-1 strain F
HSV-2 strain HG52  CCCG
```

DNA sequence alignment of HSV-1 strains 17+ and F, and HSV-2 strain HG52 in the promoter region of the gene encoding ICP34.5, starting with the proposed RL1 TATA boxes (Chou and Roizman, 1990; McGeoch et.al., 1991). The sequences are for the leftward 5′ to 3′ strands in IR_L. Nucleodide changes in HSV-1 strain F and HSV-2 strain HG52 are indicated below the HSV-1 strain 17+ sequence. All other nucleotides are identical. Absence of a base is indicated (-).The initiating methionine of RL1 is underlined.

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HSV-1 strain 17+   VRFSPHVRVRHLV-VWASAARLARRGSWARERADRARFRRRVAEAEAVIGPCLGPEARARALAR
HSV-1 strain F     VRFSPHVRVRHLV-VWASAARLARRGSWARERADRARFRRRVAEAEAVIGPCLGPEARARALAR
HSV-2 strain HG52  VCFSPRVQVRHLV-AWETAARLARRGSWARERADRDFRRRRVAAAEAVIGPCLEPEARARARAR
LMW23-NL           VYFATDDI---LIKVR-EADDIDRKGPWEQAAVDRIRFQRRIADTEKILSAVLLRKKLNPMEHR
MyD116             VHFAEKVTVHFLA-VWAGPAQAARRGPWEQFARDRSRFARRIAQAEEKLGPYLTPDSRARAWAR
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Conserved carboxy-terminal domains of HSV-1 strains 17+ and F, HSV-2 strain HG52, African Swine Fever ORF LMW23-NL and MyD116.