INVESTIGATION OF ICP34.5 AND ITS ROLE IN HSV PATHOGENICITY

BY

HOLLY A. HOLMAN

A THESIS PRESENTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

THE FACULTY OF SCIENCE AT THE UNIVERSITY OF GLASGOW

Division of Virology
Church Street
Glasgow, G11 5JR

May 2000

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Table of Contents **Page**

List of Figures

Section 6.2.

List of Tables

ABBREVIATIONS

Chemicals and Compounds

Amp	ampicillin
APS	ammonium persulphate
BSA	bovine serum albumin
$CaPO_4$	calcium phosphate
$C_2H_4O_2$	acetic acid
CH_5N_3-HCl	guanidine hydrochloride
CO_2	carbon dioxide
CS	newborn calf serum
dH_2O	distilled water
DMF	dimethyl formamide
DMSO	dimethyl sulphoxide
EDTA	ethylenediaminetetra acetic acid
EtBr	ethidium bromide
EtOH	ethanol
FCS	foetal calf serum
HCl	hydrochloric acid
H_2O	water
H_2O_2	hydrogen peroxide
HRP	horseradish peroxidase
IPTG	isopropyl-β-D-thiogalatoside
KAc	potassium acetate
$KHCO_3$	potassium bicarbonate
MeOH	methanol
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
NaAc	sodium acetate
$NaC_6H_5O_7$	tri-sodium citrate
NaI	sodium iodide
NaOH	sodium hydroxide
NaP	sodium phosphate
NP40	Nonidet P-40

PMSF	phenylmethylsulphonyl fluoride
Propan-2-ol	isopropanol
RNaseA	Ribonuclease A
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N', -tetramethylethylene diamine
Tris	tris (hydroxymethyl) aminomethane
X-gal	5-chloro-4-bromo-3-indoyl-2-D-galactoside

Descriptions

Ab	polyclonal antibody
cpe	cytopathic effect
DRG	dorsal root ganglia
MAb	monoclonal antibody
oligo	oligonucleotide
PAGE	polyacrylamide gel electrophoresis
pi	post infection
RT	room temperature
TG	trigeminal ganglia
UV	ultra violet light

Measurements

°C	degrees celsius
Ci	curie
d	day
h	hour
M	molar
mA	milliamp
mg	milligram
min	minute
ml	mililitre
mM	millimolar
ng	nonograms
nm	nanometre
OD	optical density

PBS-A	phosphate buffer saline
rpm	revolutions per minute
s	second
µl	microlitre
µm	micrometre
U	unit(s)
V	voltage
w/v	weight/volume (ratio)
w/w	weight/weight (ratio)

Tissue Culture

CS	newborn calf serum
DMEM10	Dulbecco's modified eagles medium with 10%foetal calf serum
Emet/$5C_2$	Eagle's medium with reduced methionine and serum
ETC10	Eagle's media with 10%newborn calf serum

ACKNOWLEDGEMENTS

I thank Professor R. Elliott and Professor B. Clements for use of the facilities at the Division of Virology, University of Glasgow. I also thank Professor D. McGeoch for use of the facilities of the MRC Institute of Virology.

I thank Dr. A. MacLean for his supervision and critical reading of this manuscript. I thank Professor M. Brown and Drs J. Harland and L. McKie at the Southern General Hospital for communication. The experiments carried out at the CRF were under Professor M. Brown's project licence.

I thank my second supervisor Dr. A. Davison for advising against "red-herrings" and his support throughout this project and all members of lab 309; Charles, Robin, Moira, Steve, Parvis, and Dorothy.

I would like to thank lab 308 of years past: Ross, Kathy, Alistair and lab A422 for use of equipment and friendly donations of antisera and cell lines. Thank you Jim, HA, and HB for a stimulating bench experience.

I thank Dr. H. Marsden and lab for numerous antibodies and virus strains used throughout this project. The HSV-2 ICP34.5 antisera was generated under his project licence.

I thank the washroom and media ladies for an endless supply of glassware and reagents, all the support and secretary staff.

I would like to thank all the members of the CRF and D. Cossar at the Immunocytochemistry Unit (Dept. of Pathology) for allowing me use of their facilities.

During this project I had the opportunity to learn *in vivo* techniques from Dr. N. Sawtell and Professor R. Thompson in Cincinnatti, USA. I sincerely thank them for their help and contributing knowledge of the herpes virus field. I would also like to thank Professor L. Stanberry for the financial support of this sabbatical.

I would like to thank the coffee crew for friendship and encouragement: Vic, Dave, Chris, Helen and Lindsay, and last but not least, Val and Dino.

During the writing of this manuscript I was employed at the Dept. of Biochemistry and I would like to thank all the members of the Molecular Medicine lab for support during this period. I am especially grateful to C. Murphy and Dr. P. Harrison for critical reading and advice.

I thank Dr. R. Maes in Michigan for inspiring my interest in Virology.

I dedicate this thesis to my parents and family whose encouragement and financial support throughout the past three years were immeasureable.

The author was a recipient of Glasgow University funding. Unless otherwise stated, all the results described herein were obtained by the author's own efforts.

SUMMARY

The aims of this project were to determine the role of two antisense genes, RL1 and ORF P in herpes simplex virus (HSV) virulence and to identify the HSV-2 strain HG52 ICP34.5 expression.

The first overall aim was to define individual phenotypes associated with ICP34.5 and ORF P in HSV-1 strain 17^+. HSV-1 contains an ORF in the long repeat sequences named RL1 (also called $\gamma_1$34.5) which expresses the polypeptide ICP34.5. One RL1 deletion variant, 1716, is avirulent upon intracranial inoculation in the central nervous system (CNS) and the peripheral nervous system (PNS). Replication of 1716 *in vitro* was shown to be cell type and cell state dependent.

The avirulent phenotype of 1716 based on deletion of this region of the HSV-1 genome was originally attributed solely to the RL1 gene. However, transcripts in the opposite direction and antisense to RL1 in HSV-1 were identified in 1994. From these transcripts two open reading frames (ORF O and ORF P) mapped antisense to RL1. This led to the hypothesis that ORF P may be at least partly responsible for the avirulent phenotype of ICP34.5 negative mutants.

1716 was used as the backbone to generate three recombinant viruses containing either RL1 or ORF P in the nonessential UL43 gene. Two lengths of RL1 were cloned due to uncertainty about the position of the putative RL1 promoter sequence and role of the 5' untranslated leader in expression. One RL1 fragment contained only the ORF while the other fragment contained the ORF and 134 bp additional upstream sequence. The RL1 or ORF P genes were individually sub-cloned downstream of the HSV-1 gD promoter. This promoter was used because it has similar kinetics to that of the natural ICP34.5 promoter. These RL1 or ORF P cassettes also contained the *lacZ* gene under the SV40 promoter in the opposite orientation for detection of recombinant virus.

The recombinant virus, 1622, contained only the RL1 open reading frame and demonstrated an 8-fold overexpression of ICP34.5 by Western blotting and immunoprecipitation. 1622 regained wild type replication kinetics *in vitro* in cell lines non-permissive for ICP34.5 negative viruses. In certain cell types, an *in vitro* function of ICP34.5 is to maintain protein synthesis by using a protein kinase (PKR) pathway. 1716 showed host protein synthesis shut-off in human neuroblastoma and HeLa cells confirming the previously reported data for the HSV-1 strain F ICP34.5 deletion mutant, R3616. 1622 infected cells demonstrated protein synthesis similar to 17^+ by SDS-PAGE. 1622

demonstrated a small reduction in neurovirulence compared with 17^+ by intracranial inoculation but was 40-fold more virulent than its parent following infection via the snout and trigeminal ganglia as assayed by *in vivo* replication and immunohistochemsitry.

The recombinant virus, 1623, by Western blotting expressed very low levels of ICP34.5 compared to 17^+. Based on 2-fold dilutions of 1623 infected BHK cell extracts, ICP34.5 is expressed eight times less than in 17^+ and thus 64-fold less than in 1622. Interestingly, this low level of expression still enabled 1623 to maintain wild type levels of protein synthesis in neuroblastoma and HeLa cells. However, the replication kinetics *in vitro* from 1623 was slower than 1716. This suggests that there may be a second mutation elsewhere in the genome.

The recombinant virus, 1624, contained ORF P under the HSV-1 gD promoter. Due to difficulties in cloning the ORF P gene, final purification and characterisation of this mutant was not carried out. However, expression of ORF P from 17^+ infected cells was analysed by an antiserum from a GST/ORF P fusion protein. By Western blotting, ORF P was detected in 17^+ infected cells during normal lytic infection *in vitro*. Cells infected with a RL1 epitope tagged virus, 1621, demonstrated expression of a slightly larger form of ORF P. The temperature sensitive virus, tsK, when grown at 39.5°C expresses a form of ICP4 which is non-functional and does not bind to the ORF O/P repressor site. A lower M_r form of ORF P was detected from tsK.

The second overall aim was to identify ICP34.5 in HSV-2 using two strategies. Although distinct from the first aim, this used some of the same techniques and ideas.

Based on sequence analysis by McGeoch (1991), there is 83% homology between the 63 amino acid domain found at the carboxy terminus in RL1 of HSV-2 strain HG52 and HSV-1 strain 17^+. One unique feature in the HSV-2 strain HG52 RL1 gene is the presence of a 154 bp intron. In order to identify the ICP34.5 protein two strategies were developed. First, intertypic recombinant viruses were constructed where HSV-2 RL1 was inserted under HSV-1 gD promoter in the UL43 locus of 1716. All recombinant plasmids contained the *lacZ* gene for selection of recombination as described above. One recombinant virus, 1627, contained RL1 with its natural intron and the other recombinant virus, 1628, had the intron deleted. Once these viruses were isolated, their genome structure was confirmed by Southern blotting. *In vitro* studies examining virus replication and host protein synthesis from 1627 and 1628 infected cells determined functional homology between RL1 of HSV-1 and 2.

The HSV-1 ICP34.5 protein had previously been identified using antisera raised against the HSV-1 RL1 ORF. However, these antisera did not recognise an HSV-2 ICP34.5 protein from wild type HSV-2 strain HG52, 1627 or 1628.

Second, was to raise antisera against glutathione S-transferase fused to the predicted HSV-2 RL1 ORF. Using these antisera, Western blotting and immunoprecipitation showed the presence of two proteins, 27 kDa protein and 37 kDa (ICP34.5), from HG52 and 1627 infected cells. Only the 37 kDa protein was detected following infection with 1628 and 2624, mutants without the RL1 intron. The 27 kDa novel protein has been named ICP34.5A. The relationship between these proteins is not clear, but expression of the 27 kDa ICP34.5A protein is dependent on the presence of the intron. In addition, it was found that like HSV-1 ICP34.5, HSV-2 ICP34.5 also interacts with the proliferating cell nuclear antigen (PCNA) and localises primarily in the cytoplasm.

1716 and the recombinant viruses used throughout this study demonstrated some unusual features. The genes of interest, RL1 or ORF P, were bi-directionally inserted into the UL43 *Nsi*I site. It was not previously known that this locus would have an effect on an inserted gene. The UL43 gene has been shown to be nonessential and a gene antisense to UL43, named UL43.5 was later shown to be expressed. Insertion of a cassette into the *Nsi*I site positioned the cassette at the 3' end of UL43.5 but at the 5' end of UL43. By placing the gene or genes of interest in an expression cassette which had two genes in opposite orientations, we were able to compare the effect of orientation on the levels of gene expression. Genes inserted in the same transcriptional orientation as UL43.5 were expressed better than in the opposite orientation. This led to the hypothesis that overexpression of ICP34.5 from 1622 infected cells was, at least partly, based on RL1 orientation in UL43. Two new recombinant viruses were constructed to enable a direct comparison to confirm this observation.

Finally, plaque morphology from 1716 and all recombinant viruses constructed was observed. Because 3T6 cells are non-permissive for RL1 deleted viruses 1716 gives abortive plaques in these cells. Although BHK cells are permissive for RL1 deleted viruses and allow equal replication between 17^{+} and 1716, this study found that 1716 and its recombinant viruses produced small plaques on BHK cells. The abortive plaque phenotype on 3T6 cells was originally believed to be solely a consequence of the lack of ICP34.5 expression. However, 1622 which overexpresses ICP34.5 did not restore wild type plaque morphology on 3T6 or BHK cells.

1.1. The *Herpesviridae*

Characteristic properties of *Herpesviridae* includes the ability to enter latency, a state in which on infectious progeny are produced and only limited transcription of viral genes occurs. In addition, they encode several *trans*-acting factors which regulate the temporal expression of viral genes controlling the phases of infection (Cann, 1993). *Herpesviridae* have a characteristic physical icosahedral virus structure.

1.1.1. Classification

Herpesviridae have been classified into three subfamilies based on their biological properties: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. *Alphaherpesvirinae* have the capacity to establish latent infections primarily in the ganglia. *Betaherpesvirinae* have a long reproductive cycle and the infection progresses slowly in culture. The infected cells frequently become enlarged (cytomegalo) and carrier cultures are readily established. The virus can be maintained in latent form in secretory glands, lymphoreticular cells, kidneys, and other tissues. *Gammaherpesvirinae* replicate *in vitro* in lymphoblastoid cells. Viruses in this subfamily are specific for either B or T lymphocytes. In lymphocytes, infection is frequently arrested at either a pre-lytic or lytic stage, but without production of infectious progeny. Latent virus is frequently found in lymphoid tissue.

1.2. Herpes Simplex Virion

Herpes viruses all have a similar virion structure which led to their classification into one family. Virion particles mature by budding through the altered cellular membranes of infected cells. HSV virions are 150-200 nm in size, consist of four basic morphologic elements (Fig. 1), and are described below.

The Core

The core of a mature HSV virion contains the viral DNA. The HSV-1 DNA genome is approximately 153 kbp in size and the HSV-2 DNA genome approximately 154 kbp (Dolan *et al.*, 1998). Both HSV-1 and HSV-2 genomes consists of two covalently linked components, namely, the unique long (U_L) and unique short (U_S) sequences which are flanked by terminal inverted repeats (TR and IR, respectively; Fig. 2). Most of the genetic capacity of the virus is protein coding with more than 74 HSV polypeptides being encoded (McGeoch *et al.*, 1988). Infected cell proteins (ICP), virion proteins (VP), and

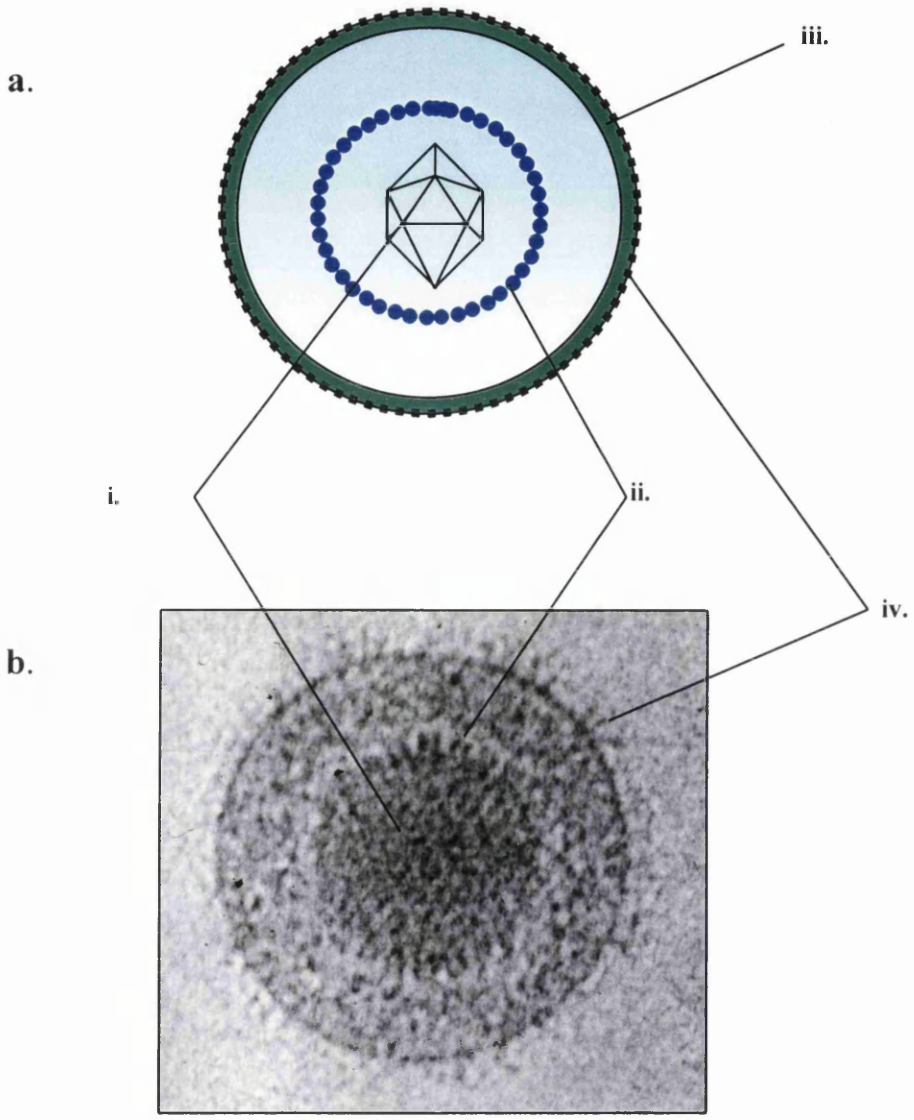


Figure 1. HSV virion.

a. Diagrammatic representation of HSV virion. **i**. capsid, **ii**. tegument, **iii**. lipoprotein envelope, and **iv**. glycoproteins. **b.** Electron micrograph image of a HSV-1 strain 17^{+} virion (photo provided by F. Rixon).

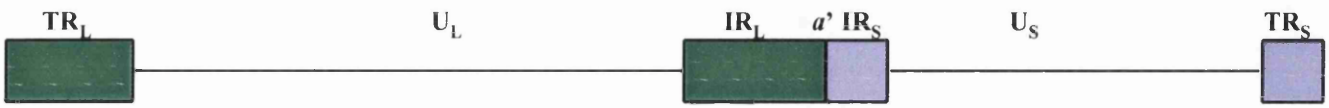


Figure 2. A schematic, linear diagram of the HSV-1 strain 17$^+$ genome (not drawn to scale).

The HSV-1 genome contains two unique regions, unique long (U_L) and unique short (U_S). These are flanked by terminal repeat sequences TR_L and and TR_S and internal repeat sequences IR_L and IR_S. The sequence where theTR_L and TR_S join is called the *a* sequence (not shown in this diagram). The junction where IR_L and IR_S meet is called the *a*' sequence.

virion molecular weight (Vmw) are the major nomenclatures for HSV proteins. The ICP numbering system is determined by order in a polyacrylamide gel (i.e. ICP1 is the heaviest and ICP47 is the lightest). A complete list of all known HSV-1 proteins, gene names and a brief description is given in Appendix A.

Capsid

Capsids are assembled in the nucleus. They are composed of protein, and are approximately 100 nm in diameter and have an icosahedral symmetry made of 162 capsomeres. Three types of capsids can be isolated from HSV-1 infected cells. They are visualised as light scattering bands in sucrose gradients and are designated A, B and C in order of increasing distance sedimented (Gibson and Roizman, 1972). A and C capsids are similar in protein content, but only the C capsid contains a genomic equivalent of DNA and presumably matures into the infectious virion. The seven proteins which compose the B capsid are VP5, VP19c, VP22a, VP21, VP23, VP24 and VP26 (Gibson and Roizman, 1972). Table 1 gives a complete nomenclature of the proteins and gene names.

Capsids are shaped like hexagonal prisms with a hollow tube through the length of the longitudinal axis (Fig. 1; Wildy *et al.*, 1960). VP5 is the major capsid protein, comprising approximately 60% of the capsid mass, and forms the hexons and pentons present in capsids. VP22a is present only in B capsids, occupies the inner space, and functions as a scaffold for assembly of the icosahedral capsid shell. VP19C and VP23 form a triplex structure that is required in the formation and stabilisation of the capsid shell. The UL26 gene product encodes an essential protease activity, VP24, specified by residues 248 to 635. VP21 shares sequence identity with the more abundant 22a from residues 307-635 and also has scaffold activity (Steven and Spear, 1996).

VP26 is the smallest capsid protein (12 kDa) and is encoded by the UL35 ORF (McNabb and Courtney, 1992a; Davison *et al.*, 1992). It is expressed late in the infectious cycle after the onset of DNA replication and has been shown to be present in multiple phosphorylated forms (McNabb and Courtney, 1992b). Desai *et al* (1998) deleted the VP26 gene and inserted a *lacZ* gene in its place and named this mutant virus KΔ26Z. They showed that VP26 is dispensable for replication in cell culture. Capsid assembly and the composition of the synthesised virions appear to be unaffected by the absence of VP26, although infectious virus yield is decreased by two-fold relative to wild type. Therefore, VP26 is the only capsid protein that is not required for replication in cell culture (Desai *et al.*, 1998). KΔ26Z had plaques which were smaller in size than wild type plaques, although

Table 1. Nomenclature of virion and capsid proteins in HSV-1.

Gene	Protein
UL18	VP23
UL19	VP5
UL26	VP21
UL26.5	VP22a
UL35	VP26
UL38	VP19C
UL46	VP11+12
UL47	VP13+14
UL49	VP22

quantitative examination was not carried out. Desai *et al* (1998) demonstrated that VP26 is important for virus replication *in vivo*. Mutant virus yields were decreased 30- to 100- fold in the trigeminal ganglia (TG). Reactivation of the mutant virus as determined by co-cultivation was also reduced (Desai *et al.*, 1998).

Tegument

The tegument is an electron dense material located between the capsid and the envelope. The amount of tegument in each virion is not equivalent. It is composed of at least 20 distinct viral proteins, some of whose properties are not yet fully understood. An example of a tegument protein is UL41 which functions as a virion associated host shut-off protein (*vhs*) (Aurelian *et al.*, 1989).

VP16, or α gene trans inducing factor (αTIF), recognises *cis*-acting response elements, the octamer-GARAT and TAATGARAT sequences, in promoters of IE genes. VP16 does not directly contact its response elements but binds to the cellular protein Oct-1 attached to the octamer or TAAT portion of the element.

VP22 is one of the structural proteins that make up this poorly understood region of the virion tegument which has been studied (Elliott and O'Hare, 1997; Dargan, 1986). VP22 is unique in its ability to enter surrounding cells with such a high level of efficiency that, after endogenous synthesis in a small subpopulation of cells, the protein forms gradients in surrounding recipient cells as reported by Elliott and O'Hare (1997). Elliott and O'Hare showed that uptake of VP22 does not involve the standard route of endocytosis, but a novel pathway that is cytochalasin D sensitive and therefore would seem to require an intact actin cytoskeleton.

Envelope

The envelope is the outermost covering of the virus and is composed primarily of lipids derived from the host cell membrane, into which are inserted the HSV glycoproteins, and are necessary for cellular recognition. Glycoproteins are individually described in Section 1.4.1. The diameter of the envelope is approximately 150-200 nm, although the exact dimensions depend on visualisation technique.

1.3. Human Herpes Viruses

To date, eight herpesviruses have been isolated from humans: herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella-zoster virus (VZV), human

cytomegalovirus (HCMV), Epstein-Barr virus (EBV), and human herpesvirus 6, 7 and 8 (HHV-6, HHV-7 and HHV-8). These herpesviruses fall into all of the three different subfamilies as described in Section 1.1.1. (Table 2).

Table 2. Known human herpesviruses and their classification (Roizman and Sear, 1996; Neipel *et al.*, 1997).

Human Herpesvirus	Official Name	Common Name	Subfamily
Herpes simplex virus 1	HHV-1	HSV-1	α
Herpes simplex virus 2	HHV-2	HSV-2	α
Varicella Zoster virus	HHV-3	VZV	α
Epstein Barr virus	HHV-4	EBV	γ
Human Cytomegalovirus	HHV-5	HCMV	β
Human herpes virus 6	HHV-6	HHV-6	β
Human herpes virus 7	HHV-7	HHV-7	β
Kaposi's sarcoma associated herpes virus	HHV-8	KSHV	γ

1.3.1. Herpes Simplex Virus

Herpes simplex virus (HSV) is a member of the subfamily *Alphaherpesvirinae* and the genus simplexvirus. Simplexviruses generally have a wide host range, a relatively short reproductive cycle, and the capacity to establish latency. They are among the most intensely studied viruses because of their ability to cause a variety of infections and to reactivate causing lesions at or near the initial site of infection (Roizman *et al.*, 1996). They serve as excellent models for studying translocation of proteins, synaptic connections in the nervous system, membrane structure and gene regulation (Roizman *et al.*, 1996).

1.3.2. Genome Structure

The members of the family *Herpesviridae* are identified by double-stranded linear DNA in the core of the virion. All herpesvirus DNAs studied to date fall into one of five

sequence arrangements (Fig. 3). A characteristic feature of herpes virus DNAs extracted from virions is the presence of nicks and gaps that cause the DNAs to fragment upon denaturation with alkali and formamide. It has been reported that packaged DNA contains ribonucleotides, but these do not account for all the sites of fragmentation of the DNAs.

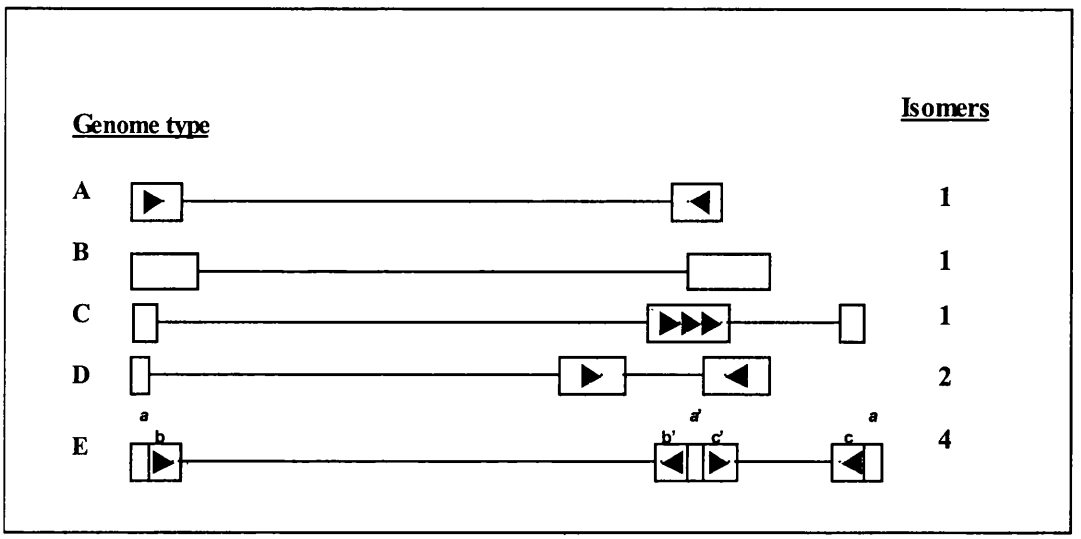


Figure 3. Diagram of the sequence arrangements in the five types of genomes of herpesviridae.

The lines represent unique regions. Reiterated sequences larger than 1 kbp in length other than terminal reiterations are shown as rectangles. The arrowhead in rectangles indicates whether the sequence is repeated in direct or inverted orientation as in genome type A, C, D and E. Where more than one set of large repeats has been reported, they are designated by letter. The shaded boxes represent terminal reiterated sequences reported in genomes types B, C, D and E. All terminal reiterations are direct repeats. In the case of the type E genomes, the terminal reiterations designated as the *a* sequence are also repeated in inverted orientations internally (*a*'). Examples of each genome type include; the channel catfish herpes virus (A), saimirine herpes virus 2 (B), EBV (C), VSV (D) and HSV (E) (Roizman and Sears, 1996).

1.3.3. The HSV-1 and HSV-2 Genome

Morphologically, HSV-1 and 2 are identical and have approximately 50% DNA homology. However, HSV-1 and 2 have some biological, biochemical, genomic and clinical differences. They differ in restriction endonuclease (RE) cleavage sites and viral protein sizes. An example of one RE which differs between HSV-1 and HSV-2 and used widely throughout this study is *Bam*HI, represented in Figure 4. Antigenic differences have been demonstrated by serologic assays (Aurelian *et al.*, 1989). An example of a poorly

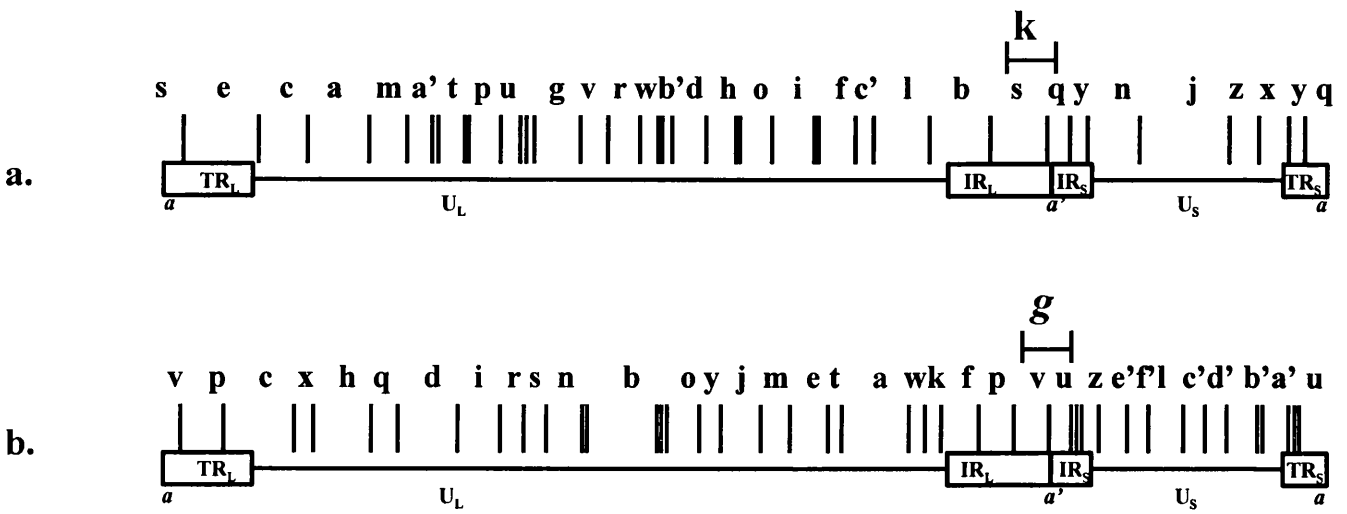


Figure 4. *Bam*HI restriction enyme digestion pattern of HSV-1 and HSV-2.

a. The HSV-1 strain 17^+ *Bam*HI digestion pattern. The fargment "k" is the L/S junction fragment containing fragments "s" and "q". This includes the RL1 and ORF P genes.
b. HSV-2 strain HG52 genome with *Bam*HI fragments indicated. The *Bam*HI fragment, *g*, is the L/S junction fragment covering the *v* and *u* sequences.

conserved gene between HSV-1 and 2 is gG (Frame *et al.*, 1986). The LAT locus of HSV-1 and 2 shares more than 80% sequence identity and contains similarly organised groups of putative *cis*-acting elements (Soares *et al.*, 1996).

1.3.4. The *a* Sequence

The *a* sequence is a direct repeat located at TR_L and TR_S and present as an inverted repeat at the L/S junction in the HSV-1 and 2 genome. The *a* sequence is approximately 500 bp in HSV-1 (strain F), but its size varies from strain to strain. The HSV-1(F) *a* sequence has a 20 bp direct repeat (DR_1), a 65 bp sequence (U_b), a 21 bp sequence (DR_2), a 37 bp sequence (DR_4), a 53 bp unique sequence (U_c) and another copy of DR_1 (Fig. 5; Roizman and Sears, 1990). The number of *a* sequences at the L/S junction and at the L terminus of the DNA varies from 1 up to 10 (Roizman, 1979) but only 1 is present at the S terminus (Fig. 5; Wagner and Summers, 1978; Locker and Frenkel, 1979).

In 1985, Chou and Roizman reported that the *a* sequence acts in *cis* to cause inversion. Deletion of DR_4 drastically reduces the frequency of inversion. Deletion of DR_2 and DR_4 abolished inversions (Chou and Roizman, 1985). Most of the DR_1 sequence can be deleted without affecting inversion.

Two highly conserved regions were identified in the U_b and U_c regions, *pac-1* and *pac-2*. These were determined to be essential for the cleavage and packaging process (Deiss *et al.*, 1986).

A mutant virus was generated by Martin and Weber (1996) to study the dispensability of the *a* sequence present at the L-S junction. The original copies of the *a* sequence were deleted from this virus by conventional marker transfer. The mutant virus which does not contain the L/S *a* sequence but had one *a* sequence inserted in the thymidine kinase (tk) gene was named HSV-1::LΔa. It had normal levels of the four isomeric forms of viral DNA in infected cells, but only two of these genomic isomers could be packaged into virions (Martin and Weber, 1996). The restriction was the result of inversion of the L component during isomerization, which prevented two of the four isomers from having cleavage and encapsidation signals of the *a* sequence in the tk gene in a packagable orientation.

1.4. HSV Lytic Cycle

During the course of its normal life cycle HSV must infect and replicate in at least two cell types, epithelial and neuronal. The HSV lytic cycle encompasses the primary

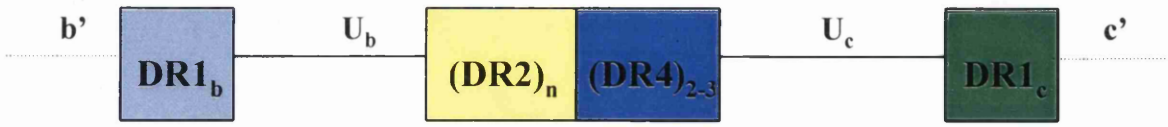


Figure 5. Diagram of the *a* sequence.

The sequence is divided by repeat domains drawn as blocks. The non-repeated sequences include U_b which is a located toward the b' region and U_c located toward the c' region. DR1b and DR1c are 20 bp elements present as direct repeats at the edge of the *a* sequence. DR2 is a directly repeated 12 bp element present in 19-22 copies. The DR4 domain is a directly repeated 37 bp element present in 2-3 copies. (Mocarski and Roizman, 1981).

infection of host cells and viral DNA replication in epithelial cells. HSV virions in the lytic cycle facilitate their replication by the preferential production of viral proteins at the expense of the host cell gene expression (Zelus *et al.*, 1996). In tissue culture, host protein synthesis and mRNA levels decline by approximately 90% within 3 hours postinfection with either HSV-1 or 2 (Zelus *et al.*, 1996). Destabilisation of mRNA is triggered by the virion induced host shut-off (vhs) protein, which is located in the virion tegument (Zelus *et al.*, 1996).

1.4.1. Glycoproteins

Membrane glycoproteins mediate HSV entry into the cell, cell to cell spread, cell fusion, envelopment, egress and immune evasion. Glycoproteins also play a role in tissue tropism and host range. HSV-1 encodes at least twelve glycoproteins, namely: gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM and gN. The precise role of all the glycoproteins has not been fully defined. Five glycoproteins: gB, gD, gH, gK and gL, have been shown to have an essential role in virus infectivity (Stannard *et al.*, 1996).

Glycoprotein B has a role in virus adsorption, penetration, cell to cell spread and cell fusion (Navarro *et al.*, 1992). It is also a specific determinant of HSV-1 neuroinvasiveness (Yuhasz and Stevens, 1993). Glycoprotein B binds heparan sulphate moieties on the cell surface. Dyer *et al* (1997) showed that dextran sulphate stimulation of infection is primarily mediated by gB. Williams and Strauss (1997) also demonstrated that HSV-2 gB interacts with cell surface glycosaminoglycans during virus attachment. Conservation of the gB gene between HSV-1 and 2 has been demonstrated by functional equivalence (Lin *et al.*, 1996). Mutations affecting the cytoplasmic C-terminal domain of gB induce a syncitial phenotype (DeLuca *et al.*, 1982, Bzik *et al.*, 1984; Cal *et al.*, 1988).

Glycoprotein C is considered nonessential for virus production in tissue culture. However, it has a function in attachment to the cell surface through heparan sulphate moieties (Padilla *et al.*, 1997). It has recently been shown that gC may not play a vital role in adsorption (Williams, 1999; Griffiths *et al.*, 1998). While gC enhances viral entry it is not required for syncitium formation and has been shown to inhibit fusion of some cultured cells (Manservigi *et al.*, 1977; Novotny *et al.*, 1996). Many syncitial HSV mutants do not express gC (Spear, 1993).

Glycoprotein D is an essential envelope glycoprotein required for viral entry (Willis *et al.*, 1998). Mutant viruses deleted in gD are severely compromised in neuronal spread (Dingwell *et al.*, 1995). It has been proposed that the role of gD in cell to cell spread

may be partially associated with its ability to bind mannose-6-phosphate receptors (MPRs) (Brunetti *et al.*, 1998).

Glycoprotein E has been shown to be non-essential (Neidhardt *et al.*, 1987). It is a Fc binding glycoprotein (Bauke and Spear, 1979) and forms a non-covalently linked heterodimer with gI. Fc binding activity may prevent complement mediated lysis of infected cells and enveloped virions and may also protect against Fc-facilitated phagocytosis (Bell *et al.*, 1990). The gE:gI complex also facilitates neurone to neurone spread of HSV-1 and its cell to cell spread (Dingwell *et al.*, 1995).

Glycoprotein H is required for membrane fusion but it is dispensable for receptor binding. Glycoprotein H negative virus binds to cells while being unable to enter them unless PEG treated (Forrester *et al.*, 1992). Adsorption of gH negative virions to cells blocks superinfection (Forrester *et al.*, 1992). Glycoprotein H may have a role in viral egress from cells, as it has been demonstrated by Buckmaster *et al* (1984) that an anti-gH antibody inhibits plaque formation when added in the overlay following virus adsorption. Mutant viruses with altered gH are retained within cells (Desai *et al.*, 1982). Targeting of gH to the rough endoplasmic reticulum results in the release of non-infectious virions from infected cells, while infectious virions containing gH are retained within the cytoplasm (Jayachandra *et al.*, 1997). Glycoprotein H may be involved in cell fusion as a gB syncitial mutant with a mutation in gH does not produce a syncitial phenotype.

Glycoprotein K, identified by Hutchinson *et al* (1992), is the product of UL53 (Debroy *et al.*, 1985; McGeoch *et al.*, 1988). It is not absolutely essential for the production of infectious virus in actively replicating cells, but is needed for efficient envelopment and transport of infectious virions to the extranuclear space. Glycoprotein K is involved in egress of HSV (Hutchinson *et al.*, 1992) and is thought to play a role in regulating membrane fusion and syncitium formation. Syncitial mutations arise more frequently within UL53 than in any other HSV gene (Ruyechan *et al.*, 1979; Bond and Person, 1984; Poguegeile and Spear, 1987; Jayachandra *et al.*, 1997). In contrast to all studied glycoproteins to date, gK accumulates in the perinuclear and nuclear membranes of cells; other glycoproteins are transported to the surfaces on infected cells (Hutchinson *et al.*, 1995).

1.4.2. Adsorption

Adsorption occurs when the glycoproteins on the virion are recognised by one or more specific cell receptor(s) which leads to binding of the cell surface. Spear (1993)

identified that cell surface heparan sulphate is a major factor in binding of HSV to the cell surface. Glycoprotein B and C are the major viral glycoproteins to play a role in adsorption (Spear, 1993).

1.4.3. Penetration

On the surface of the virion are several glycoproteins which facilitate the subsequent penetration of attached virions into the host cell. Glycoprotein B, gD, gH, and gL play an important role in penetration. When the virus has attached to the cell surface the viral envelope fuses with the cell membrane and the nucleocapsid is released into the cytoplasm.

1.4.4. Gene Expression

Gene expression involves regulatory loops controlled by signals which act either in *cis* or in *trans*. Details of each step are described below and are illustrated in context of the whole HSV lytic cycle in Figure 6. Gene expression occurs in a highly regulated order in three phases. The mRNAs produced during each of these phases corresponds to three classes of polypeptides; α (i.e. immediate early), β (i.e. delayed early), and γ (i.e. late) (Roizman, 1979). The cascade of gene expression is described in detail below.

1.4.4.1. HSV DNA Replication and Immediate Early, Early, and Late Protein Synthesis

In HSV-1, there are three *cis*-acting elements, which function as origins of DNA replication. The origin of DNA replication in the long region, ori_L, is located between the divergent transcripts for the DNA polymerase and the major binding protein (MDB) (UL29 and UL30) (Quinn and McGeoch, 1985). The origins of DNA replication in the short region, ori_S, are localised to a 90 bp fragment in R_S situated between the 5' ends of the divergent transcripts for IE3 and IE4/5 (Stow, 1982). The ori_S is present as 2 identical copies in the intact genome, one in IR_S and one in TR_S. In HSV-1 strain 17^+, ori_S contains an imperfect palindrome of 42 bases with an $(AT)_6$ region and sequence at the centre. This central $(AT)_6$ region and the sequence surrounding it are essential for viral replication (Stow, 1982). As early as 0.5 h postinfection and in the absence of protein synthesis, viral DNA accumulates in the nucleus and circularises.

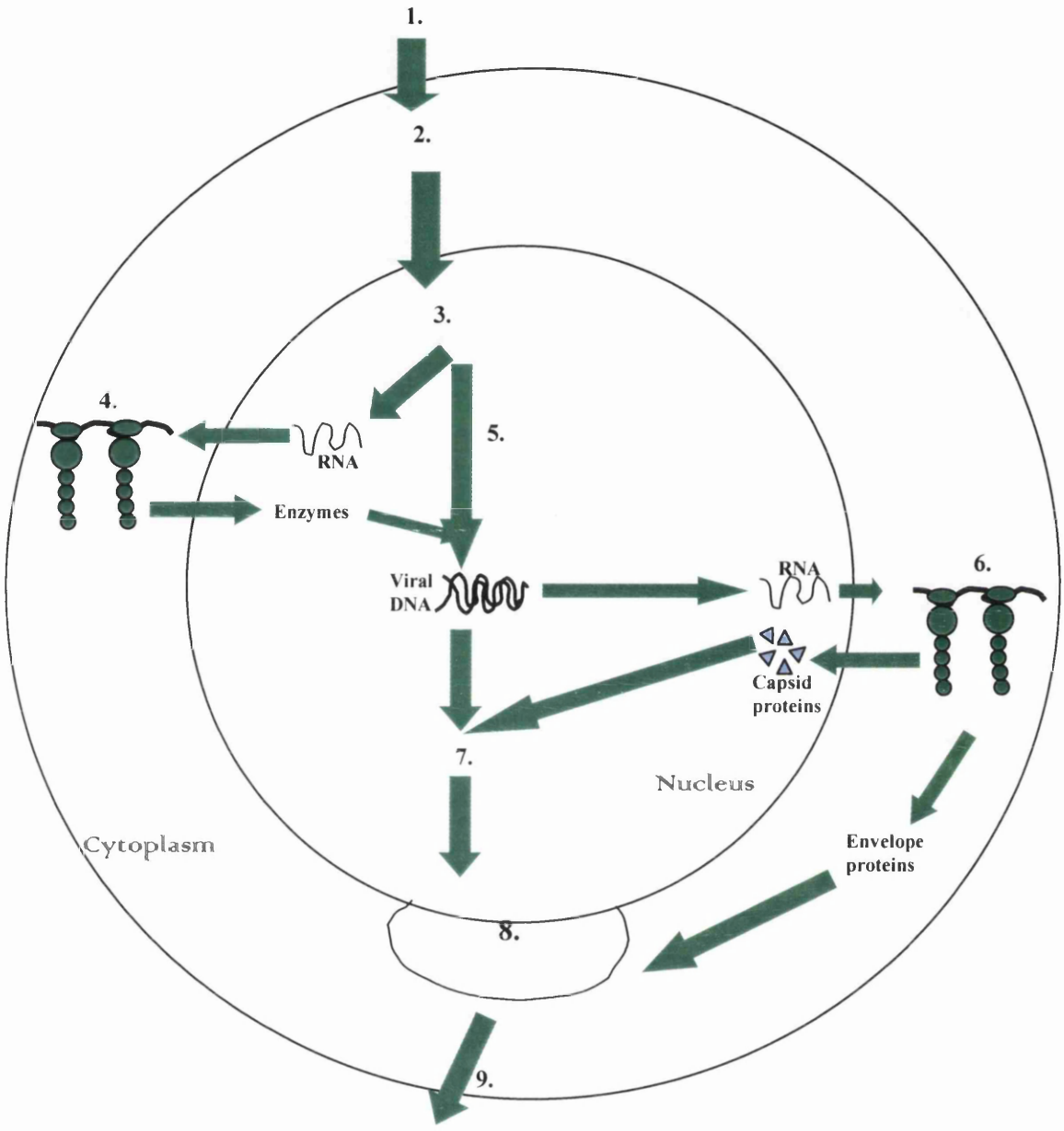


Figure 6. HSV lytic cycle replication.

The lytic cycle throughout an infected host cell is illustrated in this diagram. There are 9 stages which include the following: 1. adsorption and fusion with the cytoplasmic membrane for penetration; 2. uncoating of DNA; 3. early transcription; 4. early protein synthesis; 5. viral DNA replication, 6. late protein synthesis; 7. assembly; 8. envelopment by nuclear membrane; and 9. transport through the cytoplasm and release from the cell.

The seven HSV-1 genes which have been found to be necessary for origin-specific replication include: UL5, UL8, UL9, UL29, UL30, UL42, and UL52 (see Table 3). Once the DNA is replicated within the nucleus, the capsid is assembled then released (Lehman and Boehmer, 1999).

As described in the previous section, initially only five immediate early (IE) gene products are synthesised peaking at 2 to 4 h postinfection. Part of their function is to allow synthesis of early (E) proteins. Beta products are mostly involved in replication of viral DNA and proteins synthesis peaks 5 to 7 h postinfection. The temporal cascade of viral gene expression ends with the late (L) proteins.

1.4.5. Packaged Virions

Complete virions are transported to the cell membrane via the endoplasmic reticulum and the Golgi apparatus. Biosynthesis of herpesvirus glycoproteins follows that of eukaryotic proteins. Nonglycosylated precursors of herpesvirus membrane proteins are synthesised on polyribosomes bound to the rough endoplasmic reticulum (Roizman, 1979) and processed by addition of N/O linked sugars in the endoplasmic reticulum and Golgi apparatus. Once the DNA is replicated within the nucleus, the capsid is assembled then released. *In vivo*, following the initial lytic infection capsids are transported to the ganglia. In the ganglia, the HSV genome circularises and goes into a state known as latency.

1.5. HSV Latency

The latent phase occurs when the HSV genome maintains a quiescent infection in neurones of ganglia. It is thought that the genome takes the physical extrachromosomal shape of a circular episome (Aurelian *et al.*, 1989). It is estimated that only a small number of neurones contain latent virus with, on average, each latently infected neurone containing different number of copies of the HSV genome. The restriction of gene expression prevents cell lysis and is one of the major features of latency (Cann, 1993). Additionally, reduced gene expression diminishes recognition of infected cells by the immune system. The restricted gene expression is probably achieved by tight regulation of α gene expression which is crucial in HSV replication.

In 1997, Sawtell published a method for comprehensive quantitative analysis of latently infected tissue at the level of single cells known as CXA. It was reported that the

Table 3. HSV-1 proteins involved in DNA replication.
(concept from Lehman and Boehmer, 1999)

Protein	Function
UL9	Origin-binding protein
UL8	Helicase-primase loading protein
UL5/52	Replicative helicase
UL5/52	Primase
UL30/42	DNA polymerase
UL30	Proofreading exonuclease
UL42	DNA polymerase clamp
ICP8	Single-stranded DNA binding protein
UL30	RNase H

viral genome copy number within individual latently infected neurones is variable, ranging over three orders of magnitude from <10 to >1000. There is a direct correlation between increasing viral input titre and the number of neurones in which latency is established in the ganglion (Sawtell, 1997).

Using the latest techniques to study latency, only the latency associated transcripts (LATs) have been detected; no HSV antigens or virion particles have been detected. The LATs and their promoters (LAP1 and LAP2) are described below.

1.5.1. LAP1 and LAP2

Two LAT promoters have been identified, LAP1 and LAP2. LAP1 is situated at the 5' end of the 8.3 kbp LAT and is 700 bp upstream of the 2 kbp intron (see Section 1.5.2.). It is required to generate a series of LATs in latently infected neurones (Soares *et al.,* 1996). Sequence analysis of this region suggests the existence of several *cis*-acting elements. Soares *et al* (1996) studied the LAP1 TATA box and found it to play an essential role in transcription initiation *in vitro.* Further analysis of LAP1 by *in vitro* transcription primer extension assays indicate that the presence of upstream elements specifically contribute to LAP1 function and that sequences 620 nucleotides upstream of the transcription start site were responsible for full promoter activity (Soares *et al.,* 1996). In addition to the TATA box, the LAP1 region contains a CCAAT box homology and potential recognition sequences for several transcription factors, including Sp1, AP-2, YY1, CREB, LBF/MLTF/USF, Egr-1, a POU domain protein, and the HSV-1 immediate early transactivator, ICP4 (Soares *et al.,* 1996).

Studies have demonstrated that the LAT promoter sequence plays an important role in spontaneous reactivation in the rabbit eye model (Soares *et al.*, 1996). A mutant virus constructed with a deletion of the latency associated promoter (LAP1) at both loci, did not express LAT during latency (Soares *et al.,* 1996) and reactivated poorly. Similar findings were observed by Bloom *et al* (1996).

A second promoter which lies between LAP1 and the 5' end of the 2 kbp LAT has been identified and designated LAP2 (Soares *et al.,* 1996). There have been no studies done, to date, to demonstrate the importance of this promoter.

1.5.2. Latency Associated Transcripts (LATs)

The LAT locus is located in the long terminal repeat and is therefore present in 2 copies (Perng *et al.*, 1996b). The less abundant (i.e. minor) LAT is 8.3 kbp. The most

abundant (i.e. major) LATs are 2 co-linear, predominately nuclear poly(A)- RNAs of 2 kbp and 1.5 kbp which are believed to be introns spliced from the 8.3 kbp LAT. The major LATs appear to share 5' and 3' termini differing only in the excision of a small intron with the unusual use of GC instead of the consensus GU at the 5' cleavage site (Wagner *et al.*, 1988; Spivack *et al.*, 1991). Protein expression from LATs has not been detected during latency. LATs have been detected by Northern blot analysis and their structures analysed from cDNA clones and RT-PCR products (Spivack *et al.*, 1995). LATs can also be easily detected by *in situ* hybridisation (Maggioncalda *et al.*, 1996). The LAT transcript overlaps the ICP0 and ICP34.5 genes in an antisense direction (Fig. 7). The role of LAT in latency has yet to be fully understood.

The amount of viral DNA recovered from rabbit TG latently infected with either LAT+ or LAT- virus, when quantified, was found to be equivalent (Bloom *et al.*, 1996). However, the ability of virus to reactivate from latency was shown to reside within a 350 bp region of LAT (Bloom *et al.*, 1996). This is in contrast to the data found using the murine model (Fraser *et al.*, 1992).

Arther *et al* (1998) tested to see if the 2 kbp LAT was produced as an intron in all phases of infection by disrupting the LAT splicing signals. Mutation of the splice acceptor abrogates the 2 kbp major LAT generation during productive infection, but does not significantly influence major LAT synthesis during neuronal latency. Mutation of the splice donor also reduces the 2 kbp major LAT during productive infection.

When it was first determined that major LATs are complementary to part of the ICP0 mRNA it was hypothesised that these transcripts influenced and maintained latency by down regulating ICP0 through an antisense mechanism (Farell *et al.*, 1993). However, LAT null mutants were found to establish and maintain latency.

A role for LATs in efficient establishment of latency has been proposed (Sawtell and Thompson, 1992a; Thompson and Sawtell, 1997) and moreover, expression of the 2 kbp LAT has been shown to reduce ICP0 transactivation in dual transfection assays (Farrell *et al.*, 1993). This hypothesis was substantiated when it was found that ICP0 message levels were elevated in BHK cells infected with viruses unable to make the 2 kbp LAT (Arther *et al.*, 1998).

Chen *et al* (1997) used RT-PCR to analyse the effects of a mutation in the LAT locus on viral gene expression in latently infected mouse TG. The deletion removed the promoter, transcriptional start site, and 1,015 bp of transcribed sequences of LAT. The deletion mutant had reduced expression of the major LATs which resulted in an increase in

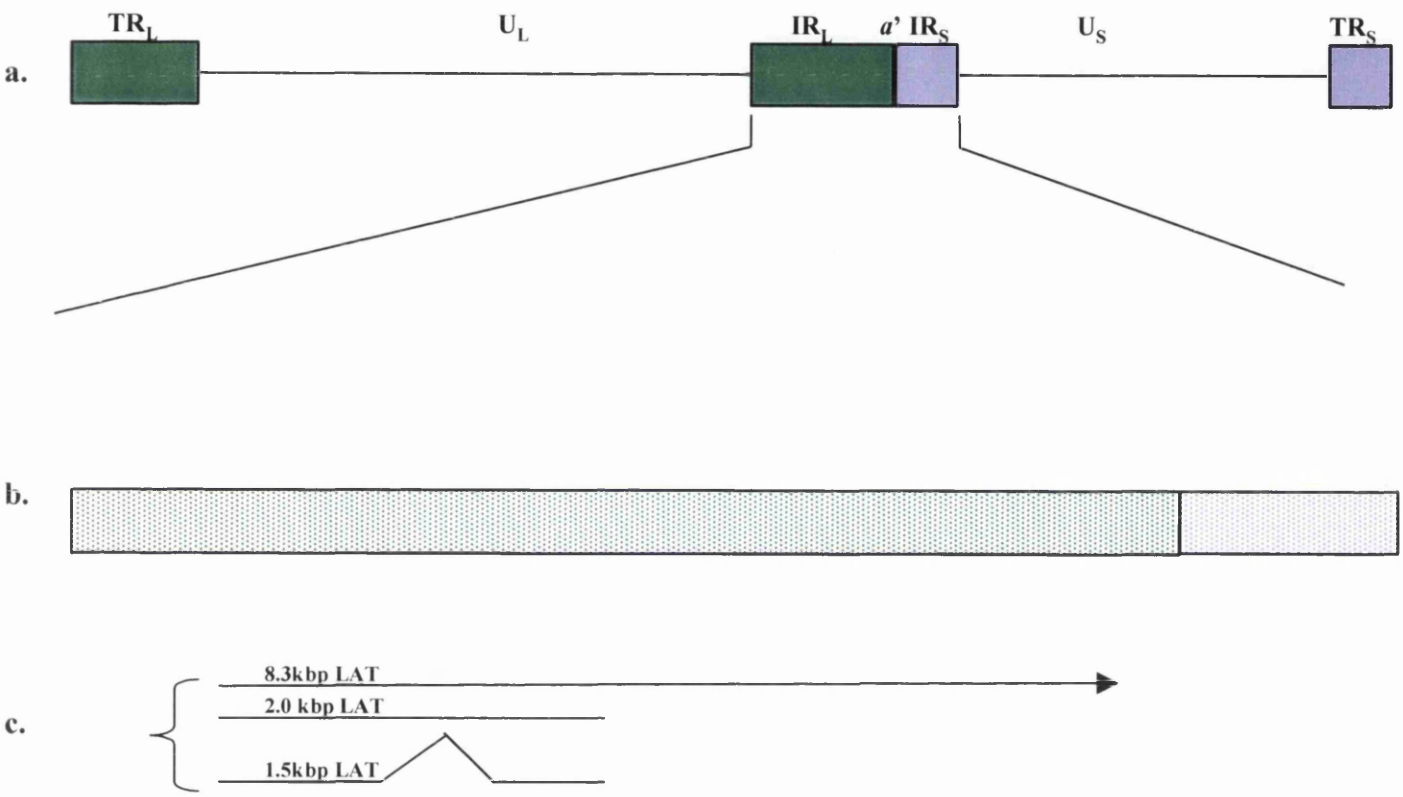


Figure 7. A schematic, linear diagram of the HSV-1, strain 17$^+$, genome (not drawn to scale).

a. The HSV-1 genome (detailed in Fig. 2). **b**. The IR_L, IR_S and *a'* sequence is expanded
c. The only transcripts which can be detected during latency (LATs) are shown here.

accumulation of transcripts from the immediate early gene encoding ICP4. The LAT deletion also resulted in an increase in the accumulation of transcripts from the early gene encoding tk, whose expression during productive infection stringently depends on ICP4. The levels of tk positively correlated with the levels of ICP4 transcripts. Chen *et al* (1997) concluded that a viral function associated with the LAT locus represses the accumulation of transcripts from at least two productive cycle genes in latently infected mouse ganglia.

Drolet *et al* (1998) sequenced the first 1.5 kbp of LAT from HSV-1 McKrae, a strain with a very high spontaneous reactivation rate. Of the HSV-1 LAT sequences available for comparison (17^+, KOS, and F) only strain 17^+ demonstrated high spontaneous reactivation in the rabbit (Perng *et al.*, 1996a,b). Unexpectedly, Drolet *et al* (1998) reported that a chimeric virus containing the KOS LAT gene in a HSV-1 McKrae genetic background had a spontaneous reactivation rate indistinguishable from McKrae. Drolet *et al* showed that the LAT function involved in spontaneous reactivation is mediated by a direct DNA or RNA mechanism rather than a protein. LAT- mutants reactivate poorly by induced reactivation in the mouse (Sawtell and Thompson, 1992a), by induced reactivation in the rabbit (Trousdale *et al.*, 1991) and by spontaneous reactivation in the rabbit (Perng *et al.*, 1996a,b). Drolet *et al* (1998) showed that expression of only the first 1.5 kbp of the primary 8.3 kbp LAT was required to restore wild type McKrae levels of spontaneous reactivation to a McKrae LAT null mutant.

Studies by Garber *et al* (1997) demonstrated that in acutely infected TG neurones, two independent isolates of a LAT- viruses exhibited expression of immediate early genes and had increased numbers of cells expressing early and late transcripts compared to wild type and LAT rescued viruses. Moreover, infections with LAT- viruses, which exhibit increased expression of productive cycle genes within sensory neurones, showed decreased frequencies of reactivation from latency (Garber *et al.*, 1997).

Goldenberg *et al* (1997) cloned the entire LAT region from strain F (10.4 kbp) under the control of a constitutive promoter and generated a neuronal cell line (NA4) stably transfected with the viral LATs region. NA4 cells produced the 2.0 kbp and the 1.5 kbp LATs. Northern blotting and RT-PCR of RNA from NA4 cells, and from TG of mice latently infected with HSV-1 strain F, revealed that the two abundant LAT species were present in the polyribosomal RNA fractions and they hypothesised that these LATs might be translated (Goldenberg *et al.*, 1997).

In 1990(a), Mitchell *et al* reported that a 17^+ mutant virus named 1704 had a 3.8 kbp deletion that starts to the left of the 5' end and extends into the coding sequence of the

IR_L LAT and a 1.2 kbp deletion that is located 5' to, but outside the coding region of the LAT in TR_L. Ganglia of BALB/c mice latently infected with 1704 had no major LATs detected by *in situ* hybridisation (Mitchell *et al.*, 1990a). The amount of viral DNA in 1704 and 17^+ latently infected ganglia was similar but there was delayed reactivation kinetics observed with 1704. This affect was believed to be the result of a biological function affected by the deletion rather than a difference in the number of genome copies present during latency (Mitchell *et al.*, 1990a).

Mitchell *et al* (1990b) established a model for HSV-2 latency in mice that is amenable to molecular studies. Data gained with this model indicated that HSV-2 transcription during latency is confined to the repeat regions of the viral genome (Mitchell *et al.*, 1990b). They identified an HSV-2 LAT and partially mapped it by *in situ* and Northern blot hybridisation experiments. This is possibly the first report identifying LATs in HSV-2 (Mitchell *et al.*, 1990b). While LAT transcription during HSV-2 latency has not been as widely studied as in HSV-1, a single 2.2 kbp LAT intron is readily detected during acute and latent infection (Yoshikawa *et al.*, 1996). In HSV-2, an equivalent to the HSV-1 1.5 kbp spliced variant has not been detected.

1.5.3. *In vitro* Latency Models

In vitro cell culture latency systems have been developed and utilised to understand the molecular mechanism of latent HSV infection. Early *in vitro* latency systems relied on infection of cells in the presence of inhibitors of virus replication for establishment, and incubation at supraoptimal temperature for maintenance of latency (O'Neill *et al.*, 1972; O'Neill 1977; Wigdahl *et al.*, 1981, 1982, 1983). Non-productive infection by HSV has also been established in a neuroblastoma cell line selected for its hyper-resistance to HSV-1 infection. In this instance, HSV-1 was retrieved by superinfection with HSV-2 (Nilheden *et al.*, 1985). A lymphoid cell line, which harbours latent HSV, has also been described (Youssouffian *et al.*, 1982).

In 1986, Russell *et al* developed an *in vitro* latency system for HSV-2. Virus replication was suppressed by infection of human foetal lung cells at the supraoptimal temperature of 42°C, and, following transfer of cells to the normal growth temperature of 37°C, infectious virus was generally not detected for at least six days. HSV-2 was reactivated by intertypic superinfection at 38.5°C with temperature sensitive mutants of HSV-1, or with HCMV.

Mutant virus *in*1814 has an insertion mutation which abolishes trans-activation of IE transcription by Vmw65 (Ace *et al.*, 1989). Using the *in vitro* latency model described above, mutant *in*1814 was retained in HFL cells following infection at low multiplicity of infection (m.o.i.) after incubation at 42°C. Reactivation occurred by superinfection of monolayers with viruses that express ICP0 (Steiner *et al.*, 1990). Latency was established by *in*1814 at 37°C and 42°C in a non-linear genome form (Jamieson *et al.*, 1995; Preston *et al.*, 1994). It was proposed that lack of Vmw65 function pre-disposes HSV to latency, as opposed to lytic growth in HFL cells, resulting in the retention of the genome similarly found *in vivo* (Harris and Preston, 1991; Valyi-Nagy *et al.*, 1991).

Danaher *et al* (1999) developed an *in vitro* model of a non-productive HSV-1 infection in neurally differentiated (Nd)-PC12 (rat pheochromocytoma) cells which allowed for inducible virus replication upon forskolin treatment or heat stress. They demonstrated that the quiescent state was characterised by the absence of cell-associated virus, capsids, and viral antigens. They also showed that heat stress (43°C for 3h) efficiently reactivated virus from quiescently infected Nd-PC12 (QIF-PC12) cells (Danaher *et al.*, 1999). This demonstrated that the proportion of cultures that activate virus production is dependent upon the m.o.i. Reactivation was a rare event limited to only a minority of the QIF-PC12 cell population (1:400-1:22,000) when latency was established at m.o.i.'s of 30 and 3, respectively. Twenty-four h post heat stress the expression of HSV-1 productive genes (ICP0, ICP4 and ICP27, UL30 and UL18) switched from undetectable or very low levels to detectable or elevated levels in a sequential order with ICP4 induction occurring as early as 3 h post induction. Viral antigen and infectious virus became detectable in cells as early as 24 h post heat stress. In this model, a quiescent state between HSV-1 and Nd-PC12 is established with the transient use of acycloguanosine (ACV). Inhibitory levels are not present in QIF-PC12 cell cultures at the time of virus induction as measured by viral growth curves. Neither heat stress nor forskolin enhanced viral growth rates or yields in Nd-PC12 cells. This indicates that these agents acts at the level of induction, rather than altering cell permissivity. Furthermore, the absence of viral structures prior to induction suggests that the block in lytic pathway is between viral uncoating and capsid maturation (Danaher *et al.*, 1999). The absence of immune based cells in the cultures indicated that induced activation of HSV-1 within neural cells can occur independent of immunological signals. The analysis by Danaher *et al* (1999) of gene expression by RT-PCR permitted the detection of as few as 9 to 140 target sequences per 1500 cells, yet gene products of ICP0, ICP4 and

UL30 could not be detected during the quiescent infection at 20 days post infection. In contrast, low levels of ICP27 and UL18 were detected during the quiescent infection. The presence of low levels of some immediate early and early gene RNAs during quiescence is not surprising as Kramer and Coen (1995) reported similar findings in murine TG latently infected with HSV-1. During the response to heat stress the immediate early HSV-1 ICP4 and host HSP72 genes demonstrated induced expression within the first 3 h of treatment. Unlike the expression of the host HSP72 gene, which peaked at 2 h post heat stress and dropped to control levels by 38 h post heat stress, the level of ICP4 transcription continually increased throughout the assay. The level of transcription of other HSV-1 genes was not enhanced during the heat stress but increased at various times following recovery at 37°C. These data implicate ICP4 in the early role of co-ordinating reactivation from latency and argue against an early role of ICP27, ICP0 and the non-stable LAT in this process. In contrast, ICP27 has been detected as early as 4 h following epinephrine induced reactivation in rabbit models (Bloom *et al.*, 1994), and ICP4 has not always been detected rapidly post-explant of latently infected murine ganglia (Minagawa *et al.*, 1994).

1.5.4. *In vivo* Latency Models

The mouse footpad model was the first *in vivo* latency system to be developed (Stevens *et al.*, 1972; Walz *et al.*, 1974). HSV inoculation of a mouse rear footpad results in a local cutaneous infection and following anterograde transfer of virus through the peripheral and central nervous systems to the brain, an acute infection develops in the nervous system. The outcome of which may be a temporary or permanent posterior paralysis and possibly death of the mouse. Mice which recover from paralysis within three weeks are used for latency studies with latency established in the lumbar/sacral ganglia which supply the nerves to the footpad. During acute infection (1 to 10 days post inoculation) HSV can be recovered from the sciatic nerve, sacrosciatic spinal ganglia, dorsal roots, spinal cord and brain. Thereafter, infectious HSV has not been detected in homogenates of nervous tissue.

In the mouse (Knotts *et al.*, 1974; Walz *et al.*, 1974) and rabbit (Stevens *et al.*, 1972) eye models, HSV resides in a latent state in the TG after infection of the cornea via scarification. Latent infections have also been established in the TG and cervical dorsal root ganglia (DRG) of mice following lip and ear inoculation, respectively (Walz *et al.*, 1974; Hill *et al.*, 1975).

Several studies have been conducted to determine transcription and/or expression of virus proteins from ganglia latently infected with HSV. To date, the LATs have been the only consistently detected transcripts (Section 1.5.2.).

1.6. Reactivation

Reactivation of HSV from ganglia results in the appearance of infectious virus at the site of the initial primary infection. In experimental animal models, reactivation has been reported to occur following traumatization of peripheral tissues (Valyi-Nagy *et al.*, 1991). Viral gene products reported to govern the establishment and maintenance of latency and facilitate reactivation include thymidine kinase, ribonucleotide reductase and the immediate early protein (ICP0) (Aurelian *et al.,* 1989). Stimuli that induce reactivation are non-specific and include axonomy, elevations in cyclic AMP (cAMP), UV light, hyperthermia, iontophoresis of epinephrine, or possible hormonal fluctuations. Reactivation of latent virus following axonal injury has been shown to occur within 3-5 days.

Several models have been developed to study *in vivo* and *in vitro* reactivation. Different animal models have been used such as guinea pigs, rabbits and mice. The guinea pig model exhibits a high level of spontaneous reactivation. In contrast, the rabbit eye model seldomly shows spontaneous reactivation. In mice, spontaneous reactivation does not occur and it is difficult to induce reactivation. The lack of spontaneous reactivation in mice is valuable and for this reason most reactivation studies have been carried out using mice. In the mouse model, effective sites for viral inoculation in the periphery include the eye, and the ear, from which latency is established in the TG. Other peripheral sites of inoculation are the flank and the footpad where latency is established in the DRG. Once latency is established different methods for inducing reactivation have been developed. For example, reactivation in the eye model is induced by UV light. Feeding heavy metals (e.g. cadmium) or heat stress can be used to reactivate virus following any route of inoculation.

The tk gene has been studied to determine its role in reactivation. After corneal inoculation of mice, tk deleted virus mutants replicated to high titres in the eye, but were severely impaired for acute replication in TG established latency but failed to reactivate from ganglia upon co-cultivation with permissive cells (Coen *et al.*, 1989). This is contrary to the popular hypothesis that tk- mutants establish latent infections and implies that neither tk activity nor ganglionic replication is necessary for establishment of latency. Rather, tk appears to be necessary for reactivation from latency (Coen *et al.*, 1989).

1.6.1. *In vitro* Reactivation Models

One method to induce reactivation *in vitro* is to explant ganglia latently infected with HSV. This reactivates the virus in culture. The *in vitro* explant culture is useful for looking at possible *in vivo* reactivation trends (Trousdale *et al.,* 1991), but is probably more a measure of latent virus than reactivation *per se.*

Moriya *et al* (1994) described a culture system which was used as an *in vitro* model of stress induced reactivation. In the presence of an antiviral drug, primary cell cultures were established from latently infected mouse TG. Latency was maintained following removal of the antiviral drug, and the investigators found that HSV-1 could be reactivated from TG cells by heat stress. Halford *et al* (1996) developed primary cultures of TG cells from HSV-1 latently infected mice to study reactivation. Infectious virus appeared in 75% of culture supernatants within 120 h post heat stress. HSV-1 lytic phase mRNA and proteins were detectable 24 h after heat stress. The effect of heat stress duration on reactivation was determined to be 0, 40 or 67% of cultures after 1, 2 or 3 h post heat stress, respectively. The results that Halford *et al* (1996) attained indicate that TG cell cultures mimic important aspects of *in vivo* latency and reactivation and this model may be useful for studying signalling pathways that lead to HSV-1 reactivation. However, this model also may have spontaneous levels of reactivating virus pre- heat stress treatment (Halford *et al.*, 1996).

The immune profile of primary TG cell cultures latently infected with HSV-1 and uninfected prior to and after stress was studied by Carr *et al* (1998) by measuring cytokine and chemokine production. Cytokines which are extracellular signalling proteins act as a local modulators in cell-cell communication. Supernatants from TG cell cultures contained detectable interleukin (IL)-6 but not IL-1β, IL-2, IL-10, interferon (IFN)-γ or tumour necrosis factor (TNF)-α as determined by enzyme linked immunosorbant assay (ELISA). IL-6 is produced from some helper T cells and macrophages and targets activated B cells or T cells by promoting B cell maturation to Ig secreting cells. IL-1 and IL-6 are also made by non-blood cells and act on many types of target cells other than blood cells; they therefore are called cytokines. Supernatants from TG cell cultures also contained detectable levels of C-10, MCP-1 and eotoxin but little (to no) MIP-1α, MIP-1β or MIP-2. While there were no differences in the basal level of MCP-1 and eotoxin in TG cell cultures from HSV-1 infected and uninfected mice, C-10 levels were significantly higher in TG cultures originating from infected mice compared to uninfected ones (Carr *et al.*, 1998).

Hyperthermic stress was used to reactivate virus and demonstrated that there was no correlation between cytokine/chemokine levels in HSV-1 reactivation. Whereas no spontaneous reactivation has been reported in mice, spontaneous reactivation occurred in 4.5% (10/220) of TG cell cultures surveyed over a 20 day period (Carr *et al.*, 1998).

Local control of the acute infection includes the presence of CD4+ and CD8+ lymphocytes (Nash *et al.*, 1987; Bonneau and Jennings, 1989) neutrophils (Tumpey *et al.*, 1996), IFN-α (Hendricks *et al.*, 1991) and IFN-γ and TNF-α (Cantin *et al.*, 1995). Cytokine profiles, following acute HSV-1 infection, suggest that the cellular immune response is primarily of the Th1 type (Stuats and Lausch, 1993; Boulet *et al.*, 1995; Kanangat *et al.*, 1996). The role of these cytokines in latent HSV-1 infection or in the reactivation of the virus from latency has not been addressed. It has been proposed that TNF-α (Liu *et al.*, 1996) and IFN-γ (Cantin *et al.*, 1995) may control reactivated HSV-1. It is also thought that humoral immunity is involved in the maintenance of HSV-1 latency (Stevens *et al.*, 1972). The lack of dendritic cells, Th1 cytokines and TNF-α which are found *in situ* may, in part, explain the increase in hyperthermic induced reactivation of HSV-1 (McKrae strain) in these cultures (50-80%) compared to the *in vivo* model of HSV-1 (McKrae strain) latency where hyperthermic induced reactivation rarely exceeds 30% (Noisakran *et al.*, 1998).

1.6.2. *In vivo* Reactivation Models

The *in vitro* methods developed which explant ganglia and induce reactivation *in vitro* have some disadvantages. These methods do not necessarily reflect a true reactivation response *in vivo*. Moreover, *in vitro* explantation maintains a constant level and supply of the reactivating stimulus. *In vivo* reactivation gives a more comprehensive analysis of the natural virus cycle and pathogenicity.

An *in vivo* stress model for inducing reactivation in Swiss Webster mice by hyperthermia induction was developed by Sawtell and Thomspon (1992b). The heat shock response includes a regulatory cascade resulting in the rapid accumulation of a specific set of proteins (e.g. HSP70) and a decrease in others. Using hyperthermia induction to reactivate HSV in mice, Sawtell and Thompson (1992b) found HSV specific antigen in isolated large ganglionic neurones within 24 hours. Work during this thesis included learning this method from Dr. Sawtell during a sabbatical and a summary of this procedure is included as Appendix B.

Using the rabbit eye model as the site of initial infection and inducing reactivation by iontophoresis with adrenaline, Trousdale *et al* (1991) showed a combination of host strain and viral strain is critical. These results differed to those done in the mouse eye model. Furthermore, not all wild type HSV strains reactivate by iontophoresis.

Transcription during *in vivo* reactivation has been analysed using RNA from latently infected rabbit TG isolated at various times following epinephrine iontophoresis. The maximum signal intensity was normally 8 h post induction and most neurones established latency again 16 hours post induction (Bloom *et al.,* 1996). Bloom *et al* (1996) used a rabbit corneal model for virus reactivation *in vivo*. LAT- and LAT+ promoter viruses were studied. LAT- reactivated with low frequency upon epinephrine induction. Transcription of lytic phase genes could be detected within 4 h following induction of rabbits latently infected with either LAT- or LAT+ virus: transcription diminished 16 h post induction. In the rabbit eye model, epinephrine induced viral reactivation was monitored by the ability to recover virus in tear film (Farrell *et al.,* 1993; Hill *et al.*, 1986 and Hill *et al.*, 1996). There is a low level of spontaneous reactivation of LAT- viruses that occurs in this model. No reproducible net increase in relative levels of viral DNA was seen in the reactivation *in vivo* suggesting that only a small proportion of stimulated neurones go on to replicate viral genomes and that any infectious virus produced in the ganglia is transported to a peripheral site and reinitiates infection very efficiently.

It has been demonstrated that ocular iontophoresis of epinephrine induced reactivation from BALB/c mice infected with HSV-1 strain McKrae (Willey *et al.*, 1984), and ultraviolet irradiation of corneal epithelium induced reactivation of McKrae strain from NIH mice (Laycock *et al.*, 1991). However, these methods were applied to only one mouse strain. Fawl *et al* (1996) studied different mouse strains in a cadmium (Cd) *in vivo* reactivation method. All mice were inoculated with HSV-1 strain F at $3x10^6$ pfu/eye, except for strain A mice which were inoculated with $5x10^5$ pfu/eye, because of the strains increased virulence. Beginning at 30 days after inoculation latently infected mice were given a footpad injection of 50 µl Cd (2 mg $CdSO_4.H_2O$/ml sterile saline solution) daily for 3 days. Within 18 to 24 h after the third Cd injection the mice were sacrificed and each TG was removed to a culture tube containing media with 5% newborn calf serum. For assay of infectious virus each TG upon removal was immediately frozen in a dry ice ethanol bath and stored at –80°C until thawed, homogenised and plated onto CV-1 monolayers (Fawl *et al.*, 1996). Cd is a toxic heavy metal which strongly induces metallothionins and heat shock proteins. When administered to mice, Cd ions rapidly accumulate in the mouse

peripheral nervous system and remain at high levels in these tissues for at least 1 week. Daily administration of Cd to latently infected CBA mice leads to reactivation of HSV from TG within 2 to 4 days of treatment (Fawl and Roizman, 1993). They also showed that Cd treatment induced reactivation of HSV-1 strain F from strain CBA, but not from BALB/c mice (Fawl *et al.*, 1996).

1.7. Genes Located in the R_L Regions

Beyond the 3' terminal of the LATs and within the L/STs there are two additional ORFs within the R_L region on the same strand which have been shown to express polypeptides ORF O and P. Two other genes within the R_L sequences on the opposite strand, RL1 and RL2, have been demonstrated to express polypeptides, ICP34.5 and ICP0, respectively (Fig. 8). The most recently published information on these proteins is detailed below.

1.7.1. ICP0

The gene which encodes ICP0 (also called Vmw110) is RL2 (McGeoch *et al.*, 1988). One of the interesting features of RL2 is that it is one of only four HSV-1 genes that contain introns (Fig. 8). RL2 lacking the first, second or both introns were constructed by site directed mutagenesis (Everett, 1991). Viruses lacking the RL2 introns were indistinguishable from the parent virus in terms of growth, particle to pfu ratio or viral polypeptide expression in a variety of cell types. The lack of introns did not affect the time course or efficiency of expression of ICP0 either during normal infection or in cyclohexamide reversal experiments. However, in transfection assays, the loss of both introns resulted in the elimination of the ability of a plasmid encoding RL2 to activate gene expression. Everett's results imply that in certain situations the introns in RL2 may contribute to the efficient expression of ICP0 but such an effect is not apparent in recombinant viruses. The role of the RL2 introns remains obscure. Since sequences upstream of the RL2 region are involved in pathogenicity (Taha *et al.*, 1989; Thompson *et al.*, 1989) and a transcript containing these sequences continues throughout the RL2 region (Chou and Roizman, 1986; Dolan *et al.*, 1992), it seemed possible that information within the RL2 introns might also be involved in pathogenicity. However, data reported by Everett (1991) indicate that recombinant viruses with RL2 deletions do not have an altered phenotype *in vitro* or *in vivo*.

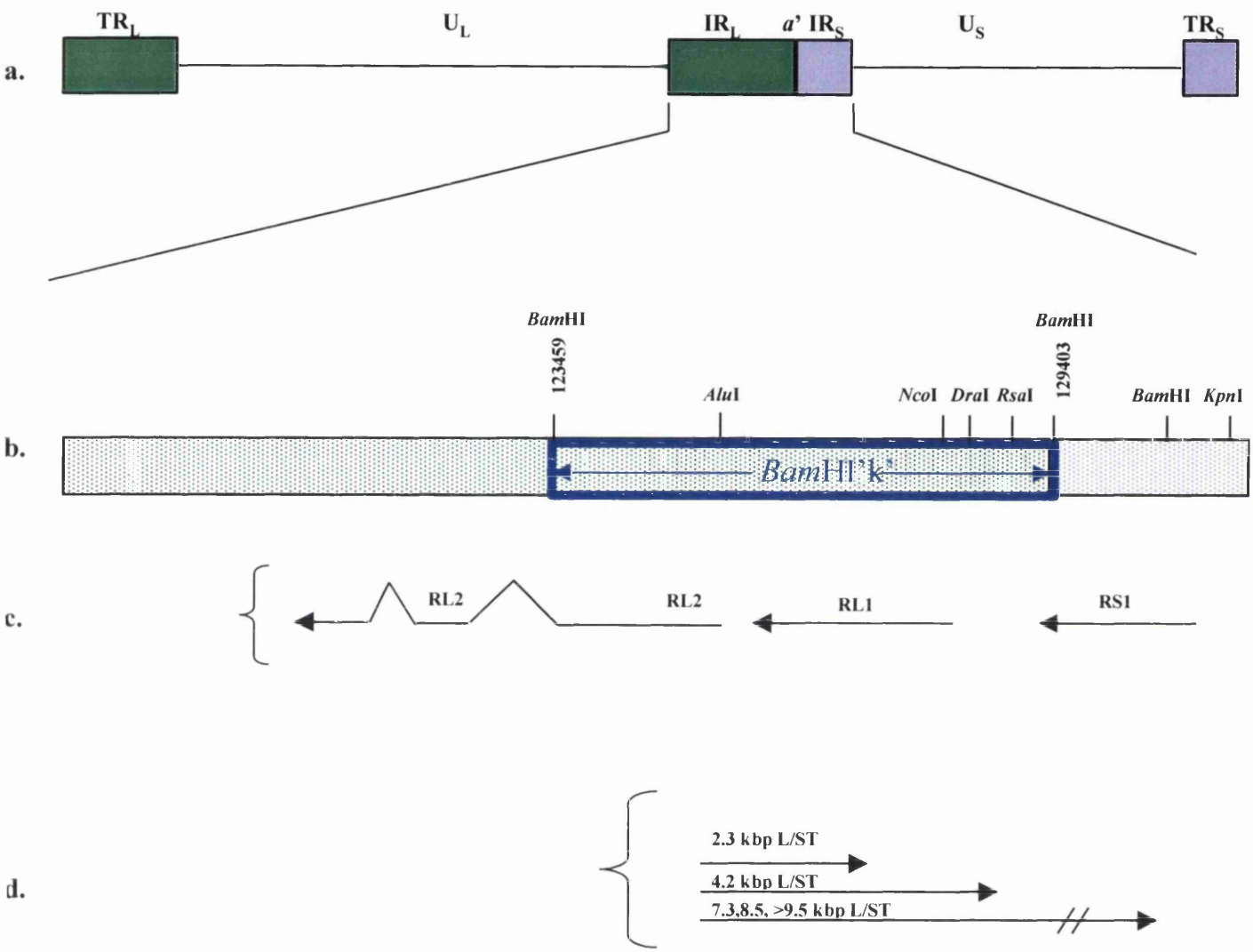


Figure 8. A schematic diagram of the HSV-1, strain 17$^+$, genome (not drawn to scale).

a. The HSV-1 genome. **b**. The IR_L, IR_S and *a'* sequence is expanded to show the relevant restriction enzyme sites within this sequence. The *Bam*HI '*k*' fragment is highlighted in blue. **c**. The genes within this sequence which encode functional proteins are, RS2, RL1 and RL2. **d**. The putative transcripts that contain the *a* sequence are called the L/ST for long/short transcripts (L/ST) .

Viruses which express inactive forms of ICP0 have a defect in the onset of the lytic cycle that is dependent on the multiplicity of infection and cell type. After low m.o.i. of human foetal lung (HFL) fibroblast cells, ICP0 mutant viruses preferentially attain a quiescent state of repressed genome expression, from which they can be reactivated by provision of exogenous ICP0 (Stow and Stow, 1986; Preston and Nicholl, 1997; Samaniego *et al.*, 1998).

An extensively studied property of ICP0 is its ability to localise to and then disrupt specific nuclear structures named nuclear domain 10 (ND10), promyelocytic leukaemia (PML) bodies or PML oncogenic domains (PODs) (Maul *et al.*, 1992; Everett and Maul, 1994; Maul and Everett, 1994). Everett *et al* (1998) showed that ICP0 binds to a member of the ubiquitin specific protease family of enzymes and that ICP0 induces the proteasome dependent loss of a number of high molecular weight isoforms of PML. Everett *et al* (1998) showed the first example of a regulator of viral gene expression functioning via the ubiquitin-proteasome pathway, suggesting a mechanism by which reactivation of latent virus might be inhibited.

A mutant HSV-1 strain KOS virus with a 350 bp deletion from –420 to –70 relative to the transcriptional start site of ICP0 was named ΔTfi (Davido and Leib, 1996). KOS, ΔTfi, and ΔTfiR replication in murine corneas and TG were comparable when assayed on a complementing cell line, but ΔTfi titres appeared 15- to 50- fold lower when measured on Vero cells. ΔTfi was correspondingly less virulent than wild type or marker rescued viruses in both immunocompetent and SCID mice. ΔTfi established and reactivated from latency with efficiencies comparable to wild type and marker rescued viruses. These results demonstrate that although the deletion in ΔTfi results in lower levels of ICP0 *in vitro* and decreased virulence *in vivo*, the establishment of and reactivation from latency are unaffected (Davido and Lieb, 1996).

The reduced expression of ICP0 RNA, protein and transactivation capability at early times pi most likely results from reduced VP16 activation due to deletion of the TAATGARAT and octamer/TAATGARAT motifs (Davido and Leib, 1996). It is possible that other cis-acting elements have been brought closer to the mRNA start site of ICP0 and that these can substitute for the deleted promoter elements during reactivation. The nearest defined viral promoter is αX (Bohenzky *et al.*, 1993; Lagunoff and Roizman, 1994; Yeh and Schaffer, 1993) which is intact in ΔTfi and has been shown to drive transcription antisense to ICP34.5, particularly in the absence of ICP4. Another region from –550 to –

129 relative to the transcriptional start site of ICP0, has transcriptional activity in transient assays following release of cells from growth arrest and has been proposed to play a role in reactivation (Ralph *et al.*, 1994). A concern regarding the reduced virulence of ΔTfi is whether ICP34.5 and ORF P have been adversely affected by the mutation (Davido and Leib, 1996).

In contrast to the above data, several studies have reported ICP0 mutants grow inefficiently *in vivo* and are impaired in their ability to reactivate from latency (Cai *et al.*, 1993; Clements and Stow, 1989; Leib *et al.*, 1989).

ICP0 is a 775 amino acid nuclear phosphoprotein which transactivates a variety of HSV-1 promoters either alone or synergistically with ICP4 (Cai and Schaffer, 1989; Everett, 1984; Gelman and Silverstein, 1986; Quinlan and Knipe, 1985). The mechanism of transactivation by ICP0 is unknown although ICP0 has been shown to interact with a 135 kDa cellular protein (Meredith *et al.*, 1994) and with ICP4 and ICP27 (Zhu *et al.*, 1994).

1.7.2. ORF O

The unspliced 8.3 kbp LAT has been shown to contain at least 16 open reading frames (ORFs) greater than 50 amino acids in length (Lagunoff and Roizman, 1996). These ORFs have been designated by the letters A through P (Chou *et al.*, 1994). ORF O and P are almost entirely antisense to the ICP34.5 transcript. Both ORF O and P are well conserved between HSV-1 strains (Chou *et al.*, 1994). In strains KOS, F, MH10, and CVG2, a single base pair substitution terminates ORF O after approximately 160 codons (Lee and Schaffer, 1998).

The role of ORF O and ORF P proteins in the establishment of latency is uncertain. The ORF O protein binds ICP4, the major regulatory protein, and prevents it from binding to DNA (Roizman, 1999).

ORF O overlaps in part with ORF P but in a different reading frame. The first and only proposed methionine codon of ORF O is located in the TATA box of ORF P (Randall *et al.*, 1997). Analysis of cells infected with a de-repressed ORF P failed to detect an RNA corresponding to ORF O. In-frame insertional mutagenesis revealed that translation of ORF O initiates at the ORF P initiator methionine and that at a point between the first and 35th codon of ORF P translation switches to the ORF O reading frame. The mechanism of the frame shift is unclear, but within the interval between the initiator and 35th codon there are no obvious splice donor or acceptor sites. The ORF O protein was found to bind ICP4 and to preclude it from binding to its response elements on DNA.

1.7.3. ORF P

A protein product has been identified from ORF P and shown not to be expressed from LAT but from the L/S transcript (Bohenzky *et al.,* 1993), whose promoter contains a high affinity binding site for ICP4 (Lagunoff and Roizman, 1995). Thus studies published to date show ORF P is expressed only in the absence of a functional ICP4 or if the ICP4 binding site is mutagenized (Lagunoff and Roizman, 1994). ORF P contains 240 amino acids in strain F (Bruni and Roizman, 1996). For sequence alignment between HSV-1 strains see Figure 9.

Lagunoff *et al* (1996) constructed an ORF P mutant virus named ORF P^{++} in which the ICP4 binding site in the promoter sequence is mutagenized and allows expression of ORF P gene throughout the lytic cycle. This virus is highly attenuated upon inoculation into mice by the intracerebral or ocular route, and the virus cannot be recovered following explantation from the TG. It is believed that either ORF P overproduction blocks the expression of some herpesvirus genes such as ICP34.5 or de-repression of the transcription of ORF P has a negative effect on the transcription of the antisense ICP34.5 RNA (Lagunoff *et al.,* 1996).

Randall and Roizman (1997) constructed a recombinant virus, R7546(P++)- which had the ICP4 binding site mutagenised to de-repress ORF P transcription and the initiation methionine codon replaced with an isoleucine codon to prevent the synthesis of ORF P protein. This mutant had reduced virulence and attenuation. It also had reduced synthesis of ICP0 and ICP22 and it was implied that ORF P expression was required for their synthesis (Randall and Roizman, 1997). Randall and Roizman (1997) indicated that ICP34.5 suppression was due to antisense transcription and not the expressed ORF P protein.

Bruni and Roizman (1996) demonstrated that the ORF P protein co-localises with splicing factors in the nuclei of infected cells which led to the hypothesis that it is a component of spliceosomes. ORF P protein co-localises and binds to p34 which is a component of the SF2/ASF splicing factor. In cells infected with viruses carrying a de-repressed ORF P, it is rapidly post-translationally modified. However, within a few hours after initial infection, the accumulation of ICP22 and of ICP0 the products of spliced mRNA are grossly reduced whereas the accumulation of ICP4 and ICP27 are unaffected (Roizman, 1999).

ORF O and P block the synthesis or function of three key immediate early proteins, ICP0, ICP22 and ICP4. Viral replication cannot ensue in the absence of these proteins.

17+	MTASASATRR	RNRARSARSR	AHEPRRARRA	AEAQTTRWRT	RTWGEKRTR.
F	MTASASATRR	RNRARSARSR	AHEPRRARRA	AEAQTTRWRT	RTWGEKRTRA
KOS	MTASASATRR	RNRARSARSR	AHEPRRARRA	AEAQTTRWRT	RTWGEKRTR.
17+	(AGV)**5**	AGGSGAPSPP	ARRRRRARCS	AVTRRRRARR	GGRRKGREGG
F	(AGV)**10**	AGGSGAPSPP	ARRRRRARCS	AVTRRRRARR	GGRRKGREGG
KOS	(AGV)**2**	AGGFGAPSPP	ARRRRRARCS	AVTRRRRARR	GGRRKGREGG
17+	WEGSAPPPGA	TPGGGGRGRG	AAAVGRASGA	**D**SGGGLSGQS	SSSSSSDADS
F	WEGLAPPPGP	APGGG**D**RGRG	AAAVGRASGA	GSGGGLSGQS	SSSSSSDADS
KOS	WEGLAPPPGP	TPGGGGRGRG	AAAVGRASVA	GSGGGLSGQ.	. SSSSSDADS
17+	GTWSHWRSSS	EQEGGGPPAG	GGGGAAAGAL	LTAGSELGVE	VTWDCAVGTA
F	GTWSHWRSSS	EQEGGGPPAG	GGGGAAAGAL	LTAGSELGVE	VTWDCAVGTA
KOS	GTWSHWRSSS	EQEGGGPPAG	GGGGAAAGAL	LTTGSELGVE	VTWDCAVGTA
17+	PVGPGGRGRR	GPRW.RRRRA	METESVPGW		
F	PVGPGGRGRR	GPRW.RRRRA	METESVPGW		
KOS	PVGPGGRGRR	GPRW.RRRRA	METESVPGW		

Figure 9. ORF P consensus sequence.

The ORF P consensus sequence from three well studied HSV-1 strains 17^{+}, F and KOS. Periods represent gaps. The only major strain specific variable lies in the number of AGV repeats (adapted from Lee and Schaffer, 1998)

Inasmuch as ORF O and P are antisense to RL1, ablation of the coding domains of these genes would also result in the deletion of the RL1 gene. Since the latter is required for viral replication in neuronal cells, the experiment would be meaningless (Roizman, 1999). Substitution of the initiator methionine of ORF O and P does not block the establishment of latency.

Lee and Schaffer constructed two mutant viruses in HSV-1 strain KOS to study the interdependent phenotypes of ORF O/P and ICP34.5. The first mutant constructed contained a nonsense mutation that terminates ORF P translation at amino acid 38 but does not alter the sequence of ICP34.5 encoded by the complementary DNA strand (Lee and Schaffer, 1998). This mutant expressed a truncated ORF P protein and had no observable phenotype relative to wild type virus *in vitro* or *in vivo*. The second mutant contained a mutation in the L/ST promoter that de-repressed L/ST expression by abrogating ICP4 binding to its cognate binding site near the transcription initiation site of the L/STs. This mutant over-expresses the L/STs and ORF P, and is highly attenuated following intracranial inoculation of juvenile mice. However, replication of this mutant was efficient in the mouse eye and TG without eliciting an adverse host response. It induced a cell type specific expression of both ICP34.5 transcripts and LATs since both types of transcripts were abundant in Vero cells but barely detectable in mouse neuroblastoma cells (NB41A3) (Lee and Schaffer, 1998). Table 4 gives an overview of the ORF P mutant viruses described above.

1.7.4. ICP34.5

The existence of a neurovirulence locus in the long repeat region of the HSV-1 genome was first identified by Thompson *et al* (1983). Following intracranial inoculation, mutants in this region are 100,000 less virulent than wild type viruses (Taha *et al.,* 1989; Chou *et al.,* 1990; MacLean *et al.,* 1991). Previous work showed that the terminal 500 bp *a* sequence acts as a γ_1 HSV promoter which led to the discovery of the protein ICP34.5. In strain F the gene was named $\gamma_1$34.5 by Chou and Roizman (1986) and the same gene in strain 17^+ named RL1 (Dolan *et al.*, 1992).

The RL1 promoter is partially located in the *a* sequence and lacks a canonical TATAA box. Two common strains of HSV-1, F and 17^+, show homology in ICP34.5 with the exception of a peptide repeat PAT present 10 times in strain F but only 5 times in strain 17^+ (Fig. 10a; Dolan *et al.*, 1992). Deletion mutants of RL1 include 1716 and R3616. Several other RL1 mutations have been made which place stop codons downstream of the

Table 4. ORF P mutant viruses.

Virus name	Mutation	Parental strain
ORF P++	ICP4 binding site mutated	F
R7546(P++)-	ICP4 binding site mutated & initiating Met replaced with Ile	F
L/ST-4BS	4 bp substitution in the ICP4 binding site in the L/ST promoter	KOS
L/ST-n38	a single bp transition mutation in ORF P at codon 38	KOS

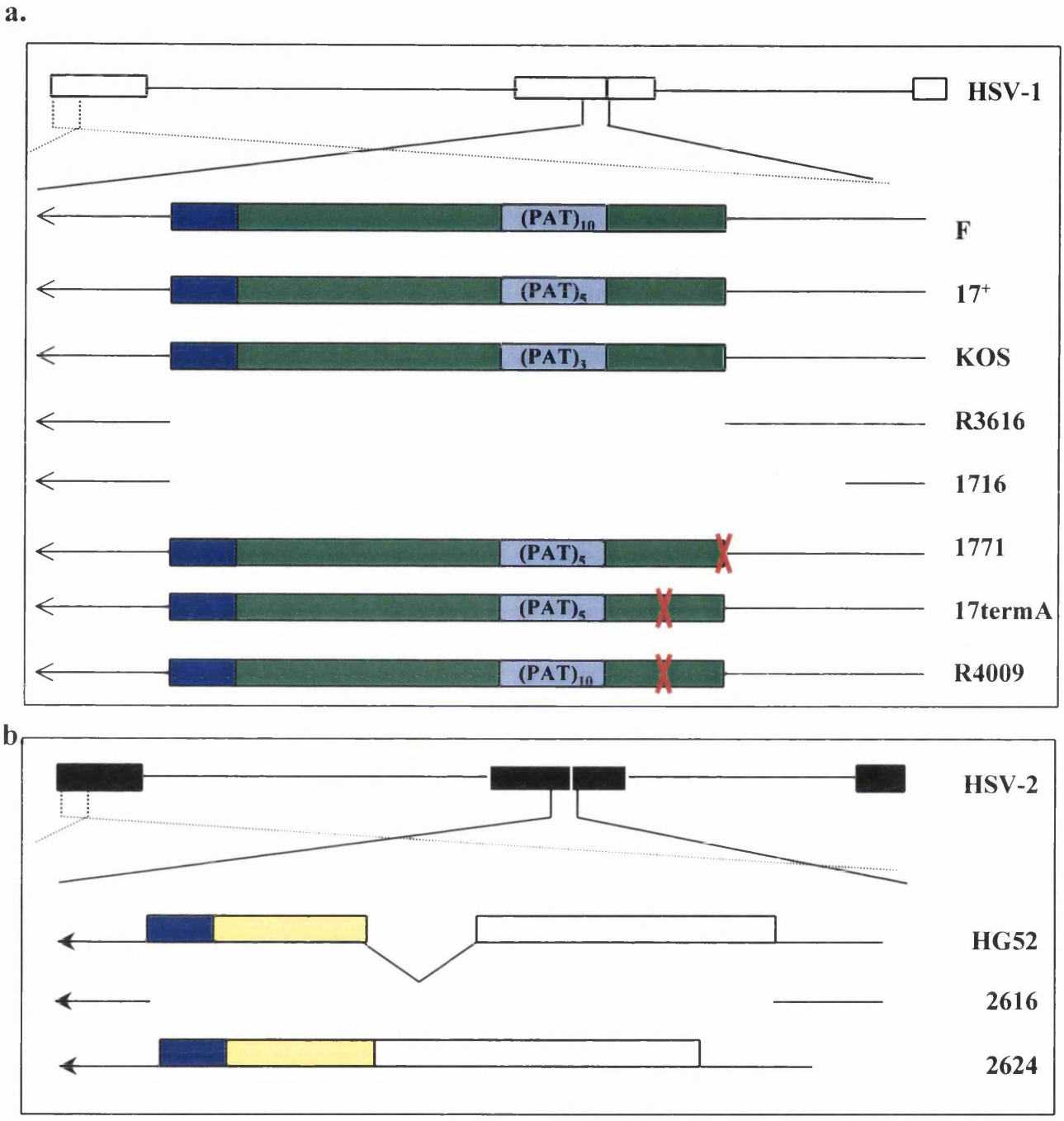


Figure 10. HSV RL1 mutant viruses.

a. Three intensely studied HSV-1 strains and their RL1 genes represented diagrammatically. Strain F has 10 copies of PAT, strain 17^+ has 5 PAT copies, and strain KOS has 3 copies. R3616 is a RL1 negative mutant in strain F. 1716 is a RL1 negative mutant in strain 17^+. 1771 contains a stop codon (9 bp) downstream of the initiating methionine. 17termA contains a stop codon inserted at the *Bst*EII site (28 amino acids) downstream of the ATG in strain 17^+. R4009 contains the same mutation as 17termA, but in strain F. **b.** The HSV-2 strain HG52 depicting the RL1 gene which contains an intron. 2616 is a RL1 deletion mutant in HG52. 2624 is a RL1 mutant virus which does not have the intron. The conserved 63 amino acid carboxy terminus is depicted by a blue box.

initiating methionine. In strain 17^+, two mutant viruses with a stop codon include 1771 which has a stop codon inserted immediately downstream of the *Nco*I site at the initiating methionine and 17termA which has a stop codon inserted at the *Bst*EII site 90 base pairs downstream of the initiating methionine (Fig. 10a). In strain F, the RL1 stop codon insertion mutant, R4009, has a mutation at the *Bst*EII site, similar to the strain 17^+ mutant 17termA (Fig. 10a).

At some sites of primary infection (e.g. footpad, vagina) the replication of ICP34.5- mutants is much less than that of wild type virus, whereas in others (e.g. eye) replication appears to be almost completely inhibited (Spivack *et al.,* 1995). ICP34.5- mutants establish latency inefficiently and have been reported to reactivate far less efficiently than wild type virus, as measured by explant *in vitro* reactivation from mouse ganglia (Perng *et al.,* 1995). The impairment in 1716 during latency is more marked in the TG rather than in the dorsal root ganglia (DRG) which may be due to replication at the site of inoculation (Spivack *et al.,* 1995).

Replication of ICP34.5 mutants in tissue culture is normal in several cell types, but is restricted in SK-N-SH neuroblastoma cells due to premature host shut-off (Chou and Roizman, 1992), murine 10T1/2 cells, stationary phase primary mouse embryo cells (Bolovan *et al.,* 1994) and 3T6 cells (Brown *et al.,* 1994a). The *in vitro* phenotype of mutant viruses will be described in more detail in Sections 1.11. and 1.12.

Dick and Rosenthal (1995) identified a tissue culture phenotype which distinguished clinical isolates from the KOS laboratory strain and mapped around the RL1 locus. The clinical isolates produced small plaques on Vero cells, were cell associated and viral glycoprotein processing appeared to be restricted to the endoplasmic reticulum (small plaque phenotype). In 1999, Bower *et al* reported that a HSV strain isolated from the brain of a neonate possessed a small plaque phenotype. The large plaque virus was isolated from the lungs and the gastro-intestinal tract. The major sequence difference between the small plaque, large plaque and KOS virus isolates was in the number of repeats of CCCGCGACC, encoding PAT. The large plaque isolate had 22 repeats the small plaque isolate had 18, while KOS has 3 repeats of the PAT domain (Bower *et al.*, 1999). Another difference between the large and small plaque isolates was a deletion of a sequence preceding the nine nucleotide repeats, causing the loss of glycine-glutamic acid glycine-alanine (GEGA). Other differences between the small, the large, and the KOS strains included a deletion of arginine near the N terminus, two conservative mutation, and two non-conservative mutations (Bower *et al.*, 1999).

Several mutant viruses have been studied to examine the RL1 phenotype *in vitro* and *in vivo* from HSV-2 strain HG52. A HG52 mutant, 2616, was isolated and determined to have a 786 bp deletion (Harland and Brown, 1991). A splice donor and splice acceptor site within the RL1 sequence was identified and by RT-PCR experiments demonstrated to be functional by Harland *et al* (1996). Ravi (1998) made a deletion mutant lacking the intron, 2624, and found that it maintained virulence upon intracranial inoculation *in vivo*. For a description of all the recombinant viruses see Figure 10b.

1.8. ICP34.5 Homologous Viral Proteins

A small number of genes to date have been shown to have homology with HSV-1 RL1. Among the herpesviridae only HSV-2 has a homologous gene which is also named RL1. The other RL1 homologous gene comes from the African Swine Fever Virus (ASFV), LMW23-NL (Sussman *et al.,* 1992).

ASFV is a large icosahedral arbovirus which contains a linear dsDNA genome of 170 to 190 kbp and replicates in the cytoplasm of infected cells (Costa, 1990). It is the sole member of an unnamed family of animal viruses. Cells of the mononuclear phagocytic system are major targets for ASFV replication *in vivo*. Viral infections with extensive necrosis of fixed macrophages of the spleen, lymph nodes, lung, and liver as well as specific lineages of reticular cells are evident following infection with highly virulent isolates (Konno *et al.*, 1971; Mebus, 1987, 1988; Moulten and Coggins, 1968). Strains of ASFV exhibiting moderate virulence also infect these cell types, but the degree of tissue involvement and the resulting damage are much less severe (Mebus, 1987, 1988). The nature of the virus-cell host interactions responsible for the differing outcomes of infection with highly virulent, moderately virulent, or avirulent ASFV strains is unknown.

1.8.1. LMW23-NL

The ASFV gene LMW23-NL is predicted to express a highly basic protein of 184 amino acids with an estimated molecular mass of 21.3 kDa (LMW23-NL). Sussman *et al* (1992) carried out a search using two protein data bases, PIR (release 7.0) and BLAST which revealed a striking similarity between LMW23-NL, MyD116 and ICP34.5 (Sussman *et al.*, 1992). All three proteins contain a centrally located acidic region with a highly conserved hydrophilic 56 amino acid domain located at the carboxy terminus. ICP34.5 and LMW23-NL both contain a highly basic terminus composed of 8 to 10 lysine and arginine residues (Sussman *et al.,* 1992; Zsak *et al.*, 1996). In the conserved domain of LMW23-NL,

comparison of this to HSV-1 ICP34.5 reveals 55% overall conservation and 30% identity (Sussman *et al.*, 1992). In the same region, comparison to the cellular gene MyD116 (see below) revealed 25 exact matches and 19 conservative matches, which correspond to 78% overall conservation with 44% identity (based on the Dayhoff Pam-250 symbol comparison table with a 0.5 cutoff (Schwartz and Dayoff, 1979).

Zsak *et al* (1996) determined that LMW23-NL is highly conserved among ASFV isolates and that it exists in either of two forms: 23-NL, or a shorter form, NL-S which is found in the European and African pathogenic isolates. Although highly conserved, the NL-S gene is nonessential for growth in pig macrophages or Vero cells *in vitro*.

1.9. ICP34.5 Homologues Cellular Proteins

1.9.1. GADD34/MyD116

Two cellular genes GADD34 and its murine homologue, MyD116, have been shown to contain a centrally located highly acidic region with a highly conserved hydrophilic 63 amino acid domain located at the carboxy terminus homologous with ICP34.5. Growth arrest and DNA damage inducible (GADD) genes were first sequenced by Fornace *et al* (1989).

Five GADD genes have been sequenced and named, GADD153, GADD45, GADD34, GADD33 and GADD7. In general, GADDs are often co-ordinately expressed genes that are induced by genotoxic stress and certain other growth arrest signals (Zhan *et al.*, 1994). GADD34 was shown to have homology with ICP34.5, specifically in the C-terminal of GADD34. The domain of ICP34.5 which binds protein phosphatase 1-α (PP1-α) is highly homologous to, and in fact replaceable by, the corresponding domain of the GADD34 gene (Chou and Roizman, 1992; He *et al.*, 1996). The function of this is discussed in Section 1.11.

Another cellular protein which shows homology with ICP34.5 is the murine myeloid differentiation primary response gene (MyD116) (Lord *et al.*, 1990; McGeoch and Barnett, 1991). Like ICP34.5, MyD116 is predicted to consist of a large amino terminal domain, a 38 amino acid sequence repeated 4.5 times and a carboxyl terminus containing sequences partially homologous to a 52 amino acid stretch in ICP34.5.

The precise function of MyD116 is currently unknown. However, it is expressed early in cellular differentiation and in terminally differentiated cells and may be involved in regulating the differentiation process or in maintaining the differentiated state in myeloid cells (Lord *et al.*, 1990; Sussman *et al.*, 1992).

Khodarev *et al* (1999) used DNA arrays containing a limited number of probes to test the accumulation of cellular transcripts in three different human cell lines. The human embryonic lung human fibroblasts were the closest to susceptible "normal" human cells *in vitro* (Khodarev *et al.*, 1999). They also used two isogenic cancer cell lines differing in the absence or presence of p53 and found that there was a significant accumulation of a small subset of cellular transcripts. The transcripts encode regulatory proteins and transcriptional factors and one stress response protein (GADD45) (Khodarev *et al.*, 1999).

MyD116, GADD45, MyD118, and GADD153 encode acidic proteins with similar charge characteristics, and have previously been shown to be regulated by the tumour suppressor, p53 (Zhan *et al.*, 1994). It is thought that the function of GADD34 or MyD116 alone or in association with GADD45 is to preclude stress responses which could result in cell death. He *et al* (1996) demonstrated that the carboxyl terminus of ICP34.5 is essential in precluding premature shut-off of protein synthesis in both neuroblastoma cells and in human foreskin fibroblasts following HSV infection. MyD116 is one of several genes cloned as cDNA from myeloid leukaemia cells induced to differentiate by IL-6 and is an immediate response gene; its expression peaks 1 h after exposure of cells to IL-6. Steady state levels of MyD116 mRNA have been detected in the terminally differentiated cells. One of the functions of the genes expressed after differentiation of the myeloid leukaemia cells is to preclude terminally differentiated cells from undergoing apoptosis.

The pathway in which RL1 may act, or the stress it precludes, appears to be different from that described to date for other virus proteins as exemplified by the involvement of *bcl2*. *Bcl2* has been shown to protect cells from apoptosis in stress or during development. However, *bcl2* does not preclude apoptosis resulting from depravation of IL-6 or the ciliary neurotrophic factor, and leukaemia inhibiting factor that share common receptors and signal transduction pathways. Chou and Roizman (1994) predict that RL1, and in particular the carboxyl domain shared with MyD116 and GADD34, may play a role in blocking apoptosis in terminally differentiated cells and that RL1 acquired this domain for its own use.

1.10. ICP34.5 Cellular Interactions

In 1997, Brown *et al* reported that ICP34.5 and the conserved 63 amino acid domain of the cellular protein MyD116 specifically complex with proliferating cell nuclear antigen (PCNA). The function of PCNA includes acting as a processivity factor for DNA polymerase gamma (Bravo *et al.*, 1987; Prelich *et al.*, 1987) and the recruitment of

replication factors to DNA replication initiation sites (Kill *et al.*, 1991). GADD45 and p21 both directly complex and interact with PCNA in competition with each other (Chen *et al.*, 1995). The site of interaction between p21 and PCNA has been defined by using small peptides to inhibit DNA replication (Warbrick *et al.*, 1995). The projected three-dimensional model of PCNA is illustrated in Figure 11.

It was proposed by Brown *et al* (1997) that after HSV infection ICP34.5 complexes with PCNA either directly or indirectly, and this complex allows cellular DNA replication to continue. When ICP34.5 is not present, PCNA complexing does not occur and the cell goes into a growth arrest state and does not provide an alternative mechanism for the virus to go through the replication cycle. It is thought that in certain cell types and cell states ICP34.5 is not required.

1.11. ICP34.5 *in vitro* Function

Viruses have evolved a variety of mechanisms to block the shut-off of protein synthesis. Poliovirus degrades double-stranded RNA protein kinase R (PKR), the influenza virus synthesises the NS1 protein which blocks the binding of double-stranded RNA to PKR. Adenovirus produces a short double-stranded RNA called Va_rRNA, which binds but fails to activate PKR. Vaccinia virus has protein K3L, which blocks the phosphorylation of eIF-2α. HSV-1 differs from other viruses in that its main defence to counter host response to infection was to capture a fragment of a cellular gene, GADD34, to dephosphorylate eIF-2α rather than to evolve mechanisms to prevent the activation of PKR or the phosphorylation of eIF-2α.

In cells infected with the HSV-1 ICP34.5- virus R3616, the double stranded PKR is activated, eIF-2α is phosphorylated, and protein synthesis is shut-off. Although PKR is activated in cells infected with the wild type virus, ICP34.5 binds protein PP1-α and redirects it to dephosphorylate eIF-2α, thus enabling sustained protein synthesis (Fig. 12; Roizman, 1999).

He *et al* (1997) showed that the carboxyl terminus of MyD116 interacts with PP1-α in yeast, and both MyD116 and ICP34.5 interact with PP1-α *in vitro*. Fractions containing the ICP34.5 and PP1 complex specifically dephosphorylate eIF-2α. Both ICP34.5 and GADD34 proteins contain the amino acid sequence motif common to subunits

Figure 11. Coiled 3-D structure of PCNA.

This is a predicted 3-D structure of human proliferating cell nuclear antigen (PCNA) by X-ray diffraction PDBid number 1AXC.
The resolution is 2.6Å (Gulbis *et al.*, 1996)

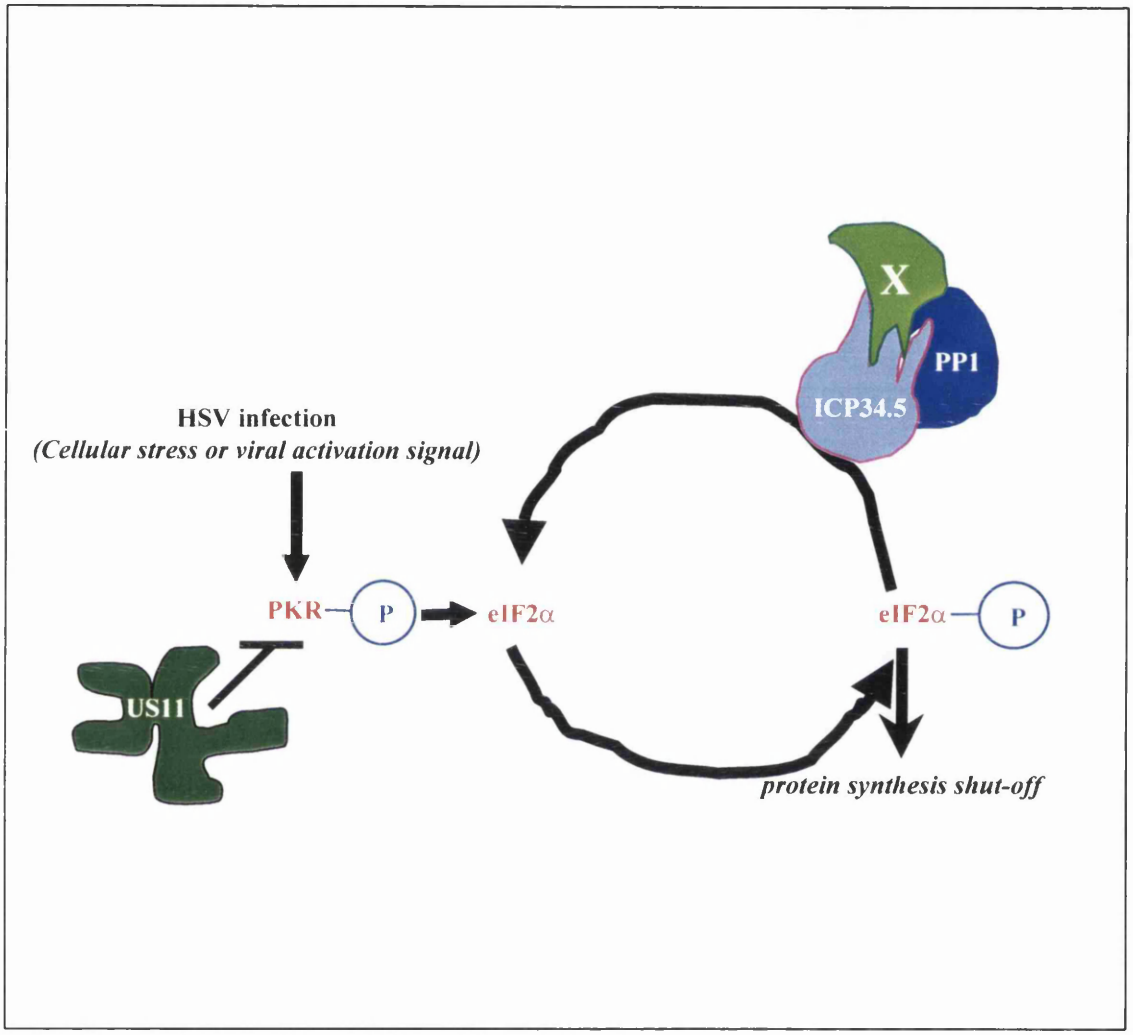


Figure 12. PKR pathway with ICP34.5.

One role of ICP34.5 *in vitro* is to preclude shut-off of host cell and virus protein synthesis in human neuroblastoma cells (SK-N-SH). HSV infection activates the double stranded protein kinase PKR. PKR phosphorylates the eukaryotic translation initiation factor eIF-2α. If ICP34.5 is not present, phosphorylated eIF-2α does not promote protein synthesis and leads to protein shut-off in SK-N-SH cells. If ICP34.5 is present it binds to protein phsophatase 1 (PP1) and potentially another factor (X) to dephosphorylate eIF-2α and maintain viral and host protein synthesis. Forced mutations of an ICP34.5 null mutant revealed expression of US11 immediately upon infection does not allow accumulation of PKR (Concept from B. Roizman and I. Mohr).

of PP1 that is required for binding to the PP1 catalytic subunit (He *et al.*, 1998). He *et al* (1998) showed that amino acids 190-203 of ICP34.5 fused to GST strongly bound and pulled down PP1. Moreover, a peptide containing amino acids 185-211 of ICP34.5 competed with intact ICP34.5 for binding PP1 and substitution of Val^{193} and Phe^{195} with Glu and Leu, respectively, abolished the activity of ICP34.5 (He *et al.*, 1998).

To summarise, PKR is activated in cells infected with both wild type and mutant viruses (Roizman, 1999). However, in wild type virus infected cells ICP34.5 acts as an accessory PP1-α factor. Specifically, it binds to PP1 through a short sequence common to all PP1 accessory factors and redirects PP1 to specifically dephosphorylate eIF-2α at the expense of other substrates of PP1.

He *et al* (1996) constructed several mutant viruses to clearly define the domain(s) responsible for blocking the host response to infection. Studies on those mutants show that the domain of ICP34.5 necessary to preclude the total shut-off of protein synthesis corresponds to the carboxyl terminal domain of ICP34.5 homologous to MyD116 and GADD34. Although, the carboxyl terminus of GADD34 can substitute for ICP34.5 in precluding the shut-off of protein synthesis, the virus carrying the chimeric gene is avirulent (Roizman and Sears, 1987).

Serial passage in human cells of a mutant virus lacking RL1 yields second site, compensatory mutations in the US11-US12 region (Mohr and Gluzman, 1996; Mulvey *et al.*, 1999). The locus where these mutations occurred have been termed SUP (Mohr and Gluzman, 1996). Analysis of the compensatory mutation revealed that a deletion in the US12 domain brought the US12 promoter 5' to the coding sequence of the US11 gene converting the latter to an immediate-early gene and reducing the accumulation of active PKR kinase (Fig. 12; Mohr and Gluzman, 1996; He *et al.*, 1997; Cassady *et al.*, 1998a; Mulvey *et al.*, 1999).

US11 is not essential for growth of HSV-1 in cultured cells and is not required for neurovirulence in animals (Brown and Harland, 1987; Longnecker and Roizman, 1986; Nishiyama *et al.*, 1993; Umene, 1986). The loss of US11 function is tolerated only in the presence of a GADD related function (i.e. ICP34.5). Both viral proteins/functions and their respective cellular counterparts may contribute to whether a given cell type is permissive for the growth of ICP34.5 mutants (Mulvey *et al.*, 1999).

Cassady *et al* (1998a) constructed a recombinant virus lacking RL1 and US8-12, R5103. The recombinant virus R5104 was derived from R5103 but contains the US10 gene and the US12 promoter fused to the coding domain of the US11 gene (Cassady *et al.*,

1998b). R5104 had a protein profile similar to that of wild type virus, whereas protein synthesis was shut-off in cells infected with R5103. Lysates of R3616, R5103 and R5104 virus infected cells lacked the phosphatase activity specific for eIF-2α characteristic of wild type virus infected cells. Lysates of R3616 and R5103 which lacked the second site compensatory mutation contained an activity which phosphorylated eIF-2α *in vitro* whereas lysates of mock infected cells or cells infected with wild type HSV-1 strain F or R5104 did not phosphorylate eIF-2α (Cassady *et al.*, 1998b).

1.11.1. PKR

The double-stranded RNA dependent protein kinase PKR is an interferon (IFN) inducible protein (Clemens and Elia, 1997). PKR, a polypeptide of 68 kDa in humans and 65 kDa in mice, is a serine/threonine kinase, which is activated by autophsophorylation upon binding to dsRNA (Clemens and Elia, 1997). Activated PKR then phosphorylates the α subunit of the translation initiation factor eIF-2α, a modification that causes inhibition of protein synthesis. PKR exhibits antiviral and antiproliferative activities. Consistent with an antiproliferative action, PKR has been shown to induce cell death by apoptosis. The mechanisms by which PKR regulates cell growth are not fully understood. Proteins capable of regulating cell growth, PKR and p53, exhibit some similar properties. Induction of both proteins results in inhibition of cell growth in mammalian or yeast cells. Mutant forms of both PKR and p53 can transform cells in culture. P53 is predominately a nuclear protein whereas PKR exhibits nuclear localisation. PKR is required for antiviral responses but p53 is not and p53 is essential for inducing cell cycle arrest upon DNA damage but it is not known whether PKR is implicated in this process. Depletion of p53 in mice induced spontaneous tumours whereas depletion of PKR is not tumorogenic (Abraham *et al.*, 1999; Yang *et al.*, 1994). Cuddihy *et al* (1999) reported that they have shown PKR to physically associate with p53 and modulate phosphorylation of human p53 on Ser^{392} *in vitro*. They showed that PKR modulates p53 transcriptional function by participating in signalling pathways leading to p53 activation (Cuddihy *et al.*, 1999).

PKR activity is dependent on dsRNA and manifests two distinct kinase activities: one for autophosphorylation and the other for phosphorylation of eIF-2α. The phosphorylation of eIF-2α blocks the exchange of GDP for GTP and consequently the inhibition of formation of the eIF-2.GTP.Met.tRNA heterotrimeric complex. PKR plays an important role during viral infection in cell growth control and differentiation. Several

reports suggest that PKR is involved in programmed cell death after viral infection and in tumour necrosis factor induced apoptosis. Donzé *et al* (1999) showed that expression of wild type PKR causes apoptosis and correlates with increased mRNA levels for the Fas receptor, a member of the tumour necrosis family of proteins. Expression of an inactive form of PKR (K296R) or the vector alone did not induce apoptosis or elevate Fas mRNA levels. Functional studies of PKR have been hampered by the toxicity of the protein in tissue culture cells (Koromilas *et al.*, 1992). Epstein-Barr virus and influenza which cause apoptosis also lead to increased expression of Fas antigen. Fas belongs to the tumour necrosis factor (TNF) and nerve growth factor (NGF) receptor family (Steller, 1995).

1.12. *In vitro* Plaque Morphology

Isolated mutants in initial HSV studies were important tools in studying virus replication, latency and oncogenic properties (Stow, 1978). In addition to temperature sensitive (*ts*) mutants, a number of specialised mutants with alterations or defects in individual genes which affect plaque morphology have been described. The usual morphology of HSV plaques is characterised by the occurrence of numerous rounded cells (*syn*+ plaques). Variants have been described which induce cell fusion and result in the production of giant multinucleate cells (*syn* plaques) (Ejercito *et al.*, 1968; Brown *et al.*, 1973; Timbury *et al.*, 1974). Incubation of cells infected with certain *syn* mutants in the presence of 2-deoxy-D-glucose results in phenotypic but not genetic reversion from *syn* to *syn*+ (Gallacher *et al.*, 1973) and inhibition of the glycosylation of virus specified glycopeptides (Knowles and Person, 1976; Keller, 1976b) suggesting that one or more fully glycosylated viral proteins are required for cell fusion. In double infected cells *syn*+ dominates *syn* (Keller, 1976a). The *syn* mutant described by Brown *et al* (1973) reverts to *syn*+ at a low frequency and has been used as an unselected marker in three-factor crosses. Mixed plaques containing discrete sectors of both morphologies have been interpreted as having heterozygous molecules during genetic recombination (Brown and Ritchie, 1975a; Ritchie *et al.*, 1977).

Brown *et al* (1994a) studied the plaque phenotype properties of 17^+ and 1716 in different cell types. In BHK cells, 17^+ produced plaques of average size with non-syncitial (*syn*+) morphology with 1716 plaque size described as slightly smaller but with the same morphology (Brown *et al.*, 1994a). In mouse embryo fibroblast 3T6 cells, 17^+ produced characteristic plaques while 1716 produced very small "abortive" plaques which failed to spread and regressed with time (Brown *et al.*, 1994a). In 1716 infected 3T6 cells, there was

a forty-fold increase in the particle:pfu with most of the virus confined to the nucleus (Brown *et al.*, 1994a). The RL1 mutant, 1771 demonstrates similar phenotype properties in 3T6 cells (Brown *et al.*, 1994b; MacLean, personal communication).

Brown *et al* (1994a) developed a method for maintaining 3T6 cells in a "stationary state". The stationary state of 3T6 cells was achieved when the serum concentration was reduced from 10% to 0.5% and the cells left undisturbed for 5 to 6 days before infection (Brown *et al.*, 1994a). Although 1716 did not replicate when 3T6 cells were in a stationary state, 17^+ displayed normal replication (Brown *et al.*, 1994a). Hence, it was considered that BHK cells were permissive for ICP34.5 negative viruses but 3T6 were "semi-permissive" when growing exponentially and "non-permissive" when in a stationary state. No other HSV-1 RL1 mutant viruses have been reported to have similar plaque phenotype properties to date.

1.13. HSV-1 and HSV-2 Virulence and Pathogenicity

The overall pathogenicity of HSV has been described above in three major stages, lytic replication, latency, and reactivation. The ability of HSV-1 and HSV-2 to replicate beyond the lytic stage successfully *in vivo* comes under the broad heading of pathogenicity and virulence.

The key difference between the pathogenicity of HSV-1 and HSV-2 is the initial site of infection. The HSV-1 portal of infection and colonisation is usually the oropharyngeal mucosa and TG. HSV-2 replication is in the genital region with colonisation at the sacral DRG (Aurelian *et al.*, 1989). Once HSV-1 and HSV-2 lytically replicate, they travel by anterograde motion to the TG and DRG, respectively, where they both establish a latent infection. The molecular mechanisms for latency and reactivation are currently thought to be the same for HSV-1 and HSV-2. Pathogenicity of both HSV-1 and HSV-2 in experimental animals is often measured by intracerebral inoculation and replication in the CNS.

Certain HSV mutants with lesions in genes that encode viral DNA replication enzymes (e.g. ribonucleotide reductase and thymidine kinase) replicate to near wild type levels in dividing cells, but do not replicate in neurones (Nichol *et al.*, 1996). This is likely due to the low levels of deoxyribonucleoside triphosphates in neurones. Several mutants have been characterised as pathogenic. Genes which have been deleted in a mutant virus and grow normally *in vitro* and by LD_{50} *in vivo* have been characterised as non-essential.

One non-essential locus in the HSV-1 genome, UL43/43.5, that is important to this study is described below. Few HSV-2 genes have been studied *in vivo* to date.

1.13.1. UL43/UL43.5

The UL43 gene is conserved among all of the sequenced *Alphaherpesviridae* subfamily. MacLean, C. *et al* (1991) showed that the UL43 open reading frame was dispensable for growth in cell culture, but attempts to demonstrate the presence of a protein were not successful. Ward *et al* (1996) demonstrated that there is an open reading frame antisense to the UL43 gene, named UL43.5. In 1996, Carter *et al* reported that the UL43 open reading frame is expressed and showed the first pair of antisense genes expressed during productive infection by wild type HSV-1 strain F (Carter *et al.*, 1996). Due the non-essential phenotype, the UL43 gene has been selected as a good candidate for inserting foreign genes.

1.14. Viral Vectors

Gene therapy and cancer therapy are among recent approaches to treat as yet, incurable genetic diseases and cancer, respectively. These therapies include the use and development of non-viral and viral vectors. Non-viral gene delivery systems currently include liposomes and "naked-DNA". These methods have the advantage of not causing disease. However, a disadvantage includes inefficiency of gene transfer. Different viruses including retroviruses, adenoviruses (AdV), adeno-associated viruses (AAV) and HSV are currently being studied as potential viral vectors for gene and cancer therapy. A mixture of the viral and non-viral therapies is also being studied using HSV with plasmids/cosmids (amplicons). Individual viral vectors are described in brief below. A selection of key advantages and disadvantages of the different viral and non-viral approaches is summarised in Table 5.

One of the most commonly studied methods of viral gene transfer is the use of retroviral vectors. Effective retroviral vectors, created by deleting essential genes, are propagated in producer cells lines. A foreign gene and a selectable marker are inserted into the vector which when packaged remains capable of entering the nucleus and recombining into the chromosome of dividing cells in culture (Miller and Miller, 1992). Retroviruses have the limitation of only being able to be purified at low titres and lack the availability of transducible cells for transplantation. The inability of these vectors to infect non-dividing cells makes them inappropriate for postmitotic gene transfer into neuronal tissue *in vivo*.

Table 5. Vectors under criticism.
(concept from Freidmann, 1997)

i.

	Liposomes	"Naked" DNA
Advantages	Have no viral gene to cause disease	Same as liposome; expected to be useful for vaccination
Disadvantages	Less effective than viruses at transferring genes to cells	Inefficient at gene transfer; unstable in most tissues

ii.

	Retroviruses	Herpes simplex viruses	Adenoviruses	Adeno-associated viruses	Amplicons
Advantages	Integrate genes into host chromo-somes; chance for long term stability	Latently infects neurones normally; does not stimulate the immune system; natural neurotropic infection	Most do not cause serious disease; large capacity for foreign genes	Integrate genes into host chromo-somes; cause no known human disease	Do not have viral proteins; causing no disease
Disadvantages	Genes integrate randomly possibly disrupting host genes; many infect only dividing cells	Reactivation mechanism is as yet not known; superinfection may cause reactivation	Genes may function transiently owing to lack of integration or to attack from immune system	Small capacity for foreign genes	Less effective than viruses at transferring genes to cells; unknown

i = viral vectors
ii = non-viral vectors

Adenoviruses have been studied for potential use in gene therapy due to its large DNA genome (approx. 38 kbp) and a capacity of maintaining large foreign DNA inserts (up to 10 kbp). Deletion of essential early genes such as E1a-E1b leads to defective viruses that can be propagated in producer cells lines. The major limitation in the development of AdV vectors is that despite deletion of E1a-E1b genes, there is a "leaky" expression of late viral genes leading to cytopathic effects and an immune response (Yang *et al.*, 1994).

Adeno-associated virus is a naturally defective small DNA virus known to be non-pathogenic (Fink *et al.*, 1996). AAV is difficult to propagate to high titres but is capable of gene delivery and expression in non-dividing cells including neurones (Kaplitt *et al.*, 1994). The long term persistence and expression of AAV has not yet been fully examined (Fink *et al.*, 1996), but its main limitation includes small packaging potential for foreign DNA.

The HSV-1 life cycle and its ability to establish a life long latent infection in neural tissue has gained attention as a potential genetic vehicle by which to deliver and express therapeutic genes to the CNS. At present two major classes of HSV-1 vectors have been developed: (1) replication defective mutants and (2) neuroattentuated mutants. The ability of HSV and these mutants to establish a life long latent infection, however, raises a critical issue in contemplating the use of these vectors in humans, especially in individuals who have already been latently infected with HSV. To exploit HSV as a vector it must be disabled to minimise the deleterious effects on the target cell. Strategies include the deletion of essential genes, allowing virus growth only in a complimenting cell line and the deletion of genes which specifically prevent growth in target cells but still allow growth in culture.

The selection of proper animal models upon which to conduct vector studies is critical, as the use of an animal system such as the rat which is non permissive to HSV replication yields inaccurate conclusions (Andreansky *et al.*, 1996). Neuronal cells are postmitotic and cannot be removed for transduction for gene therapy which is why further studies are being carried out on HSV gene delivery vectors (Fink *et al.*, 1996). *In vivo* studies by Starr *et al* (1996) demonstrated the recessive nature of mutations used in constructing the mutant viruses and the demonstration that the latent HSV-1 immediate early promoter in the rat brain can be reactivated by superinfection with replication defective HSV-1 mutants (Starr *et al.*, 1996). All currently available HSV-1 recombinants could potentially trigger or enhance outbreaks of productive viral infections in latently infected individuals.

1.14.1. Gene Therapy using HSV Vectors

Based on studies using neuronal cell cultures *in vitro*, HSV-1 has been proposed as a vector to deliver genes to mammalian neurones *in vivo* (Geller and Breakfield, 1988). A recombinant HSV capable of inhibiting its own replication as well as the replication of wild type virus would have increased safety as a general purpose vector for *in vivo* gene transfer, anti-tumour therapy, and viral vaccination against HSV infection. Preliminary studies show ICP34.5- viruses will express *trans*-genes in neuronal cells (Roizman, 1996).

1.14.1.1. Promoter/Enhancer Elements and Foreign Gene Expression

There are critical considerations when constructing HSV viral vectors for use in gene therapy. Two important aspects of selection include (1) the right locus in which the foreign gene is inserted and (2) an appropriate promoter and enhancer element for expression of the desired gene.

Lachmann *et al* (1996) examined the potential of a murine RNA polymerase (pol.1) promoter driving the reporter gene neomycin phosphotransferase (neo^R) with encephalomyocarditis virus internal entry site (EMCV-IRES) inserted into two independent loci (US5 or LAT) of HSV-1. Lachmann *et al* (1996) demonstrated that RNA pol.1 promoter can be utilised to drive trans gene expression during lytic, but not latent infection in two independent loci. Lachmann and Efstathiou (1997) examined the LAT promoter driving the *lacZ* and *lacZ*/ neo^R reporter genes inserted with an IRES in the LAT locus. β-galactosidase expression was seen during lytic and latent phases of infection using BALB/c mice *in vivo*.

Coen *et al* (1989) described tk- mutants that do not replicate in ganglia, yet express LAT efficiently and stably in mouse neurones *in vivo*. Coen *et al* suggests that tk- mutants modified to place the gene of interest under the control of LAT or neurone specific gene expression signals may offer advantages (Coen *et al.*, 1989).

1.14.1.2. "Pro-drug" Gene Therapy using HSV tk

Elshami *et al* (1996) compared pairs of tumour cell lines transfected with connexin genes that differed only in their degree of gap junctional communication. Elshami *et al* (1996) transduced tumour cells with an AdV vector containing the HSV*tk* gene and performed *in vitro* studies in which varying ratios of HSV*tk*+ to HSV*tk*- cells were mixed and subjected to a fixed concentration of GCV. Using this they compared the strength of the bystander effect. More efficient cell killing was uniformly seen in connexin

transfectants compared with the less coupled cell lines (Elshami *et al.*, 1996). These results provide direct evidence that gap junctional communication plays an important role in mediating the bystander effect of the HSV/GCV system *in vitro*.

Cool *et al* (1996) showed that long term survival of rats with 9L gliosarcoma tumours was observed only in animals with tumours transduced *in vivo* with HSVtk and GCV treatment. The results demonstrated that 9LTk subcutaneous tumours are treated more efficiently than brain tumours which might be related to the generation of more effective antitumoral immune responses when the tumours were subcutaneous (Cool *et al.*, 1996). The long term efficacy of *in vivo* gene transfer of HSV-tk, followed by GCV administration has recently been confirmed in another model, the C6 rat glioblastoma, with some of the treated animals still in remission at 8 months (Izquierdo *et al.*, 1995). The efficacy of this approach was questioned by Tapscott *et al* (1994) when they found that tumour formation was decreased in rats injected subcutaneousy or intracerebrally with cells modified to express an intracellular selectable marker alone or with the HSV-tk, even in the absence of GCV. The vector they used carried a chimeric hygromycin phophotransferase-tk fusion gene (Tapscott *et al.*, 1994). Culver *et al* (1992) first reported the ability to eliminate small brain 9L gliosarcomas in synegeneic Fischer 344 rats by sterotactically injecting in these tumours a mouse packaging cell line producing HSV-tk retroviral particles, and by administering GCV later (Culver *et al.*, 1992).

1.14.1.3. Amplicon-based Vectors

An amplicon contains a copy of an HSV replication origin (oriS) and a packaging signal sequence located in a sequence of the HSV genome (Vlazny and Frenkel, 1981; Stow, 1982; Kwong and Frenkel, 1984). The amplicon is transfected into suitable cells with helper virus which provides the required genes in *trans* for amplification and packaging of the amplicon genome. The amplicon-containing viral particles are in effect defective virions, containing multiple copies of the amplicon sequences in a concatameric form, and are able to infect mammalian cells (Zhang *et al.*, 1998).

Zhang *et al* (1998) reported using HSV tk inserted into an amplicon with a tk- and gH- mutant as a helper virus. Zhang *et al* (1998) used a BHK tk-/gH+ cell line in the presence of selection medium containing methotrexate to prepare amplicon stocks of high titres.

Studies with HSV-1 amplicons have been reported using a helper virus with a mutation in the essential IE3 gene. More recently a packaging defective vector 5dl1.2 with a deletion in the IE2 gene, resulted in higher vector titres (Starr *et al.,* 1996).

Fraefel *et al* (1996) reported using helper-virus free amplicon stocks generated from the whole HSV genome fragmented and cloned into cosmids. An amplicon plasmid was co-transfected with the HSV containing cosmids to generate a stock of helper free amplicons (Fraefel *et al.*, 1996).

1.14.1.4. Replication Restricted HSV Vectors

An HSV-1 strain 17^+ mutant *in*1312, defective for expression of VP16, ICP0, and ICP4 containing the *lacZ* gene controlled by the LAT promoter proceeded by an IRES demonstrated an ability to establish latency in DRG and latent phase expression of β-gal (Marshall *et al.*, 2000).

1.14.1.5. RL1 Mutant Vectors

Most replication defective HSV-1 vectors contain a deletion to remove one or more immediate early genes in order to prevent virus replication at the earliest time after infection. These genes must be complemented *in trans* for growth in culture. An inactivating mutation in the gene encoding ICP27 which activates immediate early gene expression after infection can be used to produce a non-pathogenic virus. Alternatively, a mutation in genes necessary for replication in particular target cells prevents replication in some fully differentiated cells (including neurones) but which still allows virus growth in actively dividing fibroblasts in culture.

For attenuated HSV growth in neurones mutations have been made in genes such as ICP6, tk, and ICP34.5. Myocardium cells are fully differentiated and cannot support a productive infection by these mutants, and thus lack of transgene expression in this cell type might suggest that apoptosis is occurring in this case. An HSV mutant, 27-lacZ, in which the essential immediate early gene Vmw65αTIF was deleted, gave high efficiency gene transfer to the cardiac myocytes *in vitro* and the rat heart *in vivo*. Viruses in which ICP34.5 (1716/lacZ) or ICP34.5 and IE63 (1764/lacZ) were inactive gave a much lower efficiency of gene transfer, mirroring the degree of cytopathic effect observed in the beating myocytes cultures (Coffin *et al.*, 1996a). In these viruses, the *lacZ* marker gene was inserted in the nonessential UL43 gene.

Enteric neurones represent a system for transplantation into the brain of patients lacking specific neurological functions, since they survive transplantation into the CNS and extend axons into surrounding brain tissue (Tew *et al.*, 1992, 1994). Viruses lacking ICP34.5 either alone (1716) or in combination with the virion transactivator Vmw65 (1764) were used to efficiently deliver a reporter gene to the enteric neurones even though it has been shown such viruses can deliver the same reporter gene to PNS and CNS neurones *in vivo* (Howard *et al.*, 1997). A virus lacking both ICP27 and ICP34.5 was shown to produce less cellular damage than one lacking only ICP27, and it was as efficient in gene transfer, where inactivation of Vmw65 reduced toxicity further (Howard *et al.*, 1997).

1.14.2. Cancer Therapy using HSV Vectors

To generate neuroattenuated mutants that will replicate only in tumour cells, HSV has been modified by either deletions in viral genes encoding enzymes for viral DNA synthesis (tk or ribonucleotide reductase) and/or deletions in RL1 (Chou and Roizman, 1990; MacLean, A., *et al.*, 1991; Martuza *et al.*, 1991, Mineta *et al.*, 1994a,b; Chambers *et al.*, 1995; McKie *et al.*, 1996; McKie *et al.*, 1998).

Methods currently used to treat malignant gliomas include surgery, radiation and chemotherapy. These approaches have not significantly enhanced the life span of patients (Mahaley *et al.*, 1989). Recent studies have used HSV-1 ICP34.5- mutants to treat glioma tumours because these viruses retain the ability to replicate in dividing tumour cells but do not replicate in the surrounding terminally differentiated cells of the CNS (Andreansky *et al.*, 1996).

1.14.2.1. "Pro-drug" Cancer Therapy using HSV tk

One approach of gene therapy for cancer purposes is the transfer of "suicide" genes into tumour cells to sensitise them selectively to drugs that are normally non-toxic. Most of these genes encode viral or bacterial enzymes converting inactive forms of the drug, designated "pro-drug" into toxic antimetabolites that inhibit nucleic acid synthesis (Moolton, 1994). One such gene from HSV-1 encodes a tk that phosphorylates antiviral nucleosides, such as GCV and acyclovir (ACV) normally metabolised at very low levels by mammalian enzymes. These drugs are transformed by viral thymidine kinase into monophosphate forms that are subsequently converted by normal endogenous cellular kinases to triphosphates. These molecules are potent inhibitors of DNA polymerases. As analogs of the natural deoxyguanosine triphosphate, they inhibit DNA synthesis and as a

consequence kill the cell (Moolten, 1994). Although untransduced cells are not sensitive to GCV at concentrations able to kill HSV tk gene modified cells, it has been found both *in vitro* and *in vivo* that HSV-tk-negative cells can in fact be killed by proximity to HSV-tk-positive tumour cells treated by GCV. This phenomenon first observed by Moolten has been designated the "bystander effect" (Moolten, 1986; Culver *et al.*, 1992).

1.14.2.2. RL1 Mutant Vectors

There are several RL1 mutant viruses and three RL1 deleted viruses (RE6, R3616, and 1716) that are actively being studied for their potential use as cancer therapies in the CNS and PNS.

The RE6 HSV-1/2 intertypic recombinant was first described in 1983 (Thompson and Stevens, 1983). Subsequently, RE6 was shown to be avirulent due to a defect in the RL1 gene which resulted in severely reduced replication in neuronal tissues (Thompson and Stevens, 1983; Thompson *et al.*, 1989). Brandt *et al* (1997) injected $1x10^6$ pfu/mouse of the neuroattenuated RE6 into the vitreous humour into LHβ-Tag transgenic mice (mice containing the SV40 large T-antigen (Tag) driven by the murine β-leutinizing hormone promoter expressing Tag in the retina), a statistically significant reduction in retinoblastoma tumour size was observed. This was the first report of the use of an HSV mutant to treat spontaneously arising tumours in an immunocompetent animal and supports the possible use of this as a cancer therapy. However, they showed that although intravitreal injection of RE6 resulted in a significant difference in retinoblastoma size, the 95% confidence intervals overlapped and they could not achieve complete regression of the tumour in any of the treated mice. This was in contrast to other reports with melanoma cell implants in which elimination of the tumours was achieved at least in a portion of the treated animals (Randazzo *et al.*, 1995).

The strain F, ICP34.5- virus, R3616, was used with fractionated ionising radiation and results suggest a synergistic action. This treatment reduced tumour volumes and gave longer survivals when compared with either treatment alone. Ionizing radiation in addition to its own cytocidal properties interacts with R3616, resulting in enhanced viral replication, improved distribution and tumour volume reduction. The viral dose was $1x10^7$ pfu/tumour (one dose in total) and the total dose of 40Gy (over the course of two weeks). R3616 has been reported to cause a limited distribution of tissue damage along the injection site (Martuza *et al.*, 1991; Markovitz *et al.*, 1997).

Epithelial ovarian cancer (EOC) remains localised within the peritoneal cavity in a large number of patients, lending itself to intraperitoneal approaches of therapy. This cancer has been studied with a number of viral vector systems. Preclinical results have been obtained as a preliminary to carrying out Phase I trials (Link *et al.*, 1996; Alverez *et al.*, 1997). Coukos *et al* (1999) investigated the effect of 1716 used as an oncolytic agent against EOC and the use of human teratocarcinoma PA-1 as carrier cells for intraperitoneal therapy. 1716 caused a direct dose dependent oncolytic effect on EOC cells *in vitro*. A single intraperitoneal administration of $5x10^6$ pfu/mouse resulted in a significant reduction of tumour volume and spread and an increase in survival in a mouse xenograft model (Coukos *et al.*, 1999). In comparison with the administration of 1716 alone, irradiated PA-1 cells, infected at two multiplicities of infection with 1716 and injected intraperitoneally at $5x10^6$ cells/animal, led to significant tumour reduction in the two models tested and the significant prolongation of mean survival in one model (Coukos *et al.*, 1999). Administration of 1716 infected PA-1 carrier cells resulted in larger areas of tumour infected by the virus (Coukos *et al.*, 1999). Kucharczuk *et al* (1997) demonstrated the efficacy of 1716 in reducing tumour burden and conferring survival advantage in an intraperitoneal model of malignant mesothelioma in the SCID mouse. Moreover, these studies suggested that extra-CNS administration of replication restricted HSV-1 might be safe.

1716 administered intraperitoneally to SCID mice was found to be completely avirulent (Kucharczuk *et al.*, 1997). There was no viral spread outside the tumours as determined by immunohistochemistry and PCR analysis of multiple murine tissues, including intraperitoneal and retroperitoneal organs as well as distant organs and the brain. This was not the result for wild type HSV-1, to which SCID mice were found to be extremely sensitive (Kucharczuk *et al.*, 1997). The administration of 1716 to normal human skin xenograft model was accompanied by no toxicity, whereas administration of wild type HSV-1 led to rapid destruction of the xenograft (Randazzo *et al.*, 1996). Application of replication restricted HSV-1 for extra-CNS malignancies was recently extended to other tumours; a ribonucleotide reductase deleted mutant was used in an experimental animal model of metastic colorectal carcinoma in the liver (Carroll *et al.*, 1993) and a replication restricted ICP34.5- mutant was used to treat experimental metastic and subcutaneous melanoma (Randazzo *et al.*, 1995; Randazzo *et al.*, 1997).

1.15. Aims

There were two aims of this project, firstly, to determine the role of two antisense genes expressing ICP34.5 and ORF P in HSV-1 virulence and secondly, to identify the HSV-2 strain HG52 ICP34.5 protein.

To achieve the first aim the following strategy was devised:

(1) Construct recombinant viruses expressing ICP34.5 and ORF P singly using 1716.

(2) Characterise recombinant viruses *in vitro* and *in vivo*.

To achieve the second aim the following strategy was used:

(1) Construct glutathione S-transferase/HSV-2 RL1 fusion protein.

(2) Purify the fusion protein.

(3) Immunise using fusion protein to generate polyclonal antisera.

(4) Analyse ICP34.5 expression by Western blotting.

(5) Construct intertypic recombinant viruses using 1716 expressing either HSV-2 RL1 with or without its intron.

2. Materials

2.1. Bacteria

The *E. coli* strain used for cloning was XL2-blue Ultracompetent (Stratagene™). For expression of ICP34.5, BL21 (DE3) [F-ompT-r$^-$b$_m^-$b] were used (Studier and Moffat, 1986). Bacteria were grown in L-broth (170mM NaCl, 10 g/l Difco bactotryptone, 5 g/l yeast extract) and 2xYT (85mM NaCl, 16 g/l Difco bactotryptone, 5 g/l yeast extract). Bacteria from glycerol stocks were plated out onto L-broth agar (L-broth containing 1.5% (w/v) agar).

2.2. Plasmids

pGEM34.5 was provided by Dr. L. McKie (McKie *et al.*, 1994). The plasmid, p35, containing the UL43 gene (n.p. 91610-96751) cloned into pUC19 (MacLean, C. *et al.*, 1991) was provided by Dr. A. MacLean. An oligonucleotide linker was inserted into p35 which became p35(PacI), which was provided by Dr. A. MacGregor.

Plasmids containing the HSV-2 RL1 gene, with and without the intron, pSB1 and pSB2 (Bdour, 1995), respectively, were provided by Dr. J. Harland.

pGEX-2T(N+1) was generously provided by Dr. R. Everett.

2.3. Cosmids

Cosmids containing the whole HSV-1 strain 17$^+$ genome were generously provided by C. Cunningham (Cunningham and Davison, 1993).

2.4. Cells and Tissue Culture Media

2.4.1. ETC10

Baby Hamster kidney 21 clone 13 (BHK) cells (MacPherson and Stoker, 1961) were grown in Eagle's medium (Gibco) supplemented with 10% new-born calf serum (Gibco), 10% (v/v) tryptose phosphate broth (Busby *et al.*, 1964).

2.4.2. DMEM10

Human neuroblastoma (SK-N-SH) cells, human cervical carcinoma (HeLa) cells, African green monkey (Vero) cells and mouse embryo fibroblast 3T6 cells (European Tissue Culture Collection) were gown in Dulbecco's modified essential medium (DMEM) supplemented with 10% foetal calf serum (DMEM10). Penicillin/streptomycin were used at appropriate concentrations.

2.4.3. Emet/5C2

When infected cells were radiolabelled, Emet/5C2 (Eagle's medium containing one-fifth normal concentration methionine and 2% newborn calf serum) was used.

2.4.4. EMC5

Eagle's medium containing 1.5% carboxymethylcellulose and 5% calf serum.

2.5. Viruses

2.5.1. HSV-1

2.5.1.2. Strain 17^+

The wild type parental virus used in this study was HSV-1 strain 17^+ (Brown *et al.*, 1973).

2.5.1.3. Mutant 1716

Strain 17^+ deletion variant 1716 was used extensively for making recombinant viruses and as an ICP34.5 negative control in many experiments (MacLean, A. *et al.*, 1991).

2.5.1.4. Mutant gD17+

Strain 17^+ mutant virus with the gD promoter driving *lacZ* expression in the UL43 locus (R. Reid, personal communication). This mutant was used as a control for insertion of a gD/*lacZ* cassette in the UL43 locus.

2.5.1.5. Strain F

An additional wild type HSV-1 strain, F, was used to compare phenotypic and ICP34.5 properties *in vitro* with strain 17^+ (Ejercito *et al.*, 1968).

2.5.2. HSV-2

2.5.2.1. Strain HG52

The wild type parental virus HSV-2 strain HG52 was extensively used in this study (Timbury, 1971).

2.5.2.2. Mutant 2616

A HG52 deletion variant in the RL1 gene, 2616, was used for protein expression and growth behavior studies (Harland and Brown, 1991).

2.5.2.3. Mutant 2624

A HG52 mutant virus in which the RL1 intron is deleted. This mutant was used to study ICP34.5 protein expression (Ravi *et al.*, 1998).

2.5.2.4. Other HSV-2 Strains

Additional HSV-2 strains which were used to look for ICP34.5 expression included: strain 333 (Seth *et al.*, 1974), G (Ejercito *et al.*, 1968), 186 (Esparza *et al.*, 1974), MS (Gudnadottir *et al.*, 1964).

2.6. Antisera

HSV-1 proteins

A peptide antibody to the $(PAT)_{10}$ repeat in ICP34.5 was provided by Dr. A. MacLean (McKay *et al.*, 1993). Two separate antisera to the ORF of RL1, namely, Rabbit 1 and 137, were provided by Dr. J. Harland (Brown *et al.*, 1997). Antisera specific to ORF P were provided by Mr. R. Reid. Dr. A. Cross generously provided monoclonal antibodies against UL42 (ZLF11) and gI (3104). Dr. H. Marsden additionally provided rabbit polyclonal antisera which recognise gC and gD and β-galactosidase.

Cellular proteins

The commercially generated PCNA antibody was supplied by Sigma.

2.7. Radiochemicals

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

[^{35}S]-methionine	[>1 000 Ci/mmol]
[^{32}P]dCTP	[3 000 Ci/mmol]

2.8. Oligonucleotides

Oligonucleotides were synthesised using a model 8600 Biosearch multiple column synthesiser at the Institute of Virology.

2.9. Enzymes

Restriction enzymes were purchased from Boehringer Mannheim and New England Laboratories. T4 DNA ligase and T4 polynucleotide kinase were purchased from Boehringer Mannheim.

Lysozyme and RNaseA were purchased from Sigma Chemical Co.

2.10. Sequencing

All sequences in this thesis was kindly carried out by Mrs. L. Taylor at the Institute of Virology and performed on an ABI 377 DNA sequencer.

2.11. Phospho-imaging

Several immunoblots were analysed on a BioRad Molecular® Imager Fx machine using a BioRad Pentium computer with QuantityOne Phospho and Multi-imager software (4.0.3). Instruction was generously provided by Mr. Colin Loney.

2.12. Solutions

2.12.1. Bacterial Culture Reagents

Promega Wizard kit:

Resuspension Solution: 50mM Tris-HCl (pH7.5), 10mM EDTA, and 100 μg/ml RNaseA

Lysis Solution: 0.2M NaOH, 1%(w/v) SDS

Neutralisation Solution: 1.32M KAc (pH4.8), 40%(v/v)propan-2-ol, and 4.2M guanidine hydrochloride

TE buffer: 10mM Tris-HCl (pH7.5), 1mM EDTA

Solution 1: 50mM Glucose, 10mM EDTA, 25mM Tris HCl (pH8) and Lysozyme (4 mg/ml)

Solution 2: 0.2M NaOH and 1% (w/v) SDS

Solution 3: 3M KAc (pH4.8)

2.12.2. Tissue Culture Reagents

HEBS: (pH7.05) 130mM NaCl, 4.9mM KCl, 1.6mM $NaHPO_4$, 5.5mM D-glucose, 21mM HEPES

Lysis buffer: 0.6% (w/v) SDS, 10mM EDTA, 10mM Tris-HCl (pH7.4) and 500µg/ml Proteinase K

NTE buffer: 10mM Tris-HCl (pH7.5), 10mM NaCl, 1mM EDTA

PBS-A: 170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4 (pH7.2)

RS buffer: 10mM KCl, 1.5mM $MgCl_2$, 10mM Tris-HCl (pH7.5)

Tris-saline: 140mM NaCl, 30mM KCl, 280mM Na_2HPO_4, 1 mg/ml glucose, 0.0015% (w/v) phenol red, 25mM Tris-HCl (pH7.4), 100U/ml penicillin, 0.1 mg/ml streptomycin.

Versene 0.6mM EDTA in PBS-A containing phenol red

2.12.3. Agarose Gel Reagents

TBE: 89mM Tris, 89mM Boric acid, and 2mM EDTA

RE stop: 100mM EDTA, 10% (w/v) Ficoll 400, 0.25% (w/v) Bromophenol blue in 5x TBE

2.12.4. Southern blot Reagents

Gel Soak 1: 200mM NaOH and 600mM NaCl

Gel Soak 2: 1M Tris-HCl (pH8) and 0.59M NaCl

20xSSC: 0.3M Na_3Citrate and 3M NaCl

Hybridization buffer: 0.5M NaP (pH7.4) and 7% (w/v) SDS

Wash solution: 2x SSC and 0.1% (w/v) SDS

2.12.5. Immunofluorescence Reagents

Permea mix: 0.5% (v/v) NP40, 10% (w/v) sucrose in PBS-A

Fix solution: 2% (w/v) sucrose, 5% (v/v) formaldehyde in PBS-A

2.12.6. SDS-PAGE and Western blot Reagents

BM: 151mM Tris-HCl (pH6.7), 6.28% (w/v) SDS, 0.15 % (v/v) β-mercaptoethanol, 0.31% (v/v) glycerol, and 0.1% (w/v) bromo-phenol blue

RGB: 375mM Tris-HCl (pH8.9) and 0.1% (w/v) SDS

SGB: 0.1M Tris-HCl (pH6.7) and 0.1% (w/v) SDS

Towbin buffer: 20% (v/v) MeOH, 25mM Tris-HCl (pH8.3), 192mM glycine

2.12.6.1. Western blot Denaturation and Renaturation Buffers.

Denaturation buffer: 7M guanidine hydrochloride, 50mM Tris-HCl, pH8.3, 50mM DTT, 2mM EDTA, 0.2% (w/v) nonfat dried milk

Renaturation buffer: 50mM Tris-HCl, pH7.5, 100mM NaCl, 2mM DTT, 2mM EDTA, 0.1% (v/v) Nonidet P-40, 0.25% (w/v) nonfat dried milk

2.12.7. Immunoprecipitation Buffers

Buffer A: 10mM HEPES, 50mM NaCl, 1mM EDTA, 0.5% (v/v) Triton, 7mM 2-mercaptoethanol, 0.5M sucrose and added immediately before use 1mM PMSF

Buffer E: 100mM Tris-HCl (pH8.0), 100mM NaCl, 2mM EDTA, 2mM EGTA, 1% (v/v) NP40, 0.5% (w/v) Na deoxycholate, and added immediately before use 0.5mM PMSF

Buffer EB: Buffer E and 2mg/ml BSA

Buffer EN: Buffer E with 500mM NaCl

2.12.8. Pull-down Reagents

Buffer C: 50mM Tris-HCl (pH8.0), 0.5mM NaCl, 1mM EDTA, 0.5% (v/v) NP40 and Boehringer Mannheim protease inhibitors

2.12.9. Fractionation Reagents

Hypotonic buffer: 1.6mM $MgCl_2$, 6mM KCl, 10mM Tris-HCl (pH8.0), 1mM dithiothreitol, 1mM PMSF, 5mM benzamidine, and 0.5%(v/v) NP40

2.12.10. Immunohistochemistry Reagents

STWS: 2%(w/v) potassium bicarbonate, 20%(w/v) magnesium sulphate in dH_2O

2.13. Chemicals

Chemicals used were analytical grade and mostly supplied from BDH chemicals UK or Sigma Chemicals Co.

APS and TEMED were supplied from Bio-Rad Laboratories. Ampicillin (Penbritin) was supplied from Beecham Research.

2.14. Other materials

2.14.1. Centrifuges

Volumes >50ml up to 10,000 rpm	Beckman Centrifuge
Volumes 1.5 ml - 45 ml up to 3,000 rpm	Benchtop Fison's Coolspin Centrifuge
Volumes <1.5ml up to 13,000 rpm	Micro-centrifuge

2.14.2. Film and computer scanning of autoradiographs

Autoradiograph XS-1 film (Southern, Northern and Western blots) was supplied from Kodak.

Film used for immunohistochemical analysis was 1600 speed from Fuji and Zoom film from Kodak.

Autoradiographs were scanned using a StudioscanIIsi scanner with 600 dpi.

Autodradiographs were developed in a Kodak X-omat model ME-3.

2.14.3. Miscellaneous Equipment

DNA was crosslinked to nitrocellulose membrane using a Stratagene UV Stratalinker™ 1800.

3. METHODS

3.1. Bacterial growth

Growth of plasmids was carried out using *E coli* and harvested by either a large scale or small scale preparation, Section 3.1.1. or 3.1.2., respectively.

3.1.1. Large Scale Isolation of Plasmid DNA: Promega© Wizard™ Maxi-prep

A 500 ml *E. coli* plasmid-containing culture was grown overnight in L-broth containing 100 μg/ml ampicillin. Cells were pelleted by centrifugation at 5,000 rpm (Sorvall GSA rotor) for 10 min. at RT. The supernatant was discarded and the pellet resuspended in 15 ml Resuspension Solution. Fifteen ml Lysis Solution was added to the pellet by gently mixing thoroughly and incubating at RT for 20 min. Fifteen ml Neutralisation Solution was added and mixed by inverting several times. The samples were centrifuged at 2,000 rpm (Fison's Coolspin) for 20 min. at RT. The clarified supernatant was poured into a fresh 50 ml Falcon tube and 0.5x propan-2-ol was added and mixed by inversion. Samples were centrifuged at 2,000 rpm (Fison's Coolspin) for 15 min. at RT. The supernatant was discarded and the pellet left to dry. Once dry, the pellet was resuspended in 2 ml TE buffer. Ten ml Wizard™ resin was added to the pellet. This solution was added to a Wizard™ maxi-column. A vacuum was applied to draw the solution through the Wizard™ maxi-column. The resin was washed by adding 80% EtOH to the Wizard™ maxi-column. The DNA was eluted by adding 1.5 ml of preheated H_2O (65-70°C) and incubating at RT for 1 min. before centrifuging the Wizard™ maxi-column at 2,500 rpm (Fison's Coolspin) for 5 min. at RT. A final step of purification of DNA was done by using a syringe with a Luer-Lok® extension and 0.22 μm filter. Once the solution went through the filter the DNA was precipitated by adding 2 volumes EtOH and 0.1 volume 3M NaAc, vortexing and placed in dry ice for 20 min. and centrifuged at 2,500 rpm (Fison's Coolpsin) for 10 min. at RT. The supernatant was discarded and the pellet rinsed with 1 ml 70% EtOH and centrifuged as above. The supernatant was discarded and the pellet left to dry at RT. The DNA pellet was resuspended in 1.5 ml dH_2O (50 μg/μl RNase A).

3.1.2. Small Scale Isolation of Plasmid DNA

A single colony that was grown overnight on an agar plate was picked with an autoclaved toothpick and placed in a Universal bottle containing 5 ml L-broth and Amp (100 μg/ml). The bottle was placed in a 37°C shaker overnight. One ml of the grown culture was centrifuged at 7,000 rpm (microfuge) for 1 min. The cell pellet was resuspended in 100 μl of Solution 1 by a brief vortex and incubated at RT for 5 min. Two-hundred μl of Solution 2 was added and mixed by inverting 4 to 6 times, and incubated at RT for 5 min. One-hundred-and-fifty μl of cold Solution 3 (3M KAc) was added and mixed by inversion and incubated at RT for 5 min. This mixture was centrifuged at 13,000 rpm (microfuge) for 5 min. and the supernatant-containing plasmid DNA transferred to a fresh tube. The 450 μl volume of supernatant (from solutions 1, 2, and 3) was clarified by adding 450 μl phenol:chloroform to each tube. Samples were vortexed and centrifuged at 13,000 rpm (microfuge) for 5 min. at RT. The aqueous layer was transferred into a fresh tube and phenol:chloroform extraction repeated as above. The aqueous layer was mixed with 1 ml EtOH and centrifuged at 13,000 rpm (microfuge) for 5 min. at RT. The pellet was washed with 70% EtOH and dried in a "speedi-vac". The pellet was resuspended in 100 μl dH_2O (50 μg/μl RNase A).

3.2. Transformation of Bacterial Cells

XL-2-Blue Ultracompetent *E.coli* (Strategene) were thawed from -70°C on ice and 20 μl aliquoted into an Eppendorf tube. One μg of plasmid DNA was added, mixed, and the tube placed on ice for 30 min. The DNA and *E.coli* was heat shocked by placing in a 42°C water bath for exactly 90 sec. followed by placing on ice for 2 min. Eighty μl 2xYT broth was added and the reaction tube incubated for 1 h at 37°C. After 1 h the mixture was pipetted and spread onto a L-broth agar plate containing appropriate antibiotics (Amp (100 μg/ml) and/or X-gal (20 μg/ml)). The 100 μl mixture was absorbed on the agar for 15 min. before the plates were inverted and placed in a 37°C incubator for 16 h.

3.3. Restriction Endonuclease Digestion

Plasmid and viral DNA was routinely evaluated by different restriction endonuclease digestion profiles. The duration and temperature conditions were carried out according to the manufacturers' instructions (Section 2.9). Enzyme activity was stopped

by placing RE stop solution in the reaction mixture. Digestion was visualized by electrophoresis through an agarose gel (Section 3.4).

3.4. Agarose Gel Electrophoresis

Agarose gels (0.6-1.5% (w/v) agarose) were prepared by boiling the appropriate quantity of agarose in 1x TBE. Ethidium bromide was added at a concentration of 0.5 μg/ml, immediately prior to pouring the gel. The solution was poured into a tray (BioRad) which held a comb. Once cool, the tray was placed in an electrophoresis kit, the comb removed and samples pipetted into individual wells. Electrophoresis of the samples was carried out until the dye front was approximately 1 cm from the end of the gel.

3.5. Elution of Restriction Enzyme Fragments from Agarose Gel

DNA was digested with the appropriate restriction enzyme and electrophoresed through an agarose gel containing 0.5 μg/ml EtBr until the fragment to be isolated was well resolved. The gel was visualised under a long-wave UV light and the desired fragment excised using a sterile scalpel.

The Gene Clean™ system was used to isolate DNA from the agarose gel. NaI was added to TBE Modifier and to 1 gel slice. The samples were placed in a water-bath at 55°C for 10 min. Glass milk beads were resuspended by vortexing and 5 μl was added to each sample. The sample containing beads was vortexed briefly and incubated at RT for 5 min. followed by centrifugation at 13,000 rpm (microfuge) for 1 min. The supernatant was discarded and the glass milk beads were washed with 0.5 ml Gene Clean Wash (-20°C) by vortexing. The beads were pelleted by centrifugation at 13,000 rpm (microfuge) for 1 min. This wash was repeated. Twenty μl dH_20 was added to the pellet which was vortexed and incubated at 55°C for 5 min followed by vortexing and centrifugation at 13,000 rpm (microfuge) for 2 min. The supernatant contained the DNA.

3.6. Phenol Chloroform Extraction and Precipitation of Plasmid DNA

An equal volume of phenol:chloroform was added to the DNA, mixed by vortexing and subjected to centrifugation for 2 min. at 13,000 rpm at RT (microfuge). The aqueous layer containing DNA was pipetted into a fresh tube. Two volumes of EtOH and 0.1 volume 3M NaAc was added to the aqueous layer, vortexed, and placed on dry ice for 20 min. The solution was centrifuged for 20 min. at 13,000 rpm at RT (microfuge). The

supernatant was discarded and 1 ml 70% EtOH added and centrifugation carried out as stated above. The supernatant was discarded and the DNA pellet left to dry. The pellet was resuspended in dH_2O (RNase A 50μg/ml).

3.7. Ligation of Plasmid DNA

Different molar ratios of plasmid DNA to vector were added to a total volume of 14 μl. Four μl 5x Ligation Buffer was added, the solution mixed and centrifuged to collect at the bottom of tube. Lastly, 2 μl T4 DNA ligase (3U/μl) was added to the solution, mixed well and placed in a water bath at 16°C overnight. The following day this was used in a transformation reaction (Section 3.2).

3.8. Purification of a Synthetic Oligonucleotide

The oligonucleotide was removed from the column by passing 1.5 ml ammonia through the column between two 1.5 ml syringes for approximately 1 h. After this time all the solution was pulled back into one syringe and transferred to a screw cap cryo-vial. Deprotection of the oligo was carried out for 5 h at 60°C. The vial was placed on dry ice for 15 min. and the cap carefully opened and a layer of parafilm with holes used to seal the top. The vial was placed in a vacuum spin overnight. The oligonucleotide was resuspended in 100 μl dH_2O by vortexing and incubated at 37°C for 30 min. The sample was centrifuged for 2 min. at 13,000 rpm (microfuge). The supernatant was placed in a fresh Eppendorf tube and OD analysis carried out.

3.9. Tissue Culture

Herpes simplex virus growth was studied *in vitro* for protein expression and replication kinetics.

3.10. Cells

Replication of HSV-1 strain 17^+ and HSV-2 strain HG52 wild types differ from RL1 deleted viruses in some (3T6) but not all cell lines (BHK): additional cell lines such as Vero, HeLa and rabbit skin cells were used to study ICP34.5 mutant viruses. An *in vitro* function of ICP34.5 is to preclude protein synthesis in certain cells, such as SK-N-SH cells.

3.10.1. BHK21/C13 Cells

Baby hamster kidney cells 21 (BHK) seeded at a 1 in 10 dilution were grown in 100 ml ETC10 in 850cm^2 roller bottles in a humidified atmosphere of 5%CO_2 at 37°C for 3 to 4 days. Confluent cells (3x10^8/roller bottle) were harvested as described in Section 3.11.

3.10.2. HeLa, Vero, Rabbit Skin and 3T6 Cells

HeLa, Vero, Rabbit Skin, and 3T6 cells were grown for 3 days in DMEM10 with 5%CO_2. Cells were passed in large flasks (T-175) by seeding at a 1 in 10 dilution in 50 ml DMEM10. Confluent cells (3x10^7 cells/T-175 flask) were harvested as described in Section 3.11.

3.10.3. SK-N-SH Cells

The human neuroblastoma (SK-N-SH) cells were grown in DMEM10 with 5%CO_2 for 5 days in a T-175 flask. Cells were split 1 in 4 for passage.

3.11. Passaging Cells

Trypsin solution (0.25% (w/v) in Tris-saline containing phenol red (pH7.5) ($NaHCO_3$)) was thawed from -20°C and mixed with versene to yield 0.06% (v/v) trypsin/EDTA solution.

T-175 flasks or roller bottles with confluent monolayers were opened in a category 2 hood, the supernatant decanted, and 10 ml or 20 ml, respectively, of a trypsin/EDTA solution was poured over the monolayer and decanted. This was repeated before the flask or roller bottle was left for 2 to 3 min. The cells were shaken into10 or 20 ml media for further use.

3.12. Cryopreservation of Cells

Confluent cell monolayers of Vero, HeLa, SK-N-SH or 3T6 cells were harvested from T175 flasks as described above by trypsinizing and resuspending in 15 ml media and pipetted into Falcon tubes. Cells were centrifuged at 3,000 rpm (Fison's Coolspin) at 4°C for 10 min. and resuspend in 5 ml of the appropriate growth medium, with 10% DMSO. Aliquots of 1 ml were pipetted into 1.5 ml cryo-vials. These were frozen overnight at -70°C and moved to a liquid nitrogen freezer for long term storage.

3.13. Growth of HSV Stocks

Roller bottles containing a fresh monolayer of BHK cells ($3x10^8$cells/bottle) were infected with 0.003 pfu/cell of HSV in 20 ml of ETC10. These were incubated at 31°C for 4 days or until c.p.e. was complete, when the cells were shaken into the medium. The cell suspension was poured into a 250 ml plastic Falcon tube and cells pelleted by centrifugation at 2,000 rpm (Fison's Coolspin) for 10 min. at 4°C. The supernatant and cell pellet was divided into 2 individual stocks: cell-associated virus and cell-released virus.

3.13.1. Cell-associated Virus

The cell pellet was resuspended in 0.5 ml medium/roller bottle, pipetted into a sterile Universal bottle, thoroughly sonicated and centrifuged at 2,000 rpm (Fison's Coolspin) for 10 min at 4°C, as described above. The supernatant was kept on ice while the pellet was treated as described above. Both supernatants were pooled to give the cell-associated virus stock. This was aliquoted into 1.5 ml sterile cryo-vials and stored at -70°C.

3.13.2. Cell-released Virus

The supernatant was poured into 250 ml sterile centrifuge bottle and spun at 12,000 rpm (Sorvall GSA rotor) for 2 h at 4°C. The supernatant was discarded and the virus pellet resuspended in 1 ml ETC10/roller bottle. The pellet was sonicated until homogeneous, and 1 ml aliquots transferred into 1.5 ml sterile cryo-vials and stored at -70°C.

3.14. Virus Titration

Virus stocks were serially diluted 10-fold in PBS-A/10%CS and 0.1 ml aliquots added to fresh monolayers of BHK cells on 60mm plates from which the media had been removed. The plates were incubated at 37°C with 5%CO_2 for 1 h to allow absorption to the cells, before overlaying with 4 ml EMC10. The plates were incubated at 37°C for 48 to 72 h. The EMC10 was poured off each plate and monolayers fixed and stained with Giemsa stain at RT for 1 h. After rinsing the plates with tap water and allowing them to dry, plaques were counted using a dissection microscope and virus titres calculated as pfu/ml.

3.15. Plaque Purification

Once c.p.e. was complete in transfection plates, cells were scraped into growth media, transferred to a bijou bottle and sonicated in a soni-bath until homogeneous. Serial 10-fold dilutions were made in PBS-A/10%CS. One-hundred µl from the 10^2 to10^6 dilutions from each were plated on fresh BHK monolayers in 60mm plates from which the media had been removed. After 1 h absorption at 37°C and 5%CO_2, the plates were rinsed once with PBS-A/10%CS and overlaid with 4 ml EMC10 and incubation continued at 37°C for a further 48 to 72 h. Using a 200 µl Gilson pipette, the plate with the fewest well separated plaques were picked, resuspended in 500 µl PBS-A/10%CS and sonicated in a soni-bath until homogeneous. This procedure was either repeated or a Southern blot performed to check the purity of the recombinant virus.

3.15.1. X-gal Staining

Recombinant viruses containing the β-galactosidase gene *lacZ* were plaque purified by staining with X-gal. BHK cells monolayers were infected with virus as described in Section 3.14 except the media overlay contained methyl cellulose with 2% calf serum and 150 µg/ml X-gal.

3.16. Sterility Checks on Virus Stocks

Brain heart infusion agar (BHI) plates were used to check for fungal contamination in viral stocks. A small aliquot of a viral stock was streaked onto BHI plates in duplicate which were sealed with parafilm and incubated at RT for 7 days. BHI plates containing 10% horse blood (BHI blood agar) were used to check for yeast or bacterial contamination by plating a small aliquot of viral stock and incubating at 37°C for 7 days. If there was no growth by 7 days stocks were considered sterile.

3.17. Preparation of HSV Virion DNA

Ten roller bottles containing a fresh monolayer of BHK cells ($3x10^8$ cells/bottle) were infected with 0.003 pfu/cell of HSV in 20 ml of ETC10. These were incubated at 31°C until c.p.e. was complete, then the cells were shaken into the medium. The cell suspension was poured into a 250 ml plastic Falcon tube and cells pelleted by centrifugation at 2,000 rpm (Fison's Coolspin) for 10 min. at 4°C. The supernatant was

kept on ice while nuclei were extracted from the cell pellet by treatment with 0.5% (w/v) NP40 in RS buffer (pH7.5) followed by centrifugation at 2,000 rpm (Fison's Coolspin) for 10 min. at 4°C. The supernatant was kept on ice and the above repeated before pooling the supernatants and centrifuging at 12,000 rpm (Sorvall SS34 rotor) for 2 h at 4°C. The supernatant was discarded and the virus pellet lysed by resuspension in NTE buffer before adding EDTA and SDS to a final concentration of 10mM and 2% (w/v), respectively. Viral DNA was extracted 3 times with saturated phenol and once with cholorform, prior to precipitating with 2 volumes of EtOH by gentle inversion. The DNA was pelleted by centrifugation in a Fison's Coolspin centrifuge at 2,000 rpm for 10 min. at RT. The DNA pellet was washed with 2/3 volume 70% (w/v) EtOH by gentle inversion and centrifuged as described above. The supernatant was discarded and the pellet air-dried for 15 min. before re-dissolving in dH_2O containing RNaseA (50 μg/ml). The quantity of DNA was determined by OD at 280nm.

3.18. Infected Cell DNA Preparation

Linbro plates with BHK cell monolayers were infected at a m.o.i. of 5 pfu/cell at 31°C. After 48 h the supernatant was removed and cells lysed by incubating with 2 ml lysis buffer for 4 h at 37°C. NaCl was added to a final concentration of 200mM and infected cell DNA extracted twice with an equal volume of phenol and once with chloroform, precipitated by the addition of 2 volumes of EtOH, and dried in a vacuum dessicator. The DNA was dissolved in 400 μl of water containing 50 μg/ml RNaseA. Restriction digests were carried out on 5% of the total sample.

3.19. Transfection of Virus DNA by $CaPO_4$ and DMSO Boost

Viral HSV DNA and plasmid DNA were diluted to the desired molar ratios and placed in an Eppendorf tube. The following were added to the DNA, 400 μl HEBS (pH7.5) and calf thymus DNA (10 μg/ml). These were mixed and spun down in a micro-centrifuge and $CaCl_2$ added to a final concentration of 130mM. The samples were left at RT for 30 min. and added to a fresh confluent monolayer of BHK cells in 60mm plates, from which the medium had been removed. Following incubation at 37°C for 1h, cells were overlaid with 4 ml ETC10 and incubated at 37°C. Four hours later the media was removed from plates and the plates washed twice with ETC10. For exactly 4 min. the cells

were overlaid with 1 ml 25% (w/v) DMSO in HEBS at RT. Four ml ETC10 was added immediately and subsequently the plates were washed twice with 4ml of ETC10, and overlaid with 4 ml ETC10. Plates were incubated at 37°C until c.p.e. was complete. Cells were scraped into the medium and pelleted by centrifugation at 2,000 rpm (Fison's Coolspin) for 10 min, resuspended in 1 ml medium, sonicated and stored the virus at -70°C until further use (Section 3.15).

3.20. One-cycle Replication Kinetics

Plates (30mm) were seeded with $2x10^6$ cells/plate in 2 ml ETC10 and grown overnight at 37°C and 5%CO_2. Growth media was poured off and 100 µl of the virus (10 pfu/cell) added. The plates were returned to 37°C and 5% CO_2 for 1 h. Plates were washed with 2 ml PBS-A/10%CS and overlaid with 2 ml ETC10. This was time point 0 h. Plates were incubated at the appropriate temperature and harvested at the designated time points (usually 0,2,4,6,8,12, and 24h) by scraping the cell monolayer into the medium and transferring the suspension to a sterile bijou bottle (Harland and Brown, 1997). The samples were sonicated and stored at -70°C until the experiment was complete (at most 72 h post infection). The samples were quickly thawed, diluted and plated as described in Section 3.15.

3.21. Multi-cycle Replication Kinetics

Thirty mm plates were seeded with $2x10^6$ cells/plate in 2 ml ETC10 and grown overnight at 37°C with 5%CO_2. Growth media was poured off and 100 µl of the virus (0.01-0.001pfu/cell) added. The plates were returned to 37°C and 5% CO_2 for 1 h. After 1 h the plates were washed with 2 ml PBS-A/10%CS and overlaid with 2 ml ETC10. This was time point 0 h. Plates were incubated at the appropriate temperature and harvested at the designated time points (usually 0,4, 8, 12, 24, 48, 72 and 96h) by scraping the cell monolayer into the medium and transferring the suspension to a sterile bijou bottle (Harland and Brown, 1997). The samples were sonicated and stored at -70°C until the experiment was complete. The samples were quickly thawed and plated as described in Section 3.15.

3.22. Southern Blot and Hybridization

Purified DNA was digested with the appropriate restriction enzyme(s) before electrophoresing on a 1% (w/v) agarose gel. The gel was visualised under a short wave UV light to confirm DNA digestion then placed in a bath containing Gel Soak I for 1 h at RT. After rinsing with dH_2O the gel was incubated in Gel Soak II for 1 h at RT and rinsed as above. Finally, the gel was incubated in 20xSSC for 1 h at RT.

A pack of 'Hi-Dry' towels was stacked on the bench followed by 3 sheets of dry Whatmann 3MM paper. On top were 3 sheets of pre-soaked 3MM paper and 1 sheet of nitro-cellulose membrane (Hybond N) in 20xSSC. The gel was placed on top and cling film was wrapped around the top. A glass plate and heavy weight were placed atop and this was left overnight to transfer DNA. To transfer DNA to two membranes this procedure was repeated on top of the gel. A UV crosslinker was used to cross link the DNA to the membrane. The membrane was allowed to dry and placed in a glass bottle and hybridization buffer added. The membrane was incubated in a BioRad oven at 65°C overnight. The membrane was washed twice in wash solution at 65°C for 30 min. Finally, it was dried for 15 min. at 80°C. The membrane was exposed to XS1 film making sure that it was 100% dry each time it was exposed.

3.22.1. Radiolabelling DNA

Twenty-five ng template DNA in dH_2O in a final volume of 11 µl was placed in a tube and denatured by heating in a boiling water bath for 10 min. followed by chilling quickly in an ice bath. The DNA was pulsed in a micro-centrifuge and placed on ice while 4 µl of High Prime enzyme and buffer was added (Boehringer Mannheim). Five µl (50µCi[α^{32}P]dCTP] was added and the tube was incubated for 15 min. at 37°C. The reaction was stopped by placing the tube in a boiling water bath for 10 min. After boiling, the radiolabelled probe was used with the blot as described in the Southern blot and hybridization section (3.22).

3.23. Indirect Immunofluorescence

Linbro wells containing coverslips were seeded at 0.5-5x10^5cells/well and grown overnight at 37°C with 5%CO_2. The cells were infected at a low moi (0.1 pfu/cell) for 6 h. After infection the cells were fixed by removing the medium and washing three times with PBS-A and adding 0.5 ml Fix solution for 10 min. at RT. Coverslips were washed 3 times

with PBS-A and a block was performed with 100 µl PBS-A/1%CS at 4°C overnight. The PBS-A/1%CS was removed and 500 µl Permea mix was added for 5 min. at RT. Coverslips were washed 3 times with PBS-A. The primary antibody was diluted to the desired concentration in PBS-A/1%CS and 20µl placed in the centre of a well on the lid of the Linbro dish. After wiping off excess fluid, the coverslips were carefully placed, cells facing down, on the antibody. Coverslips were incubated in the primary antibody for 1 h at RT. Coverslips were placed back in the wells, cells facing upwards, and washed 6 times with PBS-A. The secondary antibody FITC goat anti-rabbit IgG was diluted 1/100 in PBS-A/1%CS. Twenty µl drops were placed in the lid as described above, coverslips incubated at RT for 30 min. and washed 6 times with PBS-A.

Coverslips were mounted by dipping in H_2O and drying on a paper towel at RT until dry. A small drop of mounting fluid was placed on a glass slide and the dry coverslip, cells facing down, placed on the drop. A longer period of storage required painting clear nail varnish around the edges of the coverslips to prevent drying.

3.24. Protein Expression in Infected Cell Extracts

Virus stocks were used to infect 60mm plates containing $4x10^6$ cells with 20pfu/cell as described in Section 3.21. Plates were incubated for the desired time at 37°C. Samples were harvested by removing media and washing infected cell monolayers twice with cold PBS-A. The monolayers were taken off in 300 µl 1x BM.

3.25. SDS-PAGE

Polyacrylamde gel electrophoresis was used to visualise viral and cellular proteins. Unless otherwise stated the routine percentage of acrylamide used in this work was 10% polyacrylamide using 2.5% (w/v) crosslinker. These are single acrylamide gels, but gradient acrylamide gels were also used in this study. In the resolving gel a gradient of 5 to 12.5% acrylamide is made using a gradient mixer.

Slab gels were cast vertically in a sandwich consisting of 2 glass plates separated by 1.5 mm perspex spacers. The resolving gel was prepared using RGB with 0.06% (w/v) APS and 0.4% (v/v) TEMED. A 1 ml layer of butan-2-ol 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 rinsed with dH_2O.

The stacking gel was composed of 5% acrylamide crosslinked with the same ratio of N,N'-methylene-bisacrylamide used in the resolving gel. The SGB was mixed with 0.06% (w/v) APS and 0.04% (v/v) TEMED. This was poured on top of the resolving gel and allowed 30 min. to solidify.

Gels were electrophoresed either for 3 to 4 h at 50mA, or overnight at 10mA (Marsden *et al*, 1978).

Gels were either prepared for Western blot (Section 3.26) or fixed and stained in a solution of MeOH:$C_2H_4O_2$:dH_2O (50:7:50) with 0.2% (w/v) Coomassie Brilliant Blue R250 for 30 min. at RT. Gels were destained for a minimum of 1 h twice in a solution of MeOH:$C_2H_4O_2$:dH_2O (5:7:88). Gels were either dried under a vacuum and exposed to Kodak XS-film, or enhanced in 3 volumes of En^{3}Hance (New England Nuclear) for 1 h at RT, washed in H_2O twice for 30 min., before drying under a vacuum and exposed for flurography at -70°C.

3.26. Western blot of ICP34.5

Samples were electrophoresed through a 10% SDS-PAGE gel as described above. Nitro-cellulose ECL membrane (Amersham) and two 3MM Whatmann paper was cut to the same size as the gel and pre-soaked in Towbin buffer. The proteins in the gel were transferred to membrane for either 3 h at 250mA, or overnight at 50mA. After transferring, the membrane was blocked in PBS-A/T (PBS-A and 0.05% Tween 20) containing 5% (w/v) Marvel milk for 1 h at RT. The membrane was washed in PBS-A/T 2x 15 min. The primary antibody was diluted to the desired concentration in 25 ml PBS-A/T and incubated at either 37°C for 2 h or 4°C overnight. The membrane was washed in PBS-A/T 3 times for 15 min. at RT. The membrane was incubated in HRP conjugated secondary antibody at RT for 1h followed by 3 washes in PBS-A/T for 10 min. each. A detection reagent (ECL) was added to the membrane for 1min. and the membrane was exposed to the blot to XS-1 film for the desired amount of time.

3.26.1. Western blot Renaturation of Proteins

Proteins were separated by SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane in cold 25mM Tris, 192mM glycine (pH8.3), without MeOH. The transfer was carried out in a 4°C room. The filter was blocked by incubation in 30mM HEPES, pH 7.5, containing 100 μg/ml phenol extracted salmon sperm DNA as the

blocking agent. The filter was incubated in 100 ml denaturation buffer for 1 h at RT. The filter was washed several times in cold renaturation buffer and incubated in 500 ml renaturation buffer for 16 h at 4°C. This denaturation and renaturation procedure was adapted from the method of Hager and Burgess (1980).

3.27. Immunoprecipitation of ICP34.5

Cell monolayers, in Linbro well plates, were infected with virus at a moi of 10 and treated the same as described in the virus and host cell protein synthesis shutoff section. At the desired time(s), cells were scraped into the medium and into a 15 ml Falcon tube, centrifuged for 15 min. at 3,000 rpm (Fison's Coolspin) at RT. The supernatant was discarded, the pellet resuspended in 1 ml PBS and pipetted into a new Eppendorf tube. The tube was centrifuged at 10,000 rpm (microfuge) for 5 min. The supernatant was discarded, the pellet resuspended in 800 µl ice cold Buffer E, and placed on ice for 15 min. The sample was clarified by centrifugation at 7,000 rpm (microfuge) for 15 min. at 4°C. The pellet was kept and stored in 100 µl dH_2O at -70°C in case lysis was not complete. The supernatant was pipetted into a fresh Eppendorf and either used immediately or stored at -70°C. Fifty µl of the supernatant was added to 150 µl Buffer A and 50 µl antibody in an end-to-end mixer at 4°C overnight. After overnight mixture 75 µl protein A sepharose 50% (v/v) in Buffer E was added and the sample placed back on the mixer for 1 h at 4°C. After 1 h the sample was centrifuged at 10,000 rpm (microfuge) at 4°C for 5 min. The supernatant was removed and placed in a new Eppendorf tube. The pellet was washed twice with Buffer EB and once with Buffer EN. After the final wash the pellet was resuspended in 50 µl BM. Samples were analysed on SDS-PAGE.

3.28. Virus and Host Cell Protein Synthesis Shut-off

Replicate cultures were either mock infected or exposed to virus for 1 h at 37°C. The inocula were removed, the cells washed twice with 2 ml Emet/5C2, and overlaid with Emet/5C2 . At different times after infection the media was poured off and Emet/5C2 containing 50µCi/ml of [^{35}S]-methionine (specific activity, >1,000 Ci/mM) added to 30 mm plates for 1-2 h. The cells were then harvested, solubilized, subjected to electrophoresis in denaturing polyacrylamide gels, transferred to nitrocellulose sheet and autoradiographed.

3.29. ICP34.5/GST Fusion Protein, Growth and Purification

Bacterial cultures (NM522) were grown overnight in 5 ml. These cultures were diluted 1/10 in 2xYT broth and grown in a shaking 37°C incubator to an OD of 0.5 (3 h). Varying concentrations of IPTG were added (starting with 0.1mM) and the cultures placed back in the incubator for 2 h. After 2 h 10 µl of this culture was retained to visualize on the gel later. The remaining culture was centrifuged at 10,000 rpm (microfuge) at 4°C for 10 min. The supernatant was discarded and the pellet resuspended in 300 µl ice cold PBS-A. Cells were lysed using a soniprobe. Samples were centrifuged at 10,000 rpm (microfuge) for 5 min. at 4°C. The supernatant was transferred to a fresh Eppendorf and the pellet was stored at -70°C to visualize on a gel later. Twenty µl of 50%(v/v) Glutathione beads were added to the supernatant and mixed for 5 min. at RT. Samples were centrifuged at 10,000 rpm (microfuge) for 5 min. at 4°C. The supernatant was discarded and 100 µl of PBS-A added to the pellet, vortexed, and centrifuged for 5 min. at RT. This was repeated twice. The fusion protein was eluted by adding 10 µl glutathione elution buffer, vortexing and leaving at RT for 5 min. Samples were centrifuged at 10,000 rpm (microfuge) for 5 min. at RT. The supernatant was placed in a fresh tube and 30 µl 1xBM (Section 2.12.6.) added. BM was also added to the bead pellet, loaded in a well and analysed by SDS-PAGE.

3.30. Production of HSV-2 ICP34.5 Antisera

Two New Zealand White rabbits were immunised intramuscularly first with 2 ml of protein containing 1 mg ICP34.5 in Freund's complete adjuvant 100% (v/v) followed by 6 boosts, each 14 days apart, using the same amount of antigen but in Freund's incomplete adjuvant. Animals were bled 10 days after each boost and the antisera tested against HSV-1 strain 17^+ and HSV-2 strain HG52 infected cell extracts for reaction with ICP34.5 specific bands.

3.31. Fractionation of ICP34.5

Confluent monolayers in 90mm plates were infected with virus at a m.o.i. of 10 pfu/cell at 37°C at 5%CO_2 for 6 h and 16 h. Plates were harvested by scraping the monolayer into the media. The suspension was poured into a 15 ml Falcon tube and spun at 3,000 rpm (Fison's Coolspin) for 10 min. at 4°C. The cell pellet was rinsed with 1 ml PBS-A and put into an Eppendorf tube. Samples were centrifuged at 3,000 rpm

(microfuge) for 5 min at 4°C. The supernatant was decanted and the pellet resuspended in 1 ml Hypotonic buffer. This buffer lysed the samples which were left on ice for 5 min. After incubation the samples were briefly vortexed and the centrifuged at 10,000 rpm (microfuge) for 10 min. at 4°C to pellet the nuclei. The supernatant was left on ice while the pellet was rinsed with 1 ml PBS-A and centrifuged as described above. After centrifugation the PBS-A was decanted and the pellet resuspended in 100 µl BM. Thirty µl of the cell supernatant was pipetted into a fresh Eppendorf tube and 70 µl BM added to it. Samples were boiled for 10 min. and electrophoresed on a SDS-PAGE and Western blotted.

3.32. Pull-down

Freshly prepared glutathione agarose beads with bound ICP34.5/GST fusion protein (50 µl of a 50% (v/v) slurry) were mixed with 300 µl labeled cell protein extract and incubated for 1 h at 4°C with continuous mixing. The beads were harvested by brief centrifugation and washed 3 times in 1 ml Buffer C. The beads were mixed with 100 µl SDS-polyacrylamide gel loading buffer, boiled for 10 min., analysed by SDS-PAGE and either fixed, dried and autoradiographed, or used for Western blotting.

3.33. *In vivo* Studies

ICP34.5 mutant viruses demonstrate an avirulent phentoype *in vivo* and recombinant viruses were examined by the following *in vivo* methods.

Intracranial (Section 3.34) and footpad (Section 3.35) injections under Prof. Brown's project licence (No. PPL/60/2215) were performed under my personal licence (No. PIL/60/6414).

Snout infection (Section 3.36), harvesting snout and trigeminal ganglia for replication kinetics (Section 3.36.1), and harvesting trigeminal ganglia for immunohistochemistry (Section 3.36.2) were carried out in Dr. Sawtell's laboratory (Children's Hospital, Cincinnati, USA).

3.34. Intracranial LD_{50}

Three week old BALB/c mice were inoculated intra-cerebrally with single virus stocks. Mice were anaesthetised by inhalation of halothane and 25 µl of the appropriate virus dilution in PBS-A/5%CS inoculated into the central region of the left cerebral

hemisphere. Four mice were inoculated with each virus dilution (doses were in the range of 10^1 to 10^7 pfu/mouse). Stocks were always re-titrated on the day of inoculation to ensure correct dose administration. Mice were observed daily up to 21 days post inoculation for signs of illness or death, and the LD_{50} for each virus calculated according to the formula of Spearman-Kärber (Wardlaw, 1987).

Any mice which died within the first 16 h were assumed to have been killed by the anaesthetic/injection protocol and were excluded from the experiment. The formulae used to calculate LD_{50} was the following:

$$Log_{10}LD_{50} = m - \Delta(\Sigma p\text{-}0.5)$$

Δ = the constant interval between the log10 dilutions (= 1).

m = log10 dilution corresponding to the lowest giving death.

For instance, where there is not 100% death, it is assumed that 100% death would follow with a dose which is 10-fold higher than the largest dose.

p = the mortality fraction at each dilution as a decimal.

For instance, where mice die at the lowest dose given, it is assumed that a dose which is 10-fold lower would produce no deaths.

3.35. Footpad Infection

As described by Clements and Subak-Sharpe (1983,1988), 4 week old female BALB/c mice were inoculated in the right rear footpad with different doses of virus diluted in PBS-A/5%CS. Twenty-five µl of virus were injected into the right rear footpad. To study virus replication, at days 0,2,4,6,8,and 10, 2 mice per virus were killed by cervical dislocation, their right rear footpad washed with 70% EtOH, severed, and placed in an Eppendorf tube on dry ice.

3.35.1. Co-cultivation/Latency Reactivation

Female 4 week old BALB/c mice were infected in the right rear footpad as described above. Following inoculation, the virus stocks were re-titrated to ensure correct dosage. The mice were housed for 40 d. All surviving mice were given a lethal dose of pentobarbitone intraperitoneally and ex-sanguinated. They were dissected under a microscope (Opmid-Zeiss) and 10 ipsilateral DRG dissected and separately placed into microtitre plates containing 200 µl ETFC10/well (Fig. 13, 14). The DRG media was removed every second day and placed on fresh BHK cell monolayers and fresh 200 µl

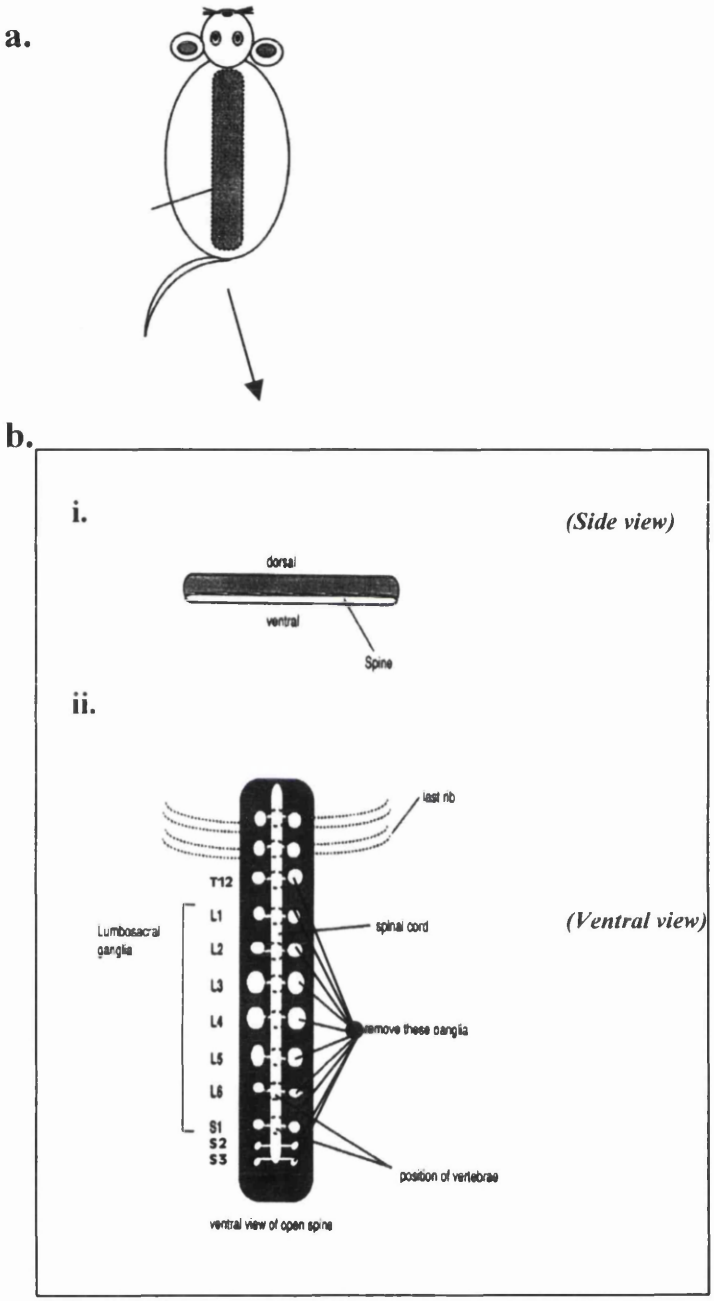


Figure 13. ***In vitro*** **reactivation method.**

a. BALB/c mice were infected with viruses in the right rear footpad. Latency was established in the DRG. Forty-one days pi mice were exsanguinated and DRG dissected. **b.** View of the spinal cord from the side (i.) and from the top (ii.). This representation of the spinal cord shows the location of individual ganglia. One of the thoracic ganglion (T12), 6 lumbar ganglia (L1-L6), and 3 sacral ganglia (S1-S3) were dissected (Adapted from Bloom, 1998).

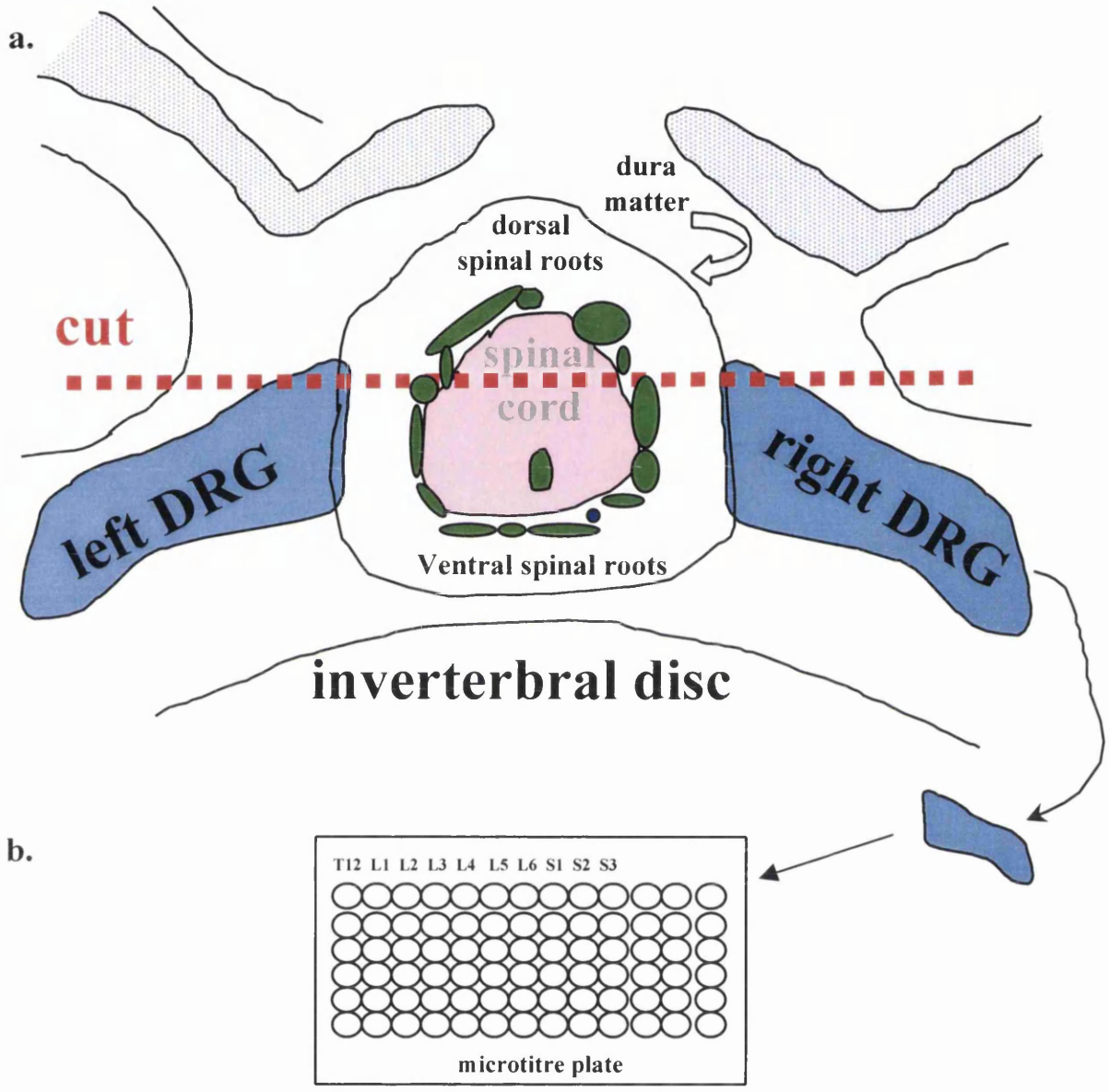


Figure 14. ***In vitro*** **reactivation method.**

a. Diagrammatic representation of a cross section from a single DRG. Only the right side DRG was dissected. **b**. An individual dorsal root ganglion was placed into a microtitre well containing media (Adapted from Engel, 1997).

pipetted onto the ganglia. The BHK cells monolayers were examined for the presence of plaques or c.p.e. Reactivation frequency was measured by percentage of total ganglia that reactivated per virus dose. Supernatant that contained reactivating virus was used to infect a 24 well plate with a fresh BHK monolayer to prepare DNA to confirm the genome structure by Southern blotting.

3.36. Snout Infection

Four to 7 week old outbred Swiss Webster mice (Harland Laboratories) were intraperitonially injected with 100 µl pentobarbitol, anesthetized, and bilaterally inoculated via snout scarification with $1x10^5$pfu in 10 µl per side.

3.36.1. Harvesting Snout and Trigeminal Ganglia for Replication Kinetic Analysis

On days 1 through 6 pi, animals (n=3 per day for each virus) were sacrificed by a lethal dose of sodium pentobarbitol intraperitoneally. Mice were decapitated and snouts disinfected by rinsing in 70% EtOH, each side removed snap frozen and stored at -70°C.

Trigeminal ganglia were dissected using sterile razor blades and forceps, snap frozen on dry ice and stored at -70°C. Once the experiment was complete, snouts were ground in a porcelain pestle and mortar with liquid nitrogen, homogenized in 1 ml glass pestle and mortar with 1 ml DMEM10 and debris pelleted by 10 min. centrifugation at 10,000 rpm (microfuge) at 4°C. Trigeminal ganglia were directly placed in a 1 ml glass pestle and mortar and homogenized in 1 ml DMEM10. Supernatants were titrated on RSC and virus yield obtained as pfu/ml.

3.36.2. Harvesting Trigeminal Ganglia for Immunohistochemistry Analysis

3.36.2.1. Perfusion

Mice were administered a lethal dose of pentobarbitol intraperitoneally. The heart rate was carefully monitored and as it slowed down the ventral abdomen was cut open and the diaphragm cut and the left atrium pierced with a needle connected to a peristaltic pump with 0.5% (v/v) paraformaldehyde. The right heart was bled out with forceps. Once the colour of the liver turned yellow, an indicator that perfusion had reached peripheral organs, the pump was turned off and the trigeminal ganglia dissected.

3.36.2.2. Embedding

Trigeminal ganglia from perfused mice were aseptically placed in 1 ml 4% (v/v) paraformaldehyde for 1 h at RT with constant mixing. This was followed by 30 min. incubation in a graded series of EtOH (90, 95%, and 100%) and two 1 h incubations in 1 ml xylene. Trigeminal ganglia were placed in a metal tray and melted paraffin was poured over the top and allowed to cool. Filling of the tray was carried out by pouring additional melted paraffin on top. A plastic tray was gently pushed on top and the unit placed on ice for 30 min. to solidify. Five μm thick sections were cut on a microtone. These were stored or deparaffinized for immediate use.

3.36.2.3. Immunohistochemistry

Slides were placed in a rack and put into a bath containing xylene for 15 min. The rack was moved to a bath containing 100% EtOH for 15 min., 70% EtOH for 15 min. and PBS-A for 15 min. The rack was placed in a bath containing H_2O to rinse the sections then transferred to a bath with MeOH (with 0.5% (v/v) H_2O_2) for 10 min. Slides were rinsed in PBS-A for 15 min and a blocking PBS-A solution (0.5% (v/v) NP40 and 5% Marvel milk) was pipetted directly onto each section and incubated in a humidified cassette for 30 min. An additional blocking step was carried out in "normal blocking serum" for 20 min. Slides were placed back in a rack and rinsed thoroughly in a PBS-A bath for 15 min. Localization of HSV antigens was performed by using a three-step biotin-avidin peroxidase system. The primary rabbit anti-HSV-1 (DAKKO) was diluted to 1/1000 in PBS-A, adsorbed with mouse liver acetone powder and pipetted directly onto each section as described above and the sections incubated for 1 h at RT. Slides were placed in a rack and washed 3 times for 15 min. in a fresh PBS-A bath. Sections were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories), absorbed with normal mouse serum diluted 1/100 with PBS-A, for 30 min. as described above. Slides were placed in a rack and washed in a fresh PBS-A bath for 15 min. 3 times. Sections were incubated in Vector® *NovaRED*™ peroxidase substrate solution for 5 min. Slides were rinsed in a H_2O bath and observed under a microscope.

Counterstaining of nuclei was performed in a bath with Mayer's Haematoxylin for 1 min. followed by rinsing in H_2O (Bancroft, 1982). Mounting of slides (Histomount) was done after taking slides through 3 graded EtOH baths (90%, 95% and 100%) for 10 min. and 2 xylene bath's for 10 min.

4. HSV-1 RESULTS AND DISCUSSION

4.1. CONSTRUCTION OF HSV-1 RECOMBINANT VIRUSES

4.1.1. Introduction

In 1983, Thompson *et al* mapped a HSV neurovirulence locus to a region in the long repeat sequences using an HSV-1/HSV-2 intertypic recombinant virus, RE6. In 1986, Chou and Roizman demonstrated that the *a* sequence contained the promoter regulatory element and transcription initiation site of a gene named RL1 in HSV-1 strain F. RL1 expresses the protein ICP34.5, which was detected using an antiserum directed against a synthetic peptide containing a PAT repeat present within ICP34.5 (Ackermann *et al.*, 1986). Several groups (see Introduction) identified that viruses which do not express ICP34.5 have a reduction in replication in certain cells *in vitro* and are avirulent upon intracranial inoculation in the murine model *in vivo*.

In 1994, Lagunoff and Roizman identified 16 open reading frames (ORF A-P) that are located in the antisense direction to RL1 within the LAT transcript in the long repeat sequences in strain F. Two ORFs (O and P) mapped almost entirely antisense to RL1 and expression of the ORF O and P proteins was subsequently demonstrated (Lagunoff and Roizman, 1995) by mutagenising the ICP4 binding site which causes repression of ORF O and P transcription.

In 1991, MacLean, A. *et al.*, isolated a spontaneous deletion variant (1716) in HSV-1 strain 17^{+} (Fig. 8) which has 759 bp of the long repeat region deleted. As the mutant 1716 lacks both RL1 and ORF P it became important to identify whether there were individual phenotypes associated with each of these genes. We hypothesized that recombinant viruses expressing either RL1 or ORF P would elucidate the individual phenotypes of each gene.

4.1.2. Construction of Mutant Viruses: 1622 and 1623

Two new recombinant viruses were constructed in a 1716 background and named 1622 and 1623. 1622 and 1623 express only ICP34.5. To generate viruses 1622 and 1623 several plasmids were sub-cloned and are described in detail below.

4.1.3. Construction of pFJ14H

The plasmid pFJ5ΔHind was altered in order to attain *lacZ* as a selection marker and the HSV-1 gD promoter to transcribe the gene of interest (Fig. 15). pFJ5ΔHind, originally constructed by Rixon and McLauchlan (1990), was digested with *SalI* and

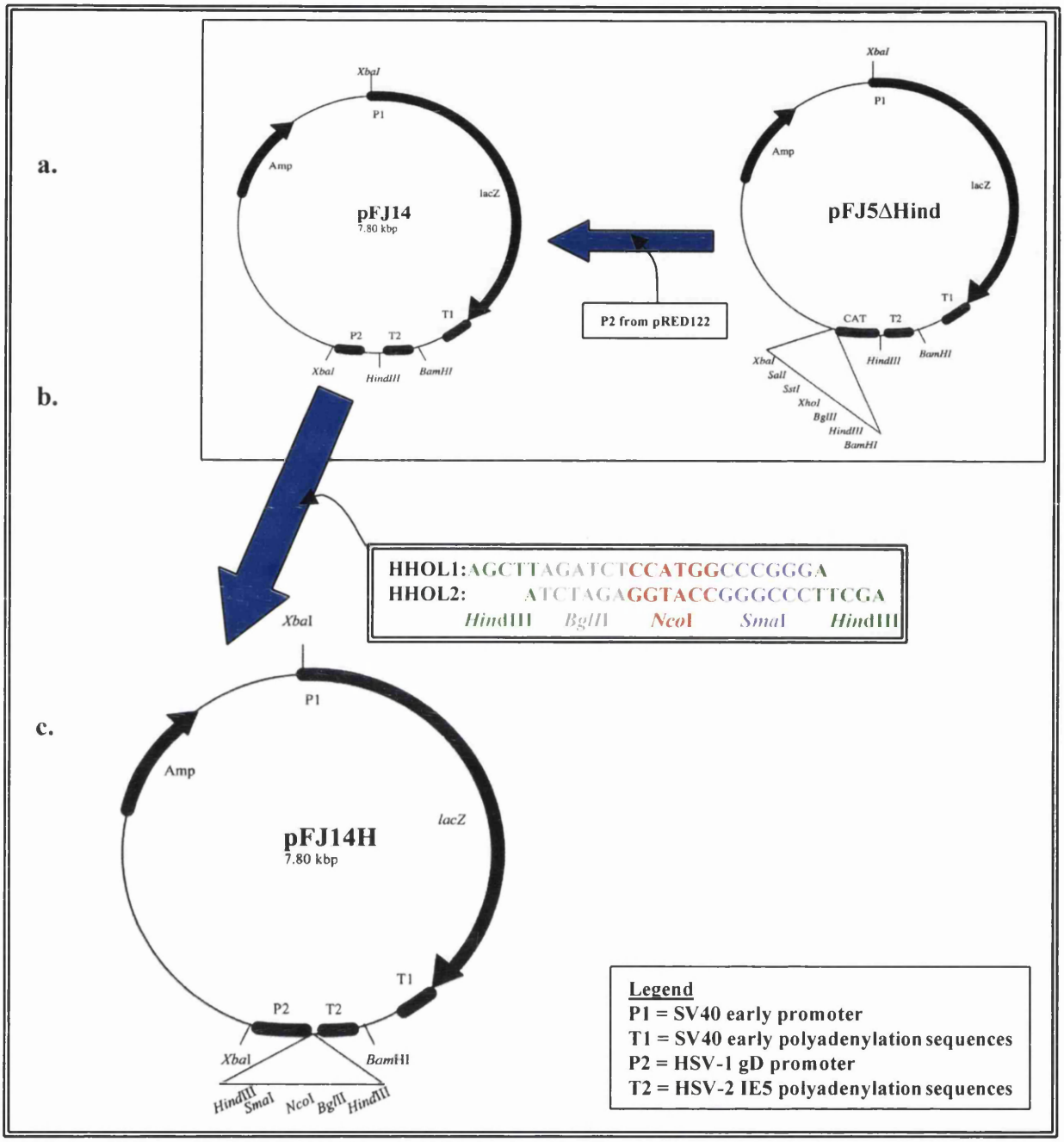


Figure 15. Construction of pFJ14H.

a. pFJ5ΔHind (Rixon and McLauchlan, 1990) was first digested with *Sal*I/*Hin*dIII to remove the CAT gene. The gD promoter was isolated from pRED122 by digestion with *Hin*dIII/*Xho*I and was ligated into pFJ5ΔHind generating pFJ14 (see text for full details). **b**. An oligonucleotide linker was synthesized whose sequence is shown. **c**. The oligonucleotide was cloned into the *Hin*dIII site to generate pFJ14H.

*Hin*dIII to remove the chloramphenicol transferase (CAT) gene (MacLean, A., unpublished) (Fig. 15a). pRED122 (Everett, 1985), was digested with *Xho*I and *Hin*dIII to isolate the HSV-1 gD promoter (403 bp) as described in Section 3.5. Ligation of the gD promoter into pFJ5ΔHind generated pFJ14 (MacLean, A., unpublished) (Fig. 15a).

Two complementary oligonucleotides containing multiple restriction enzyme sites were synthesized for sub-cloning of different genes into pFJ14. The sequence is shown in Figure 15b. The oligonucleotides were ligated into the single *Hin*dIII site of pFJ14, to yield pFJ14H (Fig. 15c).

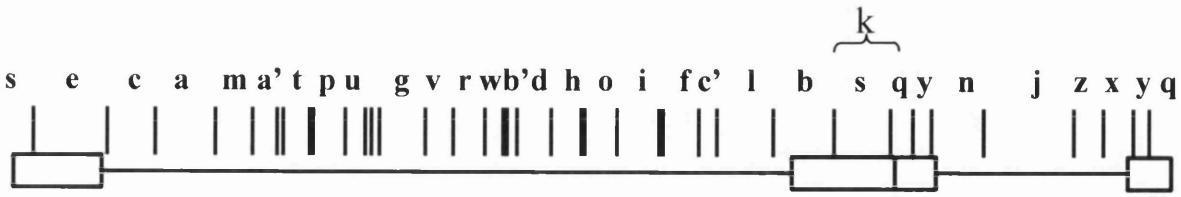
4.1.4. Cloning RL1 under the gD Promoter

The plasmid pGEM34.5 contains a 1.46 kbp *Alu*I/*Rsa*I fragment (125074 to 126530 n.p.) containing the RL1 and ORF P sequence. pGEM34.5 was originally cloned by McKie (1994). The RL1 sequence was derived from the HSV-1 *Bam*HI'k' fragment pBAM'k'/pAT153 (Davison, 1981) (Fig. 16). Two different fragment lengths of RL1 were obtained by restriction enzyme digestion and isolation by a 1% (w/v) agarose gel.

First, the 784 bp ORF of RL1 was obtained by a *Nco*I (n.p. 125859) and *Bam*HI (from pGEM34.5) digest of pGEM34.5 (Fig. 17a,e). Second, a larger RL1 fragment containing 134 bp upstream of the RL1 ORF sequence, including the 5' untranslated leader and part of promoter (Chou and Roizman, 1986) was obtained by digesting pGEM34.5 with *Dra*I (125985 n.p.) and *Bam*HI (from pGEM34.5; Fig. 17a). The difference between the predicted fragment length from the HSV-1 restriction enzyme n.p. and the actual fragment length is due to additional base pairs from pGEM34.5 which were acquired by using the *Bam*HI site downstream of *Alu*I.

The two RL1 and ORF P fragments were individually sub-cloned into pFJ14H using compatible restriction enzyme sites. In order to successfully clone the 784 bp RL1 fragment an extra step was needed. Because pFJ14H contains two *Nco*I sites it was partially digested (Fig. 18a, b). Ligation of these genes into pFJ14H positioned the gene immediately downstream of the HSV-1 gD promoter and directly upstream of the HSV-2 immediately early IE5 polyadenylation sequences. pFJ14H contains *lacZ* under the control of a SV40 promoter with its own SV40 polyadenylated sequence (Fig. 19). The *lacZ* gene is in the opposite orientation as the inserted RL1 genes. pFJ14H derived plasmids were digested with *Xba*I and RL1 or ORF P with *lacZ* were isolated from pFJ14H and gel purified.

a.



b.

*Bam*HI fragments	Molecular size (kbp)
a	12474
b	10137
c	9835
d	9273
e	8913
f	8055
g	7774
h	7539
i	6640
j	6457
k	5944
l	5372
m	5105
n	4890
o	3866
pq	3579/3436
r	3365
s t	2907/2848
uv	2294/2291
w	2210
xyz	1953/1840/1841
a'	1544
b'	1313
c'	928
d'	863
e'	797
f'g'	767/764
h'	717
i'	662
j'	517
k'	220
l'	156
m'	156
n'	90

Figure 16. Schematic representation of the HSV-1 genome *Bam*HI sites with molecular sizes.

a. A schematic representation of the forty *Bam*HI sites within the HSV-1 sequence (Davison, 1981). **b**. A quick reference to the molecular weights of each fragment. Several of the fragments are so small they are indistinguishable by regular agarose gel methods The fragments d' through n' (□) are not represented in part **a**.

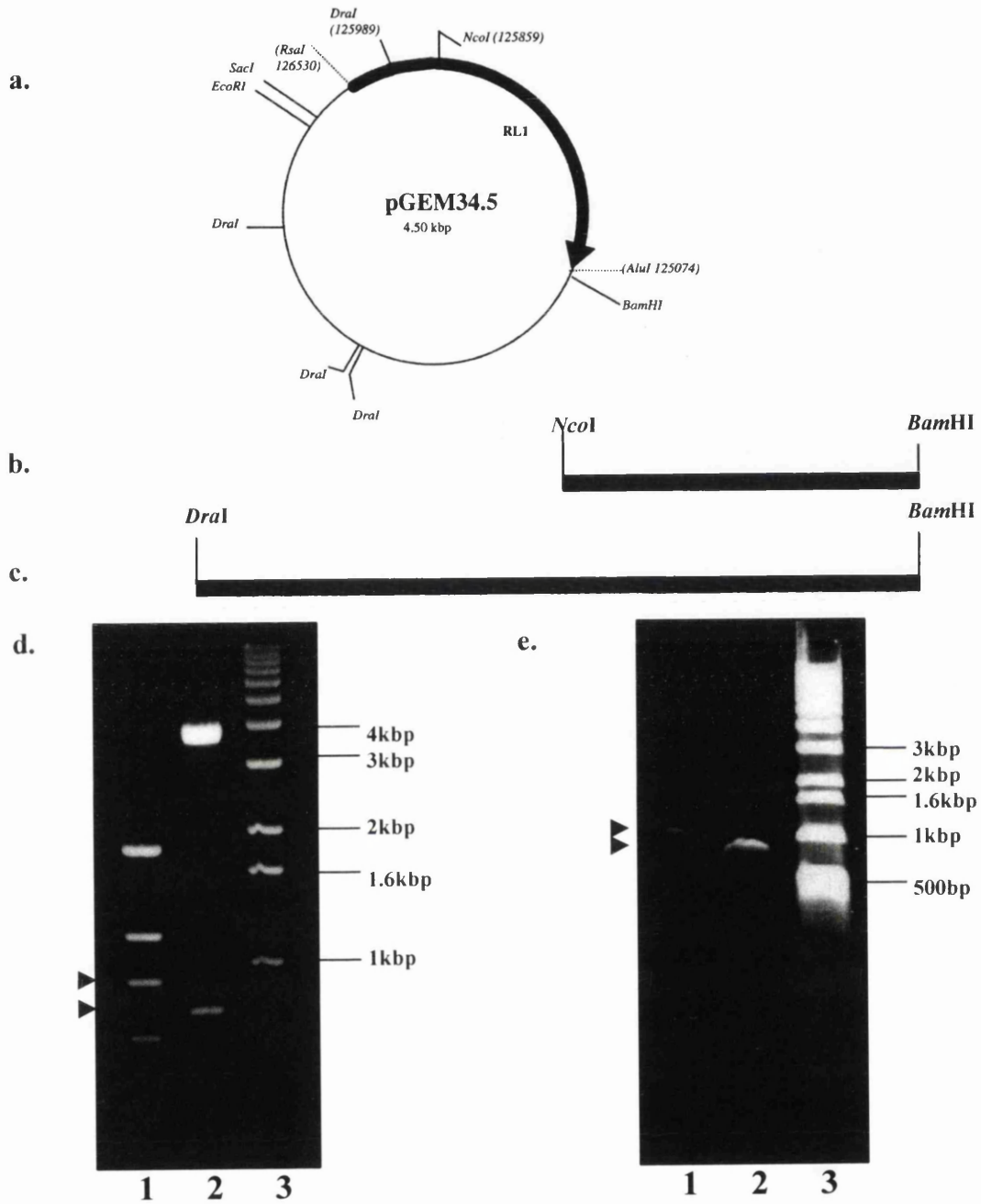


Figure 17. A schematic representation of RL1 cloning.

a. pGEM34.5 containing RL1 from HSV-1 was digested either with *Nco*I and *Bam*HI yielding a 784 bp fragment as depicted in (**b**) or with *Dra*I and *Bam*HI yielding a 918 bp fragment as depicted in (**c**). **d**. The digests were initially electrophoresed on a 1% (w/v) agarose gel with a 1 kbp DNA ladder (lane 3) and the desired fragments (►)were gel purified. Lane 1 shows the pGEM34.5 *Bam*HI and *Dra*I digest profile with 4 fragments. Lane 2 shows the pGEM34.5 *Bam*HI and *Nco*I digest profile with 2 fragments. **e**. The purified RL1 fragments, lane 1, 918 bp RL1 fragment, lane 2, 784 bp fragment and lane 3, 1 kbp DNA ladder for size assessment. These fragments were sub-cloned into pFJ14H.

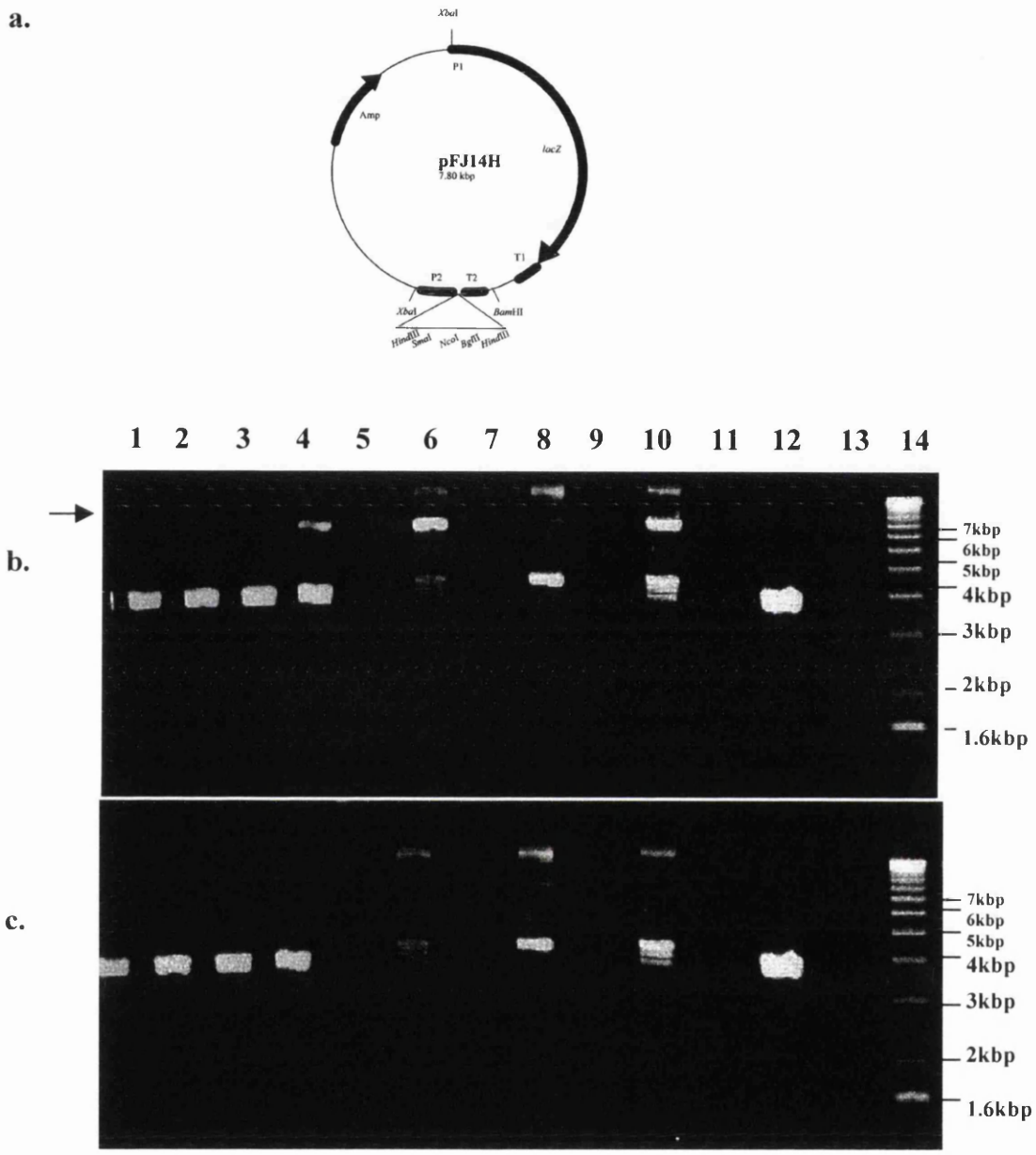


Figure 18. Partial *Nco*I digestion of pFJ14H using EtBr.

a. Schematic representation of *Nco*I sites in pFJ14H. **b**. The full range of *Nco*I digestion patterns in pFJ14H electrophoresed through a 1% (w/v) agarose EtBr stained gel. Lanes 4 through 12 have varying partial digestions from a 1 h incubation with EtBr. Lanes 1-3 and 12 are complete digestions of pFJ14H. Lanes 6, 8 and 10 are the best isolates of the correct 7.8 kbp linear pFJ14H. Lane 14 is a 1 kbp DNA ladder giving correct size comparisons. **c**. The linear pFJ14H DNA was cut out and purified. Amount of EtBr for partial digestion were as follows: lanes 1-3, 0 μg; lane 4, 0.1 μg; lane 6, 0.5 μg; lane 8, 1 μg; lane 10, 2 μg; lane 12, 5 μg.

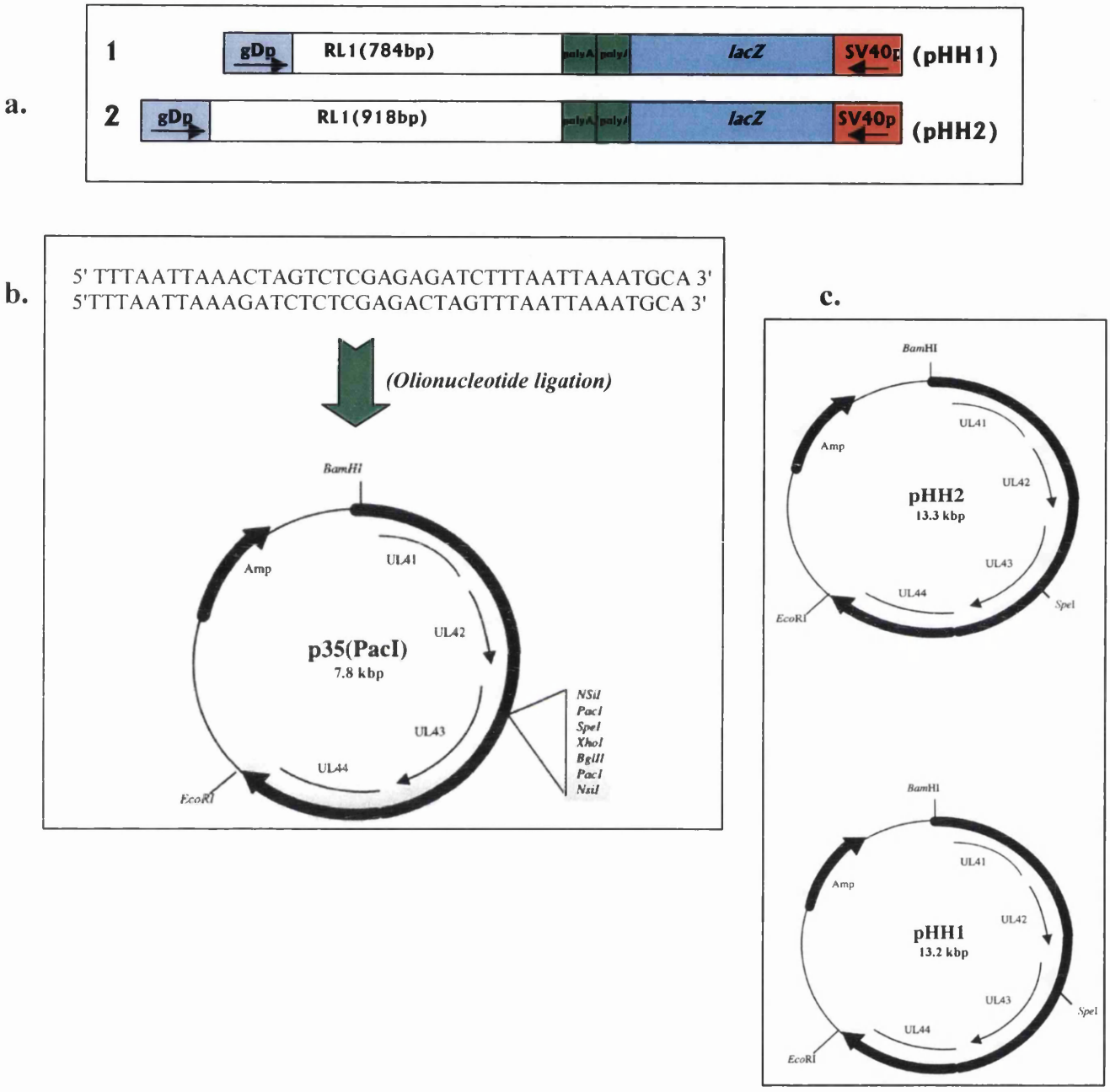


Figure 19. Diagram of pHH1 and pHH2.

a. Fragments containing the gene of interest, RL1 784 bp (1), or RL1 918 bp (2) were separately digested from pFJ14H with *Xba*I ligated into the *Spe*I site of p35(PacI). **b**. Ligation of oligonucleotides which were synthesized for additional cloning sites in the UL43 *Nsi*I locus in p35 (MacLean, C. *et al*., 1991) to generate p35(PacI). **c**. The p35(PacI) containing different *Xba*I inserts were named pHH1 and pHH2.

4.1.5. Sub-cloning pFJ14H RL1 Fragments into p35(PacI)

In 1991, MacLean C. *et al.*, cloned a 5.1 kbp (n.p. 91610- n.p. 96751) *Bam*HI-*Eco*RI fragment containing HSV-1 genes UL41 through UL45, into pUC19, to generate p35. This plasmid was digested at the *Nsi*I site located within the UL43 gene (n.p. 94911) and a double stranded oligonucleotide linker (Fig. 19a) was inserted to generate plasmid p35(PacI) (Fig. 19b. (McGregor *et al., in press*). p35(PacI) was digested with *Spe*I (in linker; compatible with *Xba*I) allowing the RL1/ *lacZ* cassettes from pFJ14H to be ligated, generating plasmids pHH1 and pHH2 (Fig. 19c).

4.1.6. Generation of 1622 and 1623

Plasmids pHH1 and pHH2 were digested with *Xmn*I and co-transfected with 1716 DNA onto BHK cells, as described in Section 3.19, to generate new recombinant viruses 1622 and 1623, respectively (Fig. 20). The transfection plates were incubated until cpe was complete. The infected cells were centrifuged, virus released by sonication, and titrated on BHK cell monolayers. Two days post titration the media was removed and a X-gal overlay applied. Detection by X-gal staining allowed several individual recombinant virus plaques to be isolated and taken through two to three further rounds of plaque purification (Section 3.15). 1622 and 1623 DNA from each plaque isolate was screened by digestion with *Bam*HI and Southern blotting (Fig. 21). 1622 has an extra *Bam*HI site from the pFJ14H insert in p35(PacI) generating fragments of 7.8 kbp and 4.5 kbp (Fig. 21a, lanes 1-4). 1623 contains a *Bam*HI site from the pFJ14H insert in p35(PacI) generating fragments of 4.6 kbp and 7.8 kbp (Fig. 21b, lanes 1-8). There is a slight variation in size from 4.5 kbp in 1622 to 4.6 kbp in 1623 due to the different lengths of RL1 (134 bp).

By the third round of plaque purification, recombinant viruses were completely pure and a ten roller bottle stock of 1622 and 1623 was grown on BHK cells (Section 3.13). A Southern blot was carried out to verify purity and DNA integrity was maintained after growth of stocks (Fig. 23). Wild type, 17^{+} and the RL1 deletion virus, 1716, have one *Bam*HI fragment of 6.6 kbp spanning the UL41 to UL44 sequence, when digested and probed with [^{32}P] radiolabelled p35(PacI) (Fig. 22). 1622 and 1623 have a *Bam*HI site from pFJ14H within the HSV sequence in p35(PacI) generating two fragments as previously described (Fig. 22).

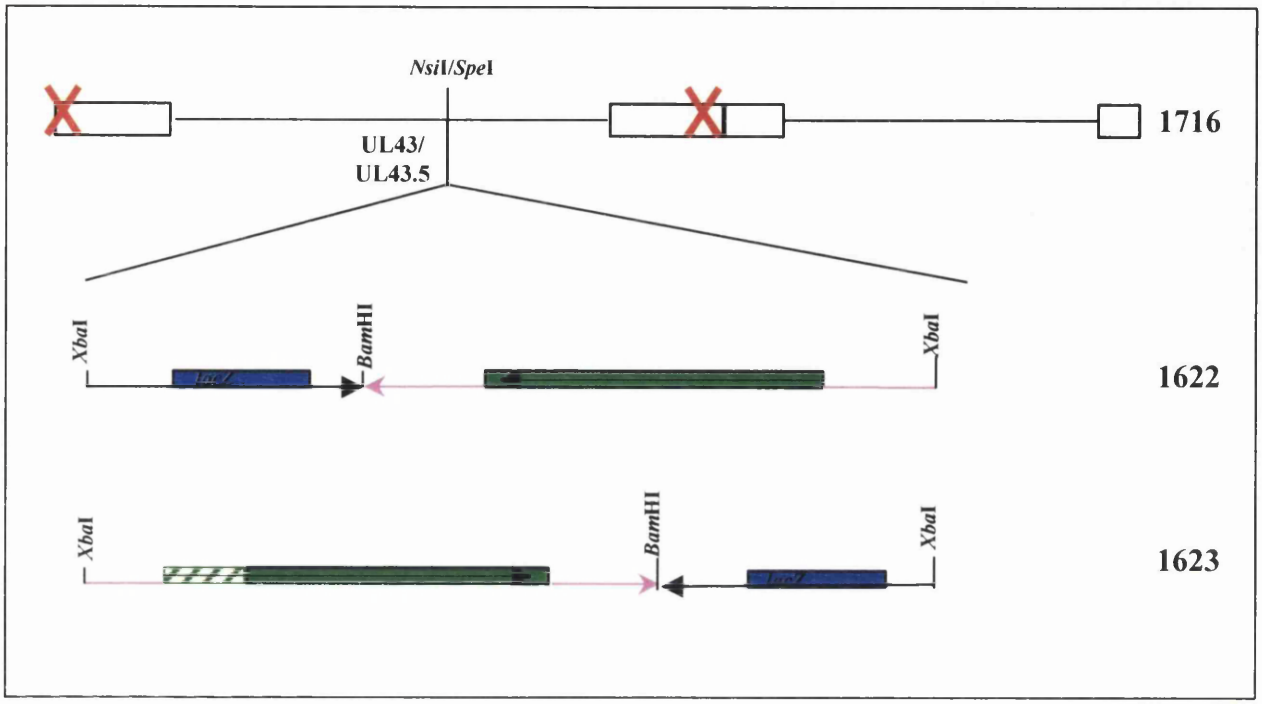


Figure 20. Schematic representation of recombinant viruses.

1716 was used as the parental strain for the listed recombinant viruses. 1622 has the HSV-1 RL1 open reading frame. 1623 has the RL1 ORF and 134 bp upstream sequence. The basic construct depicts that these genes have been inserted downstream of the HSV-1 gD promoter and upstream of the HSV-2 IE5 polyadenylation sequence. These recombinant viruses also have *lacZ* in the opposite orientation for selection upon X-gal staining. [X Denotes the RL1 deletion in 1716.]

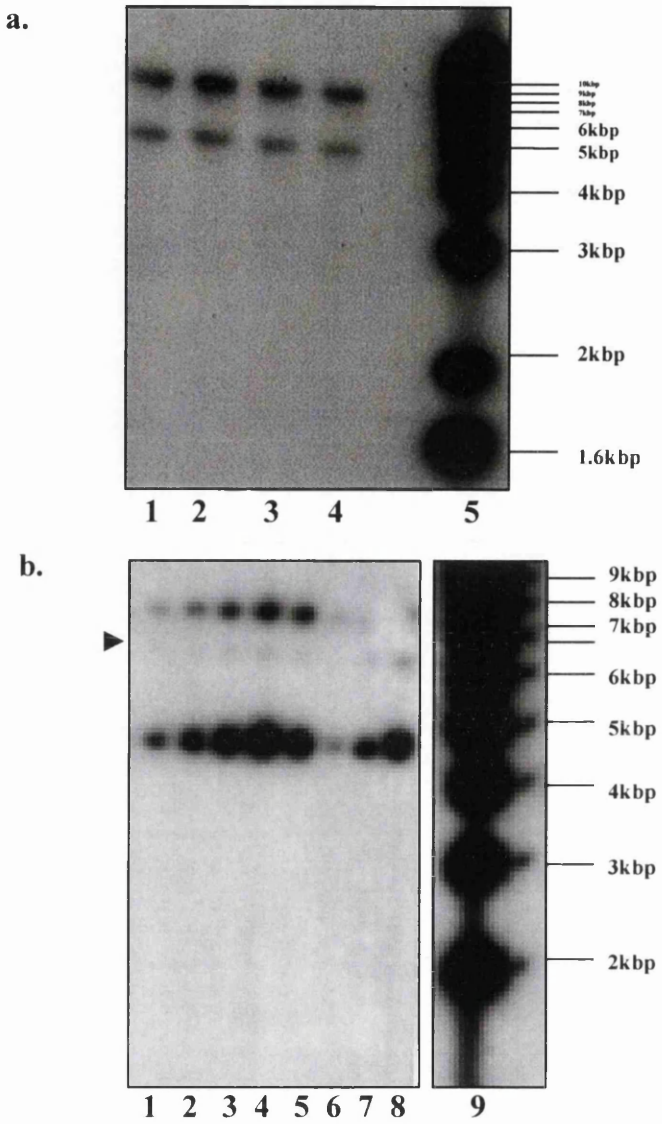


Figure 21. Southern blots of 1622 and 1623 plaque purification

a. DNA from four 1622 plaque isolates (lanes 1-4) was digested with *Bam*HI, electrophoresed through a 1% (w/v) agarose gel, Southern blotted and probed with ^{32}P labelled p35(PacI). The nitrocellulose was exposed to an autoradiograph o/n at -70°C. Band sizes are based on the 1 kbp DNA ladder (lane 5). **b**. DNA from 1623 plaque isolates was analysed by *Bam*HI digestion. Eight plaque isolates (lanes 1-8) show the presence of the wild type 6.6 kbp band (►) and the two recombinant bands of 4.6 kbp and 7.8 kbp. The fragment sizes were based on comparison with a 1 kbp DNA ladder (lane 9).

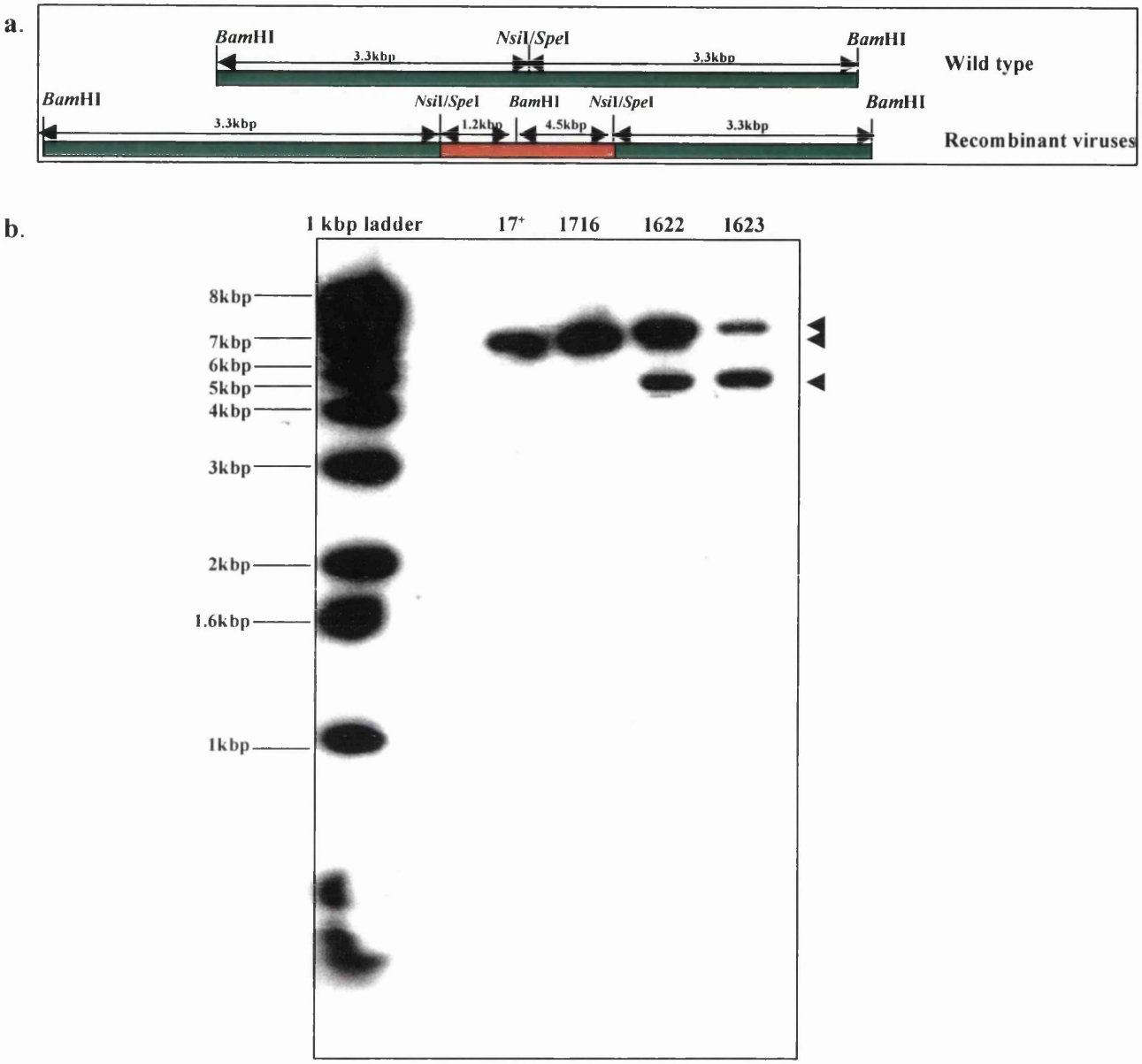


Figure 22. Southern blot of viral DNA digested with *Bam*HI.

a. A linear diagram illustrating the UL43 sequence in wild type and in recombinant viruses and sizes of *Bam*HI fragments following digestion.
b. A Southern blot of DNA prepared from recombinant viruses, 17^+ and 1716, stocks. This Southern blot shows DNA from virus stocks digested with *Bam*HI, probed with [^{32}P]dCTP p35(PacI) and exposed to an auto-radiograph for 5 h at -70°C. The single 6.6 kbp band in 17^+ and 1716 is the wild type *Bam*HI fragment from UL41 to UL44. The recombinant viruses 1622 and 1623 have RL1 inserts in the UL43 locus which introduces a new *Bam*HI site that cuts within the insert generating two bands, 4.6 kbp and 7.8 kbp. A 1 kbp DNA ladder is also shown to give accurate size comparisons.

4.1.7. Discussion

Before recombinant viruses could be constructed sub-cloning was carried out. The first step required removing the CAT gene from pFJ5ΔHind and insertion of the HSV-1 gD promoter (P2) generating pFJ14. pFJ14 contained all the genetic components needed but could not easily be used to clone the RL1 gene because it did not contain compatible restriction enzyme sites. Two oligonucleotide linkers with additional restriction enzyme sequences was synthesised and inserted into the *Hin*dIII locus of pFJ14. The linker with extra restriction enzymes gave pFJ14 the new name pFJ14H.

*Nco*I was needed for the 5' end of the 784 bp RL1 fragment. This created the complexity of having two *Nco*I sites within the pFJ14H plasmid. As a result a partial digest was carried out. The linker also contained a *Sma*I site which leaves blunt ends and yielded compatible ends for the 5' *Dra*I end of the 918 bp RL1 sequence. The plasmid pHH1 contained the 784 bp RL1 sequence which was co-transfected with 1716 DNA to generate the recombinant virus 1622. The plasmid pHH2 contained the 918 bp RL1 sequence which was co-transfected with 1716 DNA to generate recombinant virus 1623. These viruses are characterised in the subsequent sections.

4.2. CHARACTERIZATION OF 1622

4.2.1. Kinetics of ICP34.5 Expression from 1622 Infection

Although published data states that ICP34.5 is synthesised late during infection, this laboratory has demonstrated (Harland, personal communication; this section) that wild type HSV-1 17^+, expresses ICP34.5 as soon as 2 h pi. Following Southern blot confirmation that the structure of the recombinant virus, 1622, was correct, the kinetics and level of ICP34.5 expression were analysed. Western blot experiments confirm that 17^+ and 1622 express ICP34.5 at detectable levels by 2 h pi in BHK cells (Fig. 23, 24).

The levels of ICP34.5 are similar between 1622 and 17^+ up to 12 h pi (Fig. 23, 24). However, by 16 to 24 h pi there is an accumulation of ICP34.5 in 1622 infected BHK cells (Fig. 24). These Western blot results were obtained using a polyclonal antiserum, 137, which was raised against a GST/ICP34.5 fusion protein (Brown *et al.*, 1997). There was no ICP34.5 detected in 1716 infected extracts, however there are two virally induced proteins, one of 70 kDa and a second of 40 kDa detected by 137. It is not known whether these proteins are cellular or viral but they appear by 6 h pi in 17^+ and 1716 (Fig. 23) and by 12 h in 17^+, 1716 and 1622 (Fig. 24). To determine if 137 had a higher affinity for ICP34.5 expressed in 1622, different antisera were used as described in the next section.

4.2.2. Western blot Analysis of ICP34.5 from 1622 Infection

Another polyclonal antiserum, R1, generated by the same method as 137 (Brown *et al.*, 1997), used in Western blots, showed a higher level of expression of ICP34.5 in 1622 compared with 17^+ or strain F (Fig. 25a).

The ICP34.5 sequence analysis between HSV-1 strains has identified that HSV-1 strain F has 10 copies of PAT repeat, strain 17^+ has 5, and strain KOS has 3. Migration of ICP34.5 by SDS-PAGE consequently varies according to the number of repeats. An antiserum raised against a synthetic peptide, PAT_{10}, was also used in Western blots (McKay, *et al.*, 1993). This antiserum consistently reacts with and recognises ICP34.5 in HSV-1 strain F well because of the 10 PAT repeats. Anti-$(PAT)_{10}$serum does not consistently recognise ICP34.5 in 17^+ because it only has 5 PAT repeats. Western blots using anti-$(PAT)_{10}$ serum again revealed that 1622 over-expresses ICP34.5 compared to strain F and 17^+ (Fig. 25b).

Two-fold serial dilutions were made of 1622 infected BHK cells 16 h pi to ascertain a semi-quantitative level of ICP34.5 expression compared to ICP34.5 expression from 17^+ (Fig. 26). This demonstrated that the level of ICP34.5 at the 1 to 8 dilution of

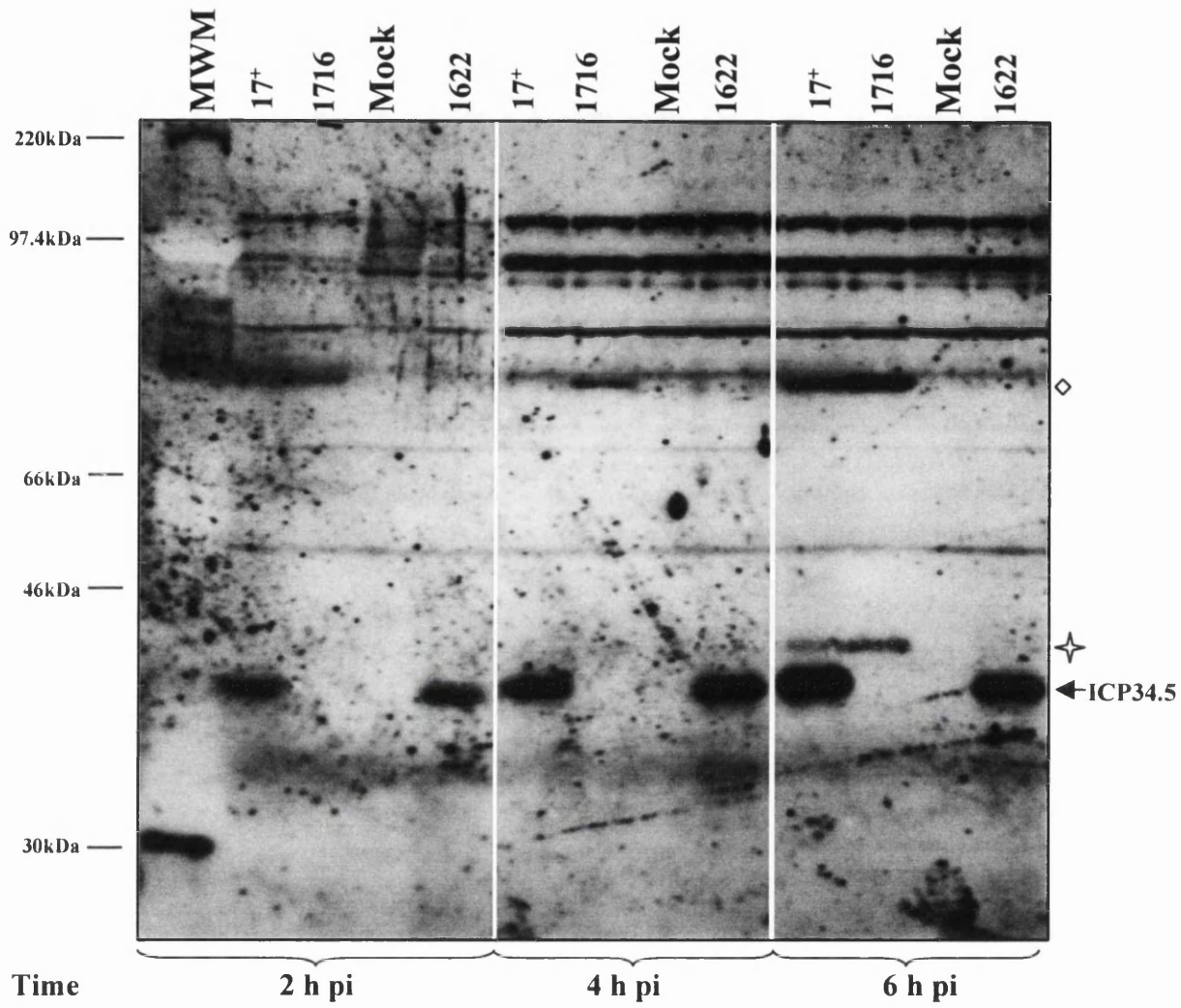


Figure 23. Western blot of ICP34.5 from infected BHK cells 2 to 6 h pi.

BHK cells were infected with viruses at a m.o.i. of 10 pfu/cell. Analysis of ICP34.5 expression was carried out on a 10% SDS-PAGE which was Western blotted with 137 antiserum and Protein A-HRP, reacted with ECL and exposed to an autoradiograph. A Rainbow™ high molecular weight marker (MWM) is shown. 17^+ and 1622 show approximately the same level of ICP34.5 accumulation at 2 h pi. The level of ICP34.5 increases by 4 h pi in both 17^+ and 1622 , and again by 6 h pi. 137 recognises two proteins induced by 1716 and 17^+ which migrate at 70 kDa (◇) and 40 kDa (✧).

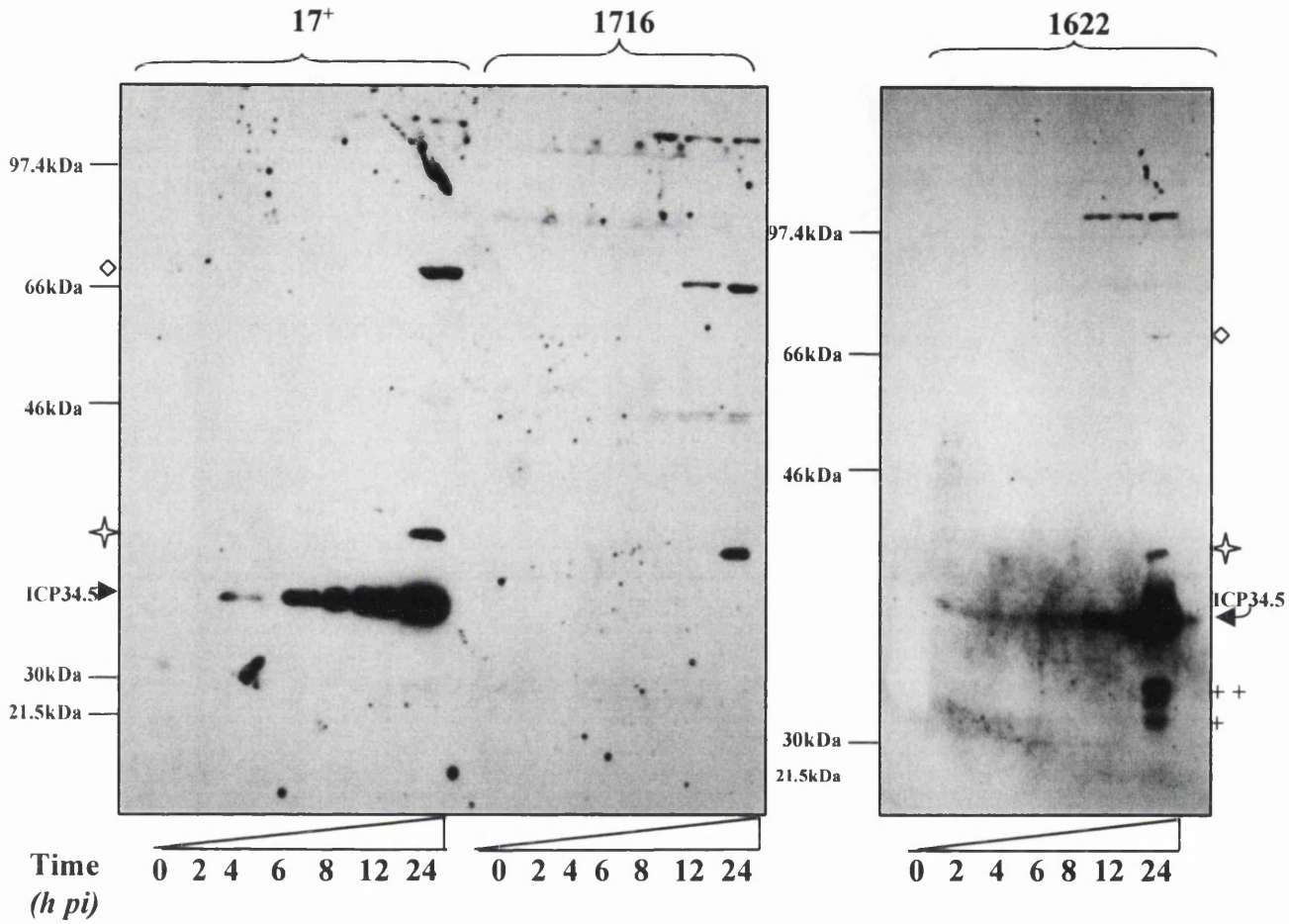


Figure 24. Western blot time course of ICP34.5 expression.

BHK cells were infected with viruses at a m.o.i. of 10 pfu/cell. Cell extracts were analysed using 10% SDS-PAGE and Western blotting with 137, Protein A-HRP and reacted with ECL. The onset of ICP34.5 expression is detectable by Western blot between 2 h pi and 4 h pi in 17^+ and 1622. Two proteins which have not yet been identified and react strongly with 137 and migrate at 70 kDa (◇) and 40 kDa (✧). There are additional bands from the 1622 infection at 24 h pi (++ and +).

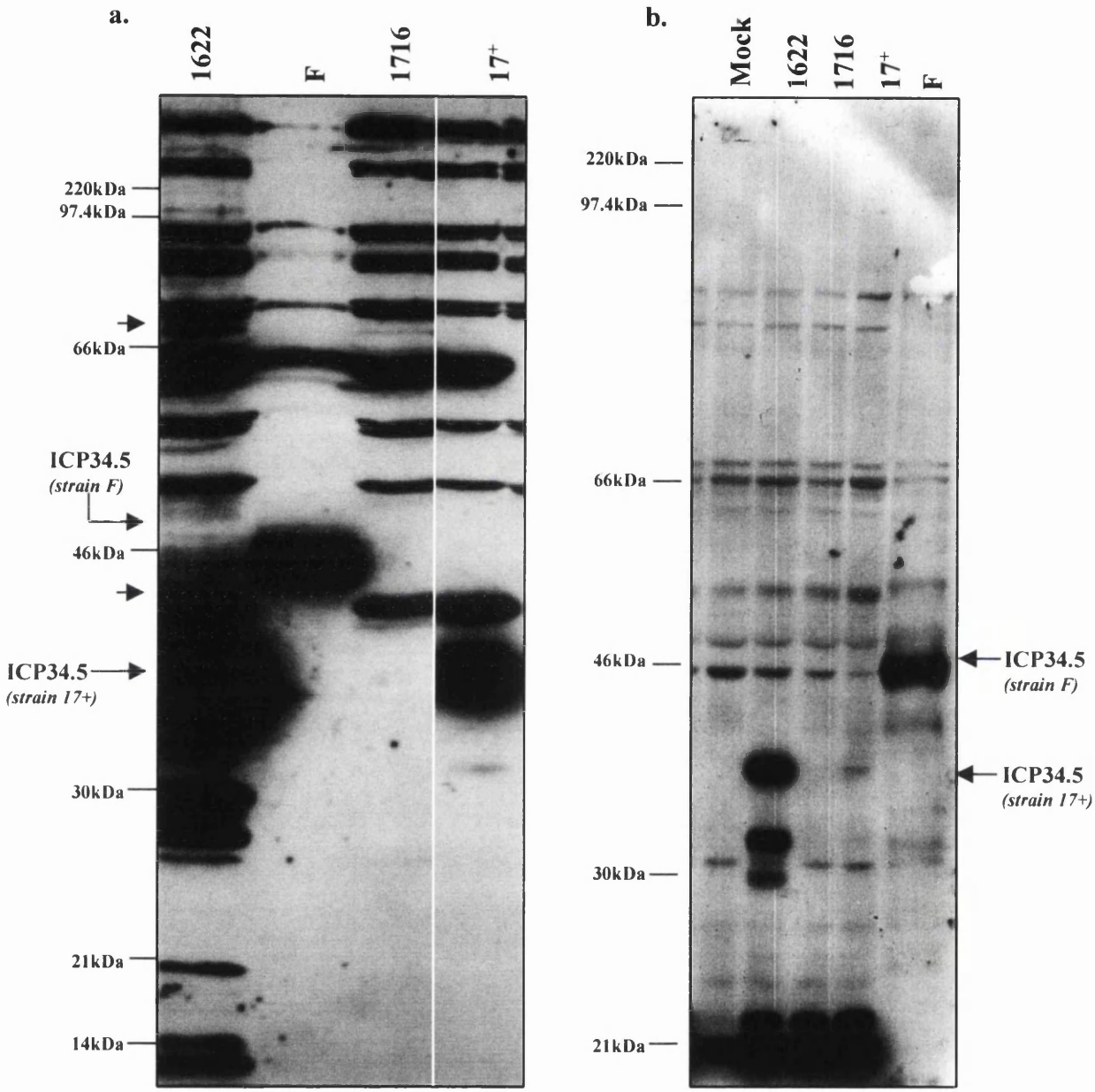


Figure 25. ICP34.5 expression by Western blot using different antisera.

BHK cells were infected with viruses at m.o.i. of 10 pfu/cell. Whole cell extracts were harvested at 16 h pi and analysed using a 10% SDS-PAGE, Western blotted and reacted with different ICP34.5 antisera. **a**. Western blot using R1 antiserum against GST/RL1 ORF fusion protein shows over-expression of ICP34.5 from 1622 compared with F and 17^+. **b**. Western blot using $(PAT)_{10}$ polypeptide antiserum generated against a synthetic peptide of 10 (PAT) repeats naturally occurring in HSV-1 strain F.

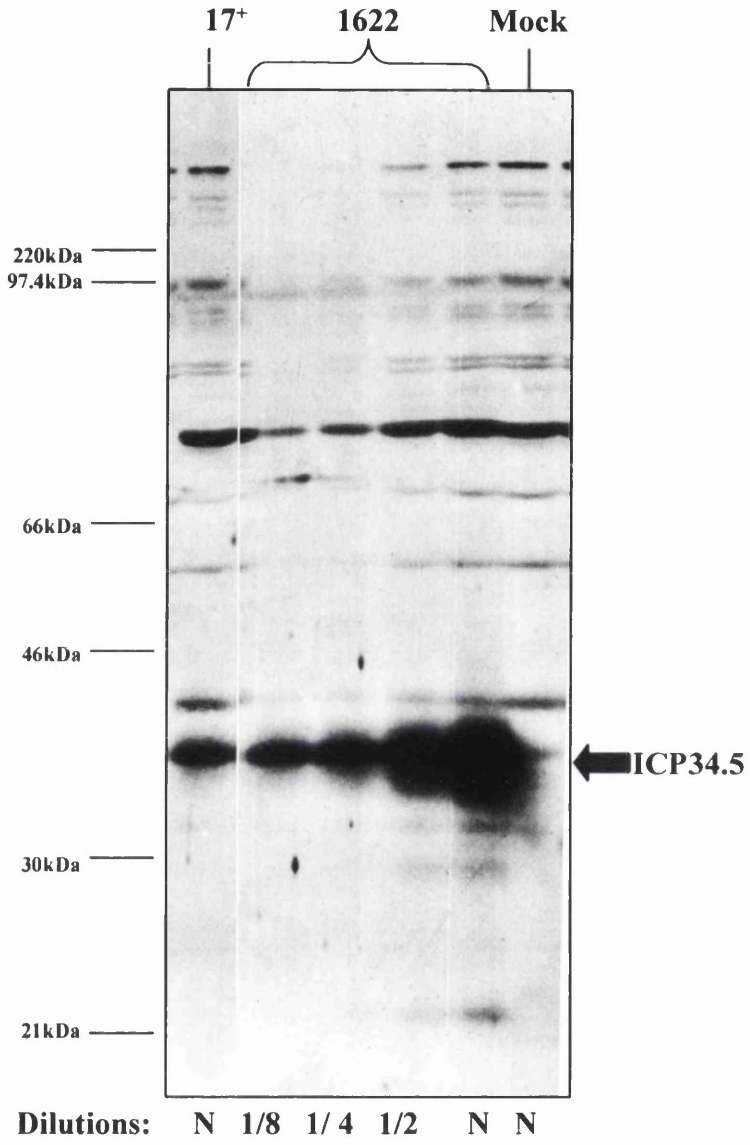


Figure 26. Semi-quantitative comparison of ICP34.5 from 1622 and 17^+ infected BHK cells 16 h post infection.

A serial 2-fold dilution was made of 1622 infected BHK cell extracts 16 h pi. 17^+ and 1622 infections were carried out at a m.o.i. of 10 pfu/cell. Samples were analysed using 10% SDS-PAGE Western blotted and reacted with 137. The dilution showing the closest level of ICP34.5 to 17^+ from 1622 is 1 in 8.

1622 was close to the neat 17^+ infected extract. This result yields an estimation that 1622 expresses approximately 8 times more ICP34.5 than 17^+.

As a further test that ICP34.5 expression in 1622 was genuinely more than in 17^+ and not a reflection of a better infection, a Western blot was carried out using an antiserum to another HSV protein. Expression of the 65 kDa DNA binding protein (UL42) was analysed by Western blot (Fig. 27). The infected cell extracts on this gel are the same neat extracts that were used in the Western blot of ICP34.5 in the previous figure (Fig. 27). The level of UL42 expression is slightly higher in 17^+ compared to 1716, 1622 and F. This suggests that the overexpresssion of ICP34.5 from 1622 is genuine and not a reflection of a better infection. It also shows that the level of another viral protein is similar between wild type 17^+, 1716 and 1622.

4.2.3. Detection of Native ICP34.5 Expression

To look at expression of ICP34.5 from 1622 infection in a more native form *in vitro*, two experiments were carried out: 1) immunopreciptation and 2) blot renaturation.

4.2.3.1. Immunoprecipitation of ICP34.5 from 1622 Infection

To examine the expression level of ICP34.5 from 1622 an IP was carried out using 137. Two different cell types, BHK and 3T6, were examined (Fig 28 and 29). A brief explanation of the fractions kept during IP will be given here followed by Western blot data. Cells were infected with 17^+, 1716, 1622 and mock infected in duplicate at a m.o.i. of 10 pfu/cell and harvested at 16 h pi. One set of infections was exposed to [^{35}S]methionine containing media from 14 to 16 h pi. The second set of infected plates was unlabelled. All infected and mock infected cells were harvested and immunoprecipitated with 137 as described in Section 3.29. There were five fractions kept and visualised on these autoradiographs. First a fraction of the infected whole cell extract (WCE). Second, following lysis of the cells a fraction of the cellular pellet was kept in case ICP34.5 was insoluble and protein was lost in the debris (PELLET). Third, was a fraction of the supernatant which did not bind to the Ab (SN). Fourth, an IP was performed on a sub-fraction of the pellet (PELLET BEADS). The fifth fraction was an IP performed with the original supernatant (SN beads) (as per protocol; Section 3.29).

BHK infected extracts were immunoprecipitated with 137 (Fig. 28). The samples which had been labelled with [^{35}S]methionine were analysed using a 10% SDS-PAGE. A

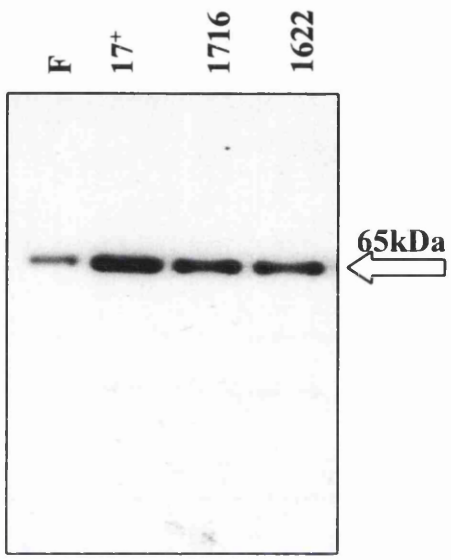


Figure 27. Western blot of BHK infected whole cell extracts using an anti-UL42 serum.

A monoclonal antibody against the UL42 65 kDa DNA binding protein was used in a Western blot to confirm that the virus infections were equal and accuracy of loading.There was no alteration in the expression of this protein in the recombinant viruses. These were the same extracts used in Figure 24.

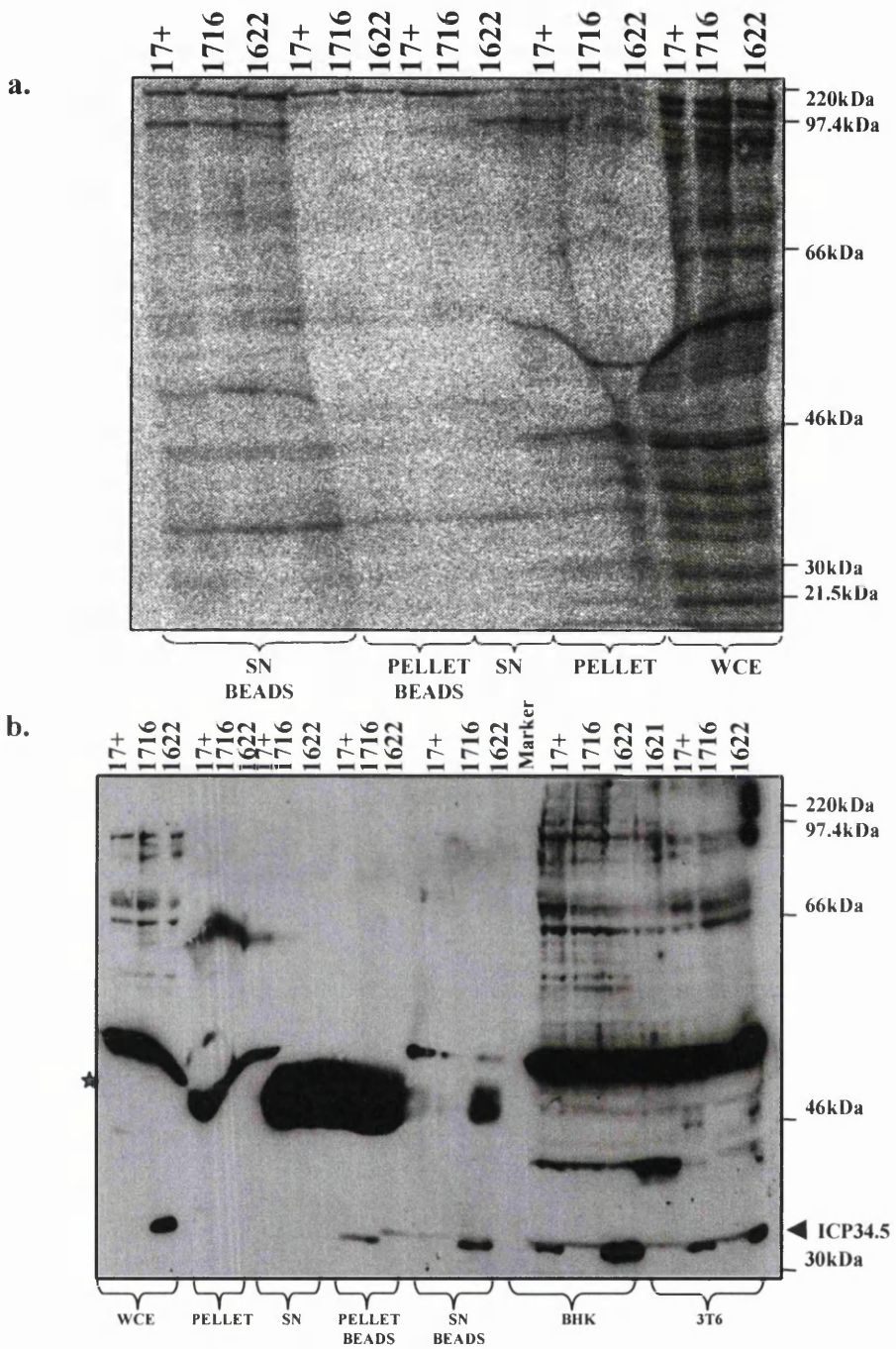


Figure 28. Immunoprecipitation of infected BHK cell extracts using 137.

Samples were electrophoresed through a 10% SDS-PAGE. One set of samples were radiolabelled with ^{35}[S]methionine until 16 h pi (**a**), the second set of samples were not radiolabelled (**b**). **a**. The gel containing radioactive samples was dried down and exposed to a phospho-imaging screen o/n. **b**. The gel containing non-radioactive IP samples was Western blotted with 137. A fraction of the infected extracts was saved directly after harvest (WCE). The harvested samples were lysed and a fraction of the cellular pellet kept for analysis (PELLET). A fraction of the supernatant which did not bind to beads was kept (SN). Sepharose beads were added to another fraction of the initial pellet (PELLET BEADS) and the lysed supernatant (SN BEADS). Separate infected BHK and 3T6 cells were included in this blot.★ Indicates the strong recognition of the heavy chain IgG

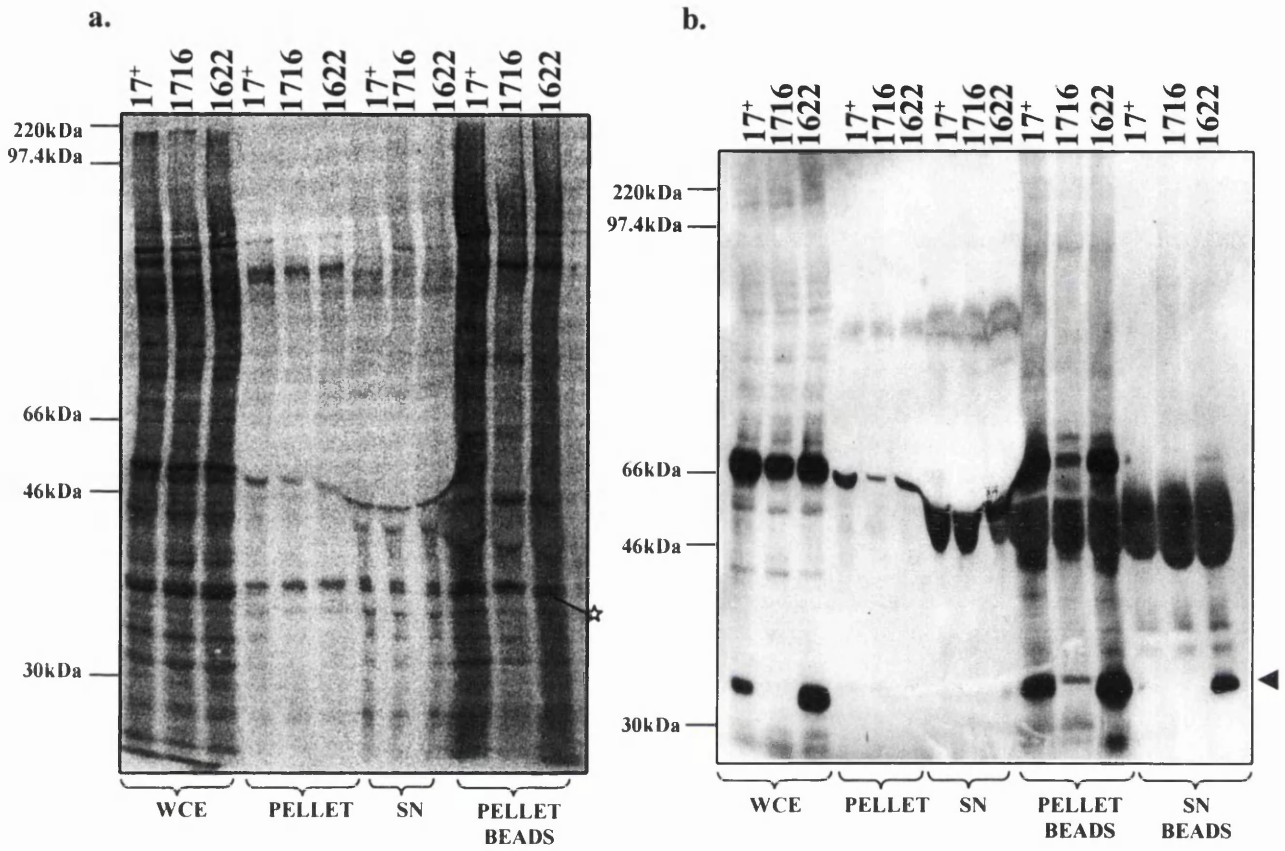


Figure 29. Immunoprecipitation of infected 3T6 cell extracts using 137.

3T6 cells were infected with a m.o.i. of 10 pfu/cell in duplicate and harvested 16 h pi for IP. One set of samples were radiolabelled with ^{35}[S]methionine for 14-16 h pi and immunoprecipitated with 137 (**a**). The second set of samples was not exposed to radioactivity but immunoprecipitated and used in a Western blot with 137 (**b**).
a. The gel containing radioactive samples was dried down and exposed to a phospho-imaging screen o/n. ICP34.5 (☆) was identified from 17$^+$ and 1622 infected cells from the IP, PELLET BEADS fraction. A cellular band migrates closely to ICP34.5 in 17+, 1716 and 162
b. The gel containing non-radioactive samples was blotted using 137. A fraction of the WCE was saved directly after harvest. The harvested samples were lysed and a fraction of the cellular pellet kept for analysis (PELLET). Sepharose beads were added to another fraction of the initial pellet (PELLET BEADS) and the lysed supernatant (SN BEADS). ICP34.5 from 1622 is detected in the SN BEADS fraction (◀).

dried gel containing electrophoretically separated radiolabelled samples was exposed to a Phospho-imaging screen overnight. Autoradiography of the IP using radiolabelled infected BHK cell extracts showed very little incorporation of [^{35}S]methionine and no specific precipitation of ICP34.5 (Fig. 28a). Samples which were not labelled with [^{35}S]methionine were analysed by 10% SDS-PAGE and Western blotted with 137. The Western blot shows precipitation of ICP34.5 in 1622 (SN BEADS) but not in 17^+ and 1716 infected cells (Fig. 28b). Recognition of ICP34.5 was detected from separate 17^+ and 1622 infected BHK and 3T6 cell extracts (BHK and 3T6, respectively).

3T6 cells were immunoprecipitated as described for BHK cells above. Infected 3T6 cells were analysed using a 10% SDS-PAGE, the gel was dried and exposed to a Phospho-imaging screen o/n. There was little incorporation of [^{35}S]methionine from infected 3T6 cells (Fig. 29a). It appears that there may be a small amount of [^{35}S]methionine labelled ICP34.5 detected in the 17^+ and 1622 (PELLET BEADS), samples which were immunoprecipitated. A cellular protein migrates very closely to ICP34.5 and is present in 17^+, 1716 and 1622 (Fig. 29a).

Samples which were not labelled with [^{35}S]methionine were immunoprecipitated and Western blotted with 137. The Western blot shows recognition of ICP34.5 in 1622 and 17^+ but not in 1716 in the WCE and the PELLET BEADS fraction. A protein migrating at 70 kDa is also recognized in 17^+, 1716 and 1622 in the WCE, but only from 17^+ and 1622 in the PELLET BEADS fraction (Fig 29b). Expression of ICP34.5 from 1622 is greater than in 17^+. 1622 is also recognised in the SN BEADS fraction (Fig. 28b).

4.2.3.2. Immunofluorescence using 137 Antiserum

The polyclonal antiserum 137 was used in immunofluorescence experiments to visualise and localise ICP34.5 from 1622 infection *in vitro* (Fig. 30). However, there was no specific identification of ICP34.5. A distinct punctate nuclear staining is present in 17^+ and 1622 but also was detected in mock and 1716.

4.2.3.3. Western blot Renaturation of ICP34.5 from 1622 Infection

Western blot renaturation of ICP34.5 from 1622 infected cells was also performed to examine ICP34.5 expression. BHK cells were infected with 17^+, 1716 or 1622 at a m.o.i. of 10 pfu/cell and harvested 16 h pi in SDS lysis buffer. Samples were completely denatured and analysed using 10% SDS-PAGE. The gel was blotted in specific conditions

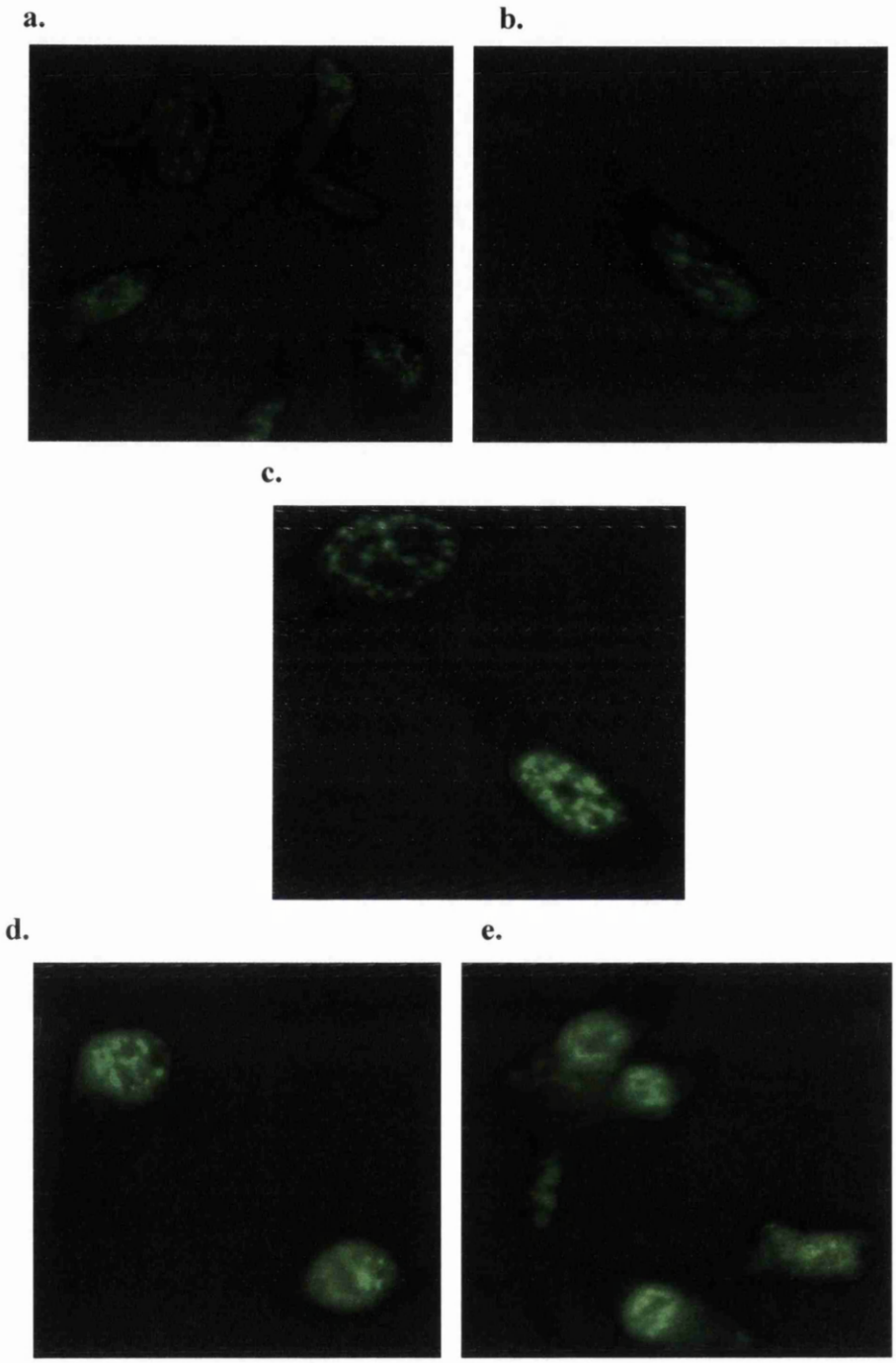


Figure 30. Immunofluorescence of infected BHK cells using 137.

BHK cells were mock (a), 1716 (b) 17^{+} (c) or 1622 (d, e) infected and fixed at 5 h pi. There is punctate nuclear staining in all samples.

to allow renaturation of enzymatic proteins as described in Section 3.28.1. Although no apparent changes took place in the level of ICP34.5, detection of the 40 kDa protein was lost post-renaturation from 17^+, 1716 and 1622 (Fig. 31).

4.2.4. Host Protein Synthesis from 1622 Infection

Roizman and others (Chou and Roizman, 1992, 1994) have characterised that one of the roles of HSV-1 ICP34.5 is to maintain host and viral protein synthesis late in infection in a human neuroblastoma cell line (SK-N-SH). To determine whether ICP34.5 expression from 1622 rescued this phenotype, BHK, SK-N-SH and HeLa cells were infected at a m.o.i. of 10 pfu/cell and pulse labelled with [^{35}S]methionine for 2 h (14 to 16 h pi). In BHK cells, all viruses gave the same protein synthesis profile (Fig. 32a). However, in 1716 infected SK-N-SH cells, as predicted, little protein synthesis occurred (Fig. 32b). The protein synthesis profile in 1622 infected cells was identical to that from 17^+ in SK-N-SH cells (Fig. 32b). Interestingly, another human cell line, HeLa cells, also showed a decrease in protein synthesis following 1716 infection (Fig. 32c) with protein synthesis again the same in 1622 and 17^+ infected cells (Fig. 32c).

In BHK cells, 1716 demonstrates no impairment in viral replication, but in 3T6 cells it is impaired and the *in vivo* avirulent phenotype is mimicked (Brown *et al.*, 1994a). To determine if this phenotype could be attributed to host protein synthesis shut-off the following experiment was carried out (Fig. 33). BHK and 3T6 cells were infected at a m.o.i. of 10 pfu/cell and radiolabelled with [^{35}S]methionine for 2 h (14-16 h pi). At 16 h pi cells were harvested in SDS lysis buffer. Samples were electrophoresed through a 10% SDS-PAGE, the gel dried and exposed to an autoradiograph film for 5 h at –70°C. Viral induced proteins were visible indicative of protein expression from all viruses (17^+, 1716 and 1622). There was no overall difference in protein synthesis between wild type 17^+, 1716 or 1622 (Fig. 33), indicating that host protein synthesis shutoff does not play a role in the lack of 1716 replication in 3T6 cells.

4.2.5. *In vitro* Replication Kinetics of 1622

One role of ICP34.5 is the involvement in viral replication. Deletion of the RL1 gene dramatically reduces the virus's ability to replicate in some, but not all, cell lines.

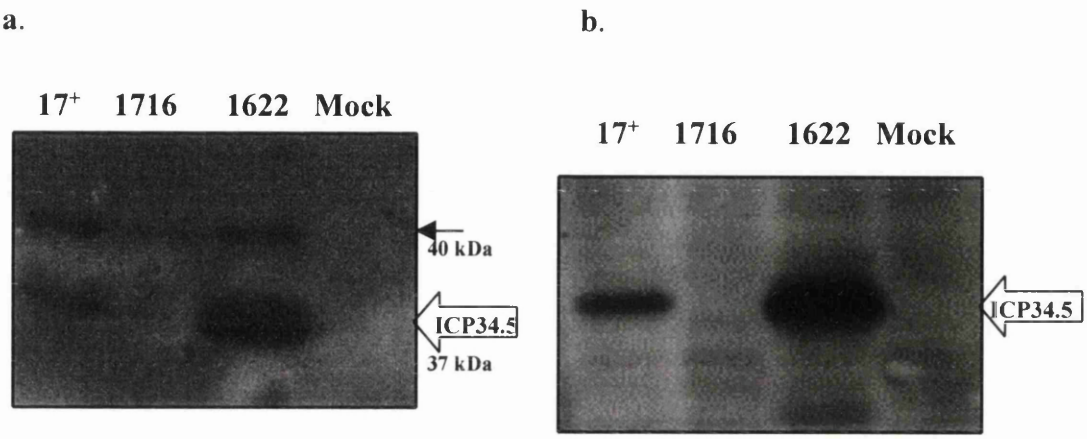


Figure 31. Blot renaturation of 17+, 1622 and 1716 infected BHK cells 16 h pi.

BHK cells were infected at a m.o.i. of 10 pfu/cell and harvested at 16 h pi in SDS lysis buffer. Samples were analysed using a 10% SDS-PAGE and Western blotted with 137. Samples were loaded in duplicate lanes for regular Western blot methods (**a**) and renaturation in the absence of methanol at 4°C. (**b**). Both blots were reacted with 137 antiserum. The 40 kDa protein is not recognised after renaturation, but ICP34.5 is recognised at the same level. The appearance of ICP34.5 size difference is due to uneven electrophoresis through the gel.

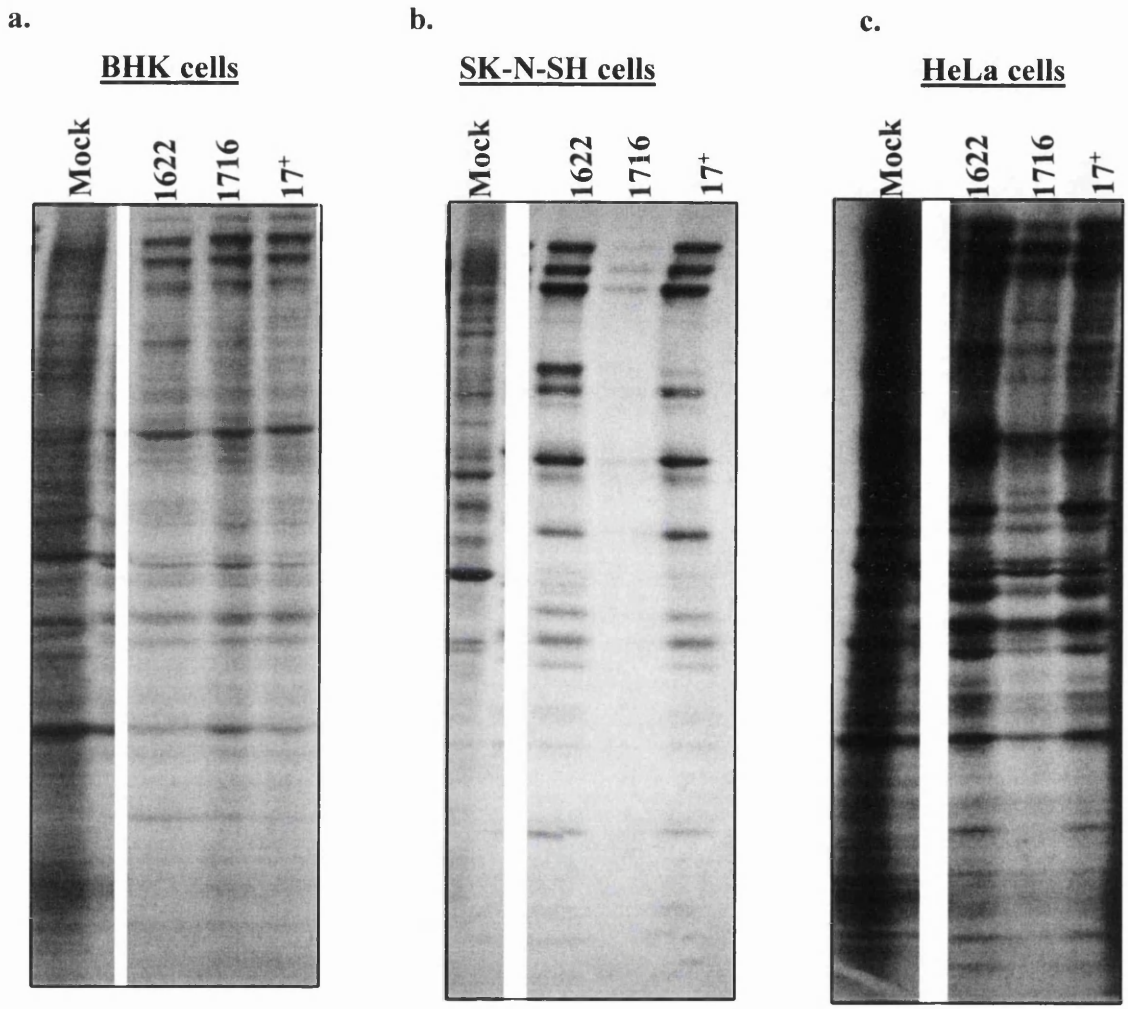


Figure 32. Host cell protein synthesis shut-off in BHK, SK-N-SH and HeLa cells.

BHK, SK-N-SH and HeLa cells were infected with a m.o.i. of 10 pfu/cell and at 14 h pi pulse [^{35}S]methionine labelled until 16 h pi. The infected cells were harvested and analysed by 10% SDS-PAGE and exposure to an autoradiograph o/n at -70°C. **a**. In all BHK cell infections, there is no difference in protein synthesis. **b**. In SK-N-SH cell infections, there is protein synthesis shut-off from 1716, but no shut-off in protein synthesis from 1622 and 17^{+}. **c**. Infection in HeLa cells show protein synthesis shut-off from 1716 only.

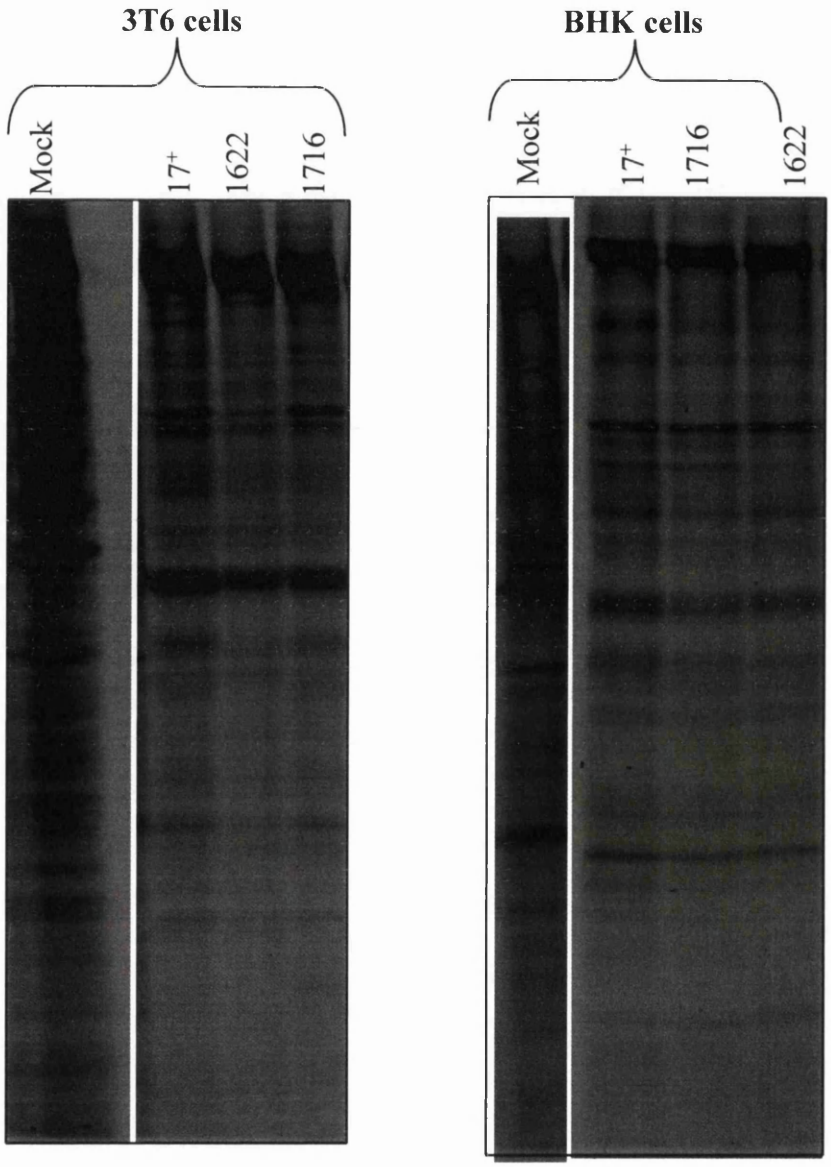


Figure 33. Host cell protein synthesis in BHK and 3T6 cells.

Infected BHK and 3T6 cells were radiolabelled with [^{35}S]methionine for 2 h (14 to 16 h pi). Samples were analysed using 10% SDS-PAGE.
The gel was dried and exposed to autoradiograph film o/n at –70°C. There is no difference in protein synthesis in BHK and 3T6 cells between the different virus infections.

4.2.5.1. Single Cycle Replication Kinetics

To study single cycle replication kinetics, BHK cells were infected at a m.o.i. of 10 pfu/cell and no difference between the replication kinetics of 17^+, 1716 or 1622 was observed (Fig. 34a; Table 6). These were the results from a single experiment. In non-permissive 3T6 cells, infection at a m.o.i. of 10 pfu/cell showed that 1716 does not replicate to the same titre as 17^+ (Fig. 34b; Table 6). 1716 yields a maximum titre at 36 h pi which is approximately 70-fold less ($1.2x10^6$ pfu/10^6 cells) than 1622 and 17^+ ($8x10^7$ pfu/10^6 cells and $7x10^7$ pfu/10^6 cells, respectively).

4.2.5.2. Multi Cycle Replication Kinetics

The first experiment to look at viral replication used primate Vero cells and human HeLa cells (Fig. 35; Table 7), two cell lines which had not previously been studied for 1716 replication. The following was carried out in three independent experiments and yielded similar results. Due to the surprising result of HeLa host cell protein synthesis shut-off from 1716, replication kinetics were examined in these cells (Fig. 33c; Section 4.2.4.). Vero and HeLa cells were infected with a m.o.i. of 0.001 pfu/cell.

Infected Vero cells samples were harvested at 0, 56, 72 and 96 h pi. Samples were sonicated and titrated in triplicate on BHK cells. 1716 showed a reduction in its ability to replicate. The peak titre of 1716 was $5.0x10^5$ pfu/10^6cells at 72 h pi. 17^+, 1622 and gD17+ had similar kinetics with peak titres of $1.1x10^7$ pfu/10^6 cells, $8x10^6$ pfu/10^6cells and $1.1x10^7$ pfu/10^6 cells, respectively at 96 h pi. This is a difference between wild type 17^+ and 1716 of approximately 15 to 20-fold (Fig. 35a).

HeLa cells were infected and at times 0, 24, 72 and 126 h pi samples were harvested, sonicated and titrated in triplicate on BHK cells. 17^+ and 1622 had similar kinetics reaching a peak titre of $9x10^5$ pfu/10^6cells at 126 h pi. 1716 did not replicate in these cells (Fig. 35b).

An experiment with additional RL1 mutant viruses including 17termA, 17termAR, 1771 and 1771R, were added as controls on BHK and rabbit skin cells (Table 8). 17termA contains a stop codon insertion near the *Bst*EII site and 17termAR is its rescuant virus. 1771 contains a stop codon insertion near the *Nco*I site and 1771R is its rescuant virus. In BHK cells, there was no significant replication difference between these viruses (Fig. 36a; Table 9).

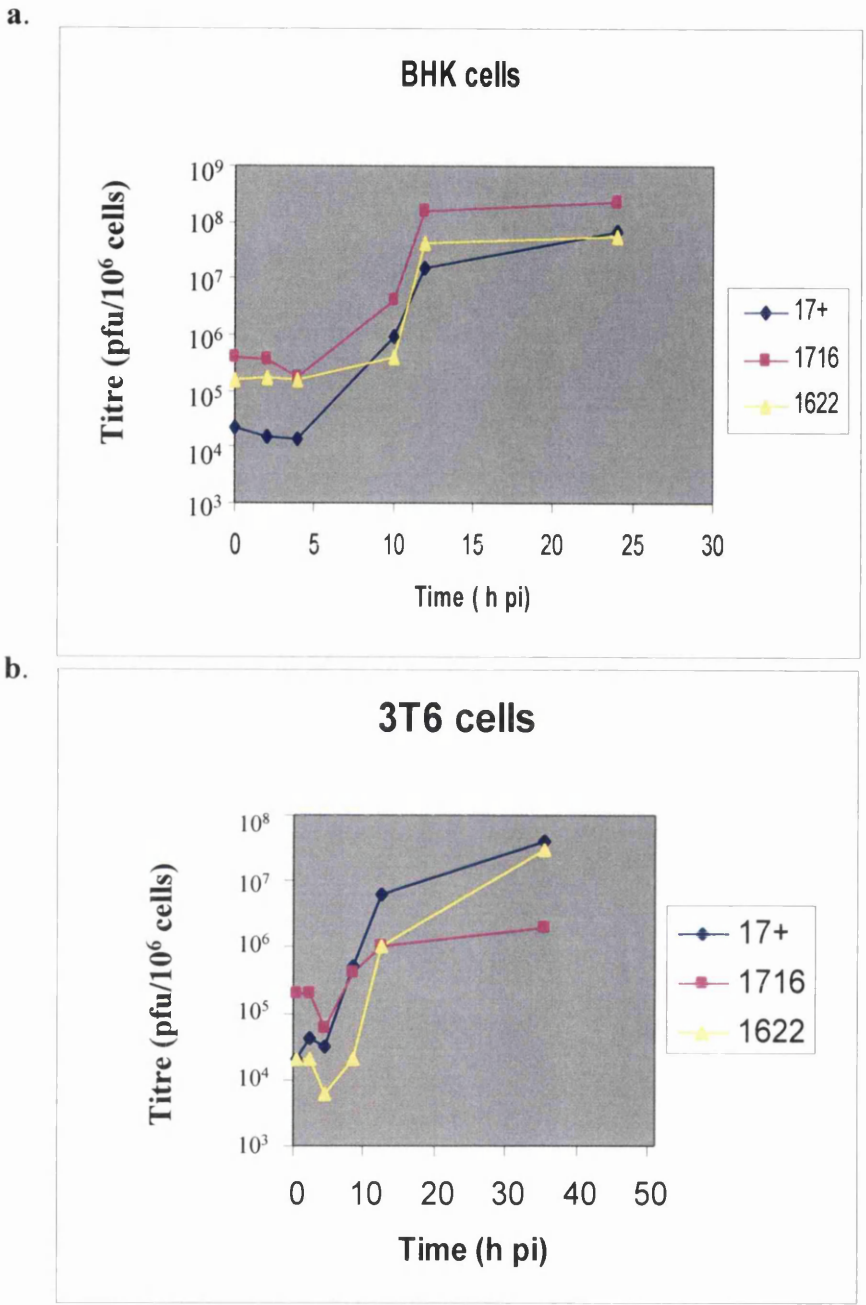


Figure 34. Single-cycle replication kinetics in different animal cells.

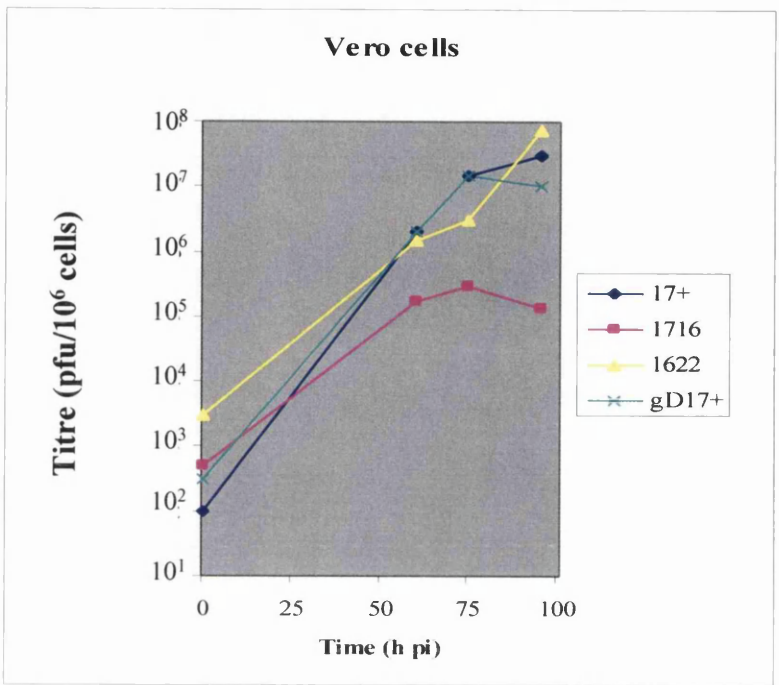
BHK and 3T6 cells were infected at a m.o.i. of 10 pfu/cell.
a. At times 0, 2, 4, 10, 12 and 24 pi infected BHK cells were scraped into the medium, sonicated and titrated onto BHK cell monlayers. All viruses grow with similar kinetics 17^+ (◆), 1716 (■) and 1622 (▲). **b**. At times 0, 2, 4, 12 and 36 h pi 3T6 cells were scraped into the medium, sonicated and titrated onto a BHK monolayer. In 3T6 cells, 1716 (■) does not replicate as well as 17^+ (◆) and 1622 (▲), which replicate to reach titres of $7x10^7$ pfu/10^6 cells and $8x10^7$ pfu/10^6 cells, respectively, at 36 h pi.

Table 6. BHK and 3T6 titres for single cycle growth kinetics.

BHK cells			
	17^+	1716	1622
0	$2.2x10^4$	$4.0x10^5$	$1.5x10^5$
2	$1.5x10^4$	$3.5x10^5$	$1.7x10^5$
4	$1.4x10^4$	$1.7x10^5$	$1.5x10^5$
10	$8.7x10^5$	$4.0x10^6$	$4.0x10^5$
12	$1.5x10^7$	$1.5x10^8$	$4.2x10^7$
24	$6.9x10^7$	$2.3x10^8$	$5.3x10^7$

3T6 cells			
	17^+	1716	1622
0	$2.0x10^4$	$2.0x10^5$	$2.0x10^4$
2	$4.0x10^4$	$2.0x10^5$	$2.0x10^4$
4	$3.0x10^4$	$6.0x10^4$	$6.0x10^3$
8	$5.0x10^5$	$4.0x10^5$	$2.0x10^4$
12	$6.0x10^6$	$1.0x10^6$	$1.0x10^6$
35	$4.0x10^7$	$2.0x10^6$	$3.0x10^7$

a.



b.

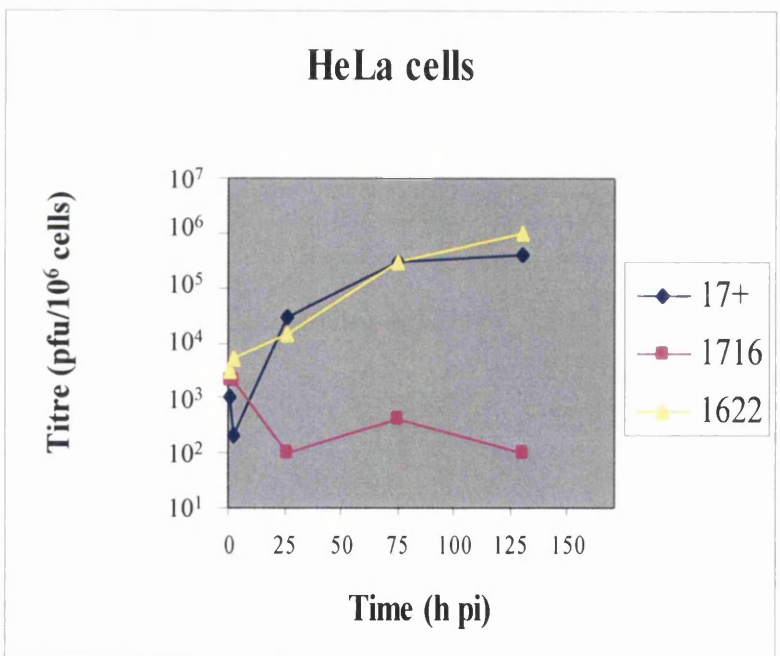


Figure 35. Multi-cycle replication kinetics in primate and human cells.

Vero and HeLa cells were infected at a m.o.i. of 0.001 pfu/cell. Infected cells were scraped into the medium, sonicated and titrated onto a BHK monolayer. **a**. At 0, 56, 72 and 96 h pi infected cells were harvested. Vero cells show that 1716 (■) does not replicate with the same kinetics as 17^{+} (◆), 1622 (▲) or gD17+ (×). gD17+ (×) was used as a control for insertion in the UL43 locus. **b**. At 0, 24, 72 and 126 h pi samples were harvested from infected HeLa cells.

Table 7. Vero and HeLa titres for single cycle growth kinetics.

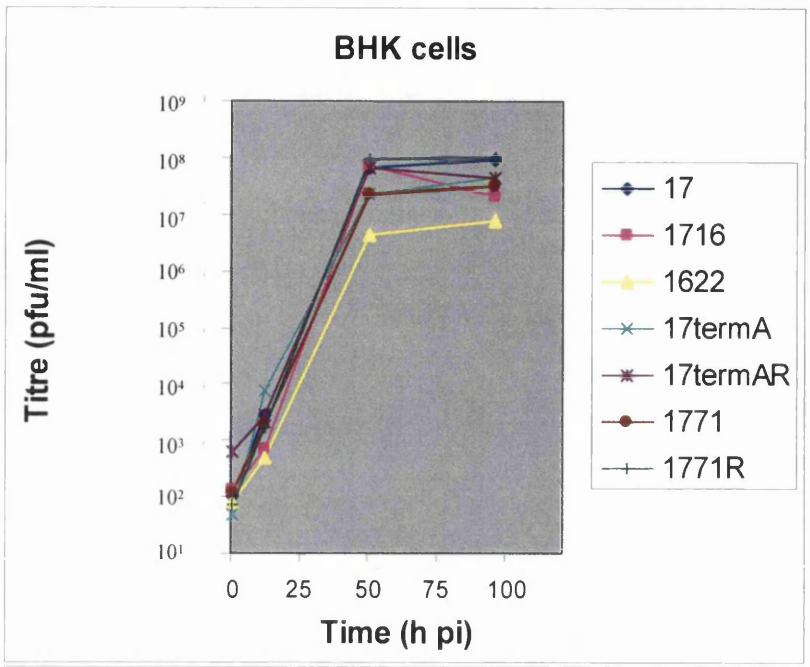
Vero cells				
	17^+	1716	1622	gD17+
0	$1.0x10^2$	$5.0x10^2$	$3.0x10^3$	$3.0x10^2$
60	$2.0x10^6$	$1.7x10^5$	$1.5x10^6$	$2.0x10^6$
75	$1.5x10^7$	$3.0x10^5$	$3.0x10^6$	$1.5x10^7$
95	$3.0x10^7$	$1.3x10^5$	$7.3x10^7$	$1.0x10^7$

HeLa cells				
	17^+	1716	1622	gD17+
0	$1.0x10^3$	$2.0x10^3$	$3.0x10^3$	$4.0x10^2$
2	$2.0x10^2$	$2.0x10^3$	$5.0x10^3$	$4.0x10^2$
26	$1.8x10^4$	$1.0x10^2$	$1.4x10^4$	$8.0x10^3$
75	$3.0x10^5$	$1.2x10^2$	$3.0x10^5$	$2.0x10^5$
130	$5.0x10^4$	$1.0x10^2$	$1.0x10^6$	$5.7x10^5$

Table 8. The names of viruses and a brief description.

Virus name	Description
17^{+}	wild type RL1 and ORF P
1716	759 bp deletion of RL1and ORF P at both loci.
1716R	1716 rescuant; wild type.
1771	stop codon 9 bp downstream of initiating ATG *Nco*I locus in RL1.
1771R	1771 rescuant; wild type RL1.
17termA	stop codon near the *BstE*II locus in RL1.
17termAR	17termA rescuant; wild type RL1.
gD17+	gD promoting *lacZ* in UL43 locus of 17+.

a.



b.

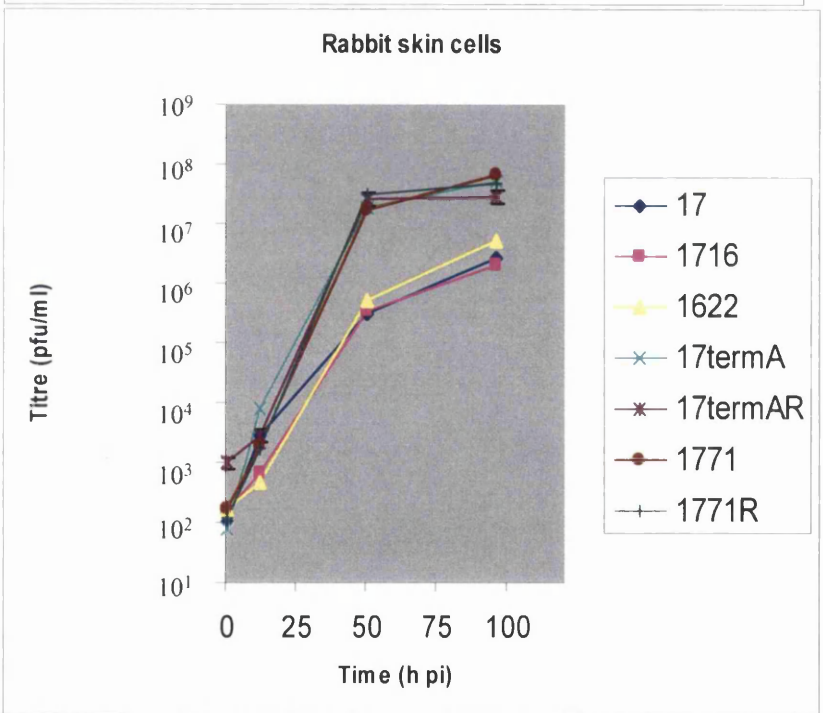


Figure 36. Multi-cycle replication kinetics in different animal cells.

BHK and rabbit skin cells were infected at a m.o.i. of 0.001 pfu/cell. At regular intervals cells were scraped into the medium, sonicated and titrated onto a BHK monolayer in triplicate. **a**. All viruses replicated on BHK cells with similar kinetics 17^+ (◆), 1716 (■), 17termA (x), 17termAR (*), 1771 (●) and 1771R (▌) , with a slightly lower replication from 1622 (▲) **b**. In rabbit skin cells 17^+ (◆) 1716 (■) and 1622 (▲) replicated poorer than 17termA (x), 17termAR (*), 1771 (●) and 1771R (▌).

Table 9. BHK and Rabbit skin cell titres for single cycle growth kinetics.

BHK cells					
	17^+	1716	1622	17termA	17termAR
0	$1.0x10^2$	$1.3x10^2$	$8.0x10^1$	$5.0x10^1$	$5.0x10^1$
12	$2.8x10^3$	$6.5x10^2$	$4.8x10^2$	$7.5x10^3$	$2.8x10^3$
50	$6.3x10^7$	$7.1x10^7$	$4.3x10^6$	$2.3x10^7$	$6.7x10^7$
96	$8.8x10^7$	$2.0x10^7$	$7.7x10^6$	$4.2x10^7$	$4.2x10^7$

Rabbit skin cells					
	17^+	1716	1622	17termA	17termAR
0	$1.0x10^2$	$1.5x10^2$	$1.7x10^2$	$8.0x10^1$	$9.8x10^2$
12	$2.8x10^3$	$6.5x10^2$	$4.8x10^2$	$7.5x10^3$	$2.8x10^3$
50	$3.1x10^5$	$3.3x10^5$	$5.2x10^5$	$1.8x10^7$	$2.6x10^7$
96	$2.5x10^6$	$2.0x10^6$	$5.2x10^6$	$5.0x10^7$	$2.8x10^7$

Another permissive cell line, rabbit skin cells, showed no difference in replication between 17^+, 1716 or 1622 or 17termA, 17termAR, 1771 or 1771R (Fig. 36b; Table 9). However, there were two distinct groups of replication: group 1, 17^+, 1716 and 1622; and group 2, 17termA, 17termAR, 1771 and 1771R. This will be discussed in more detail in Section 6.2. It is important to note here that 17^+, 1716 and 1622 stocks were grown in BHK cells whereas group 2 (17termA, 17termAR, 1771 and 1771R) mutant virus stocks were generated on rabbit skin cells.

4.2.6. Intracranial LD_{50} of 1622 Infection

Intracranial experiments to confirm that the wild type phenotype had been restored to 1622 *in vivo* were carried out by estimation of LD_{50} values in three-week old, female, BALB/c mice (Table 10). In the first experiment mice were inoculated intracranially with virus stocks of 17^+, 1716R, or 1622 (Section 3.36). 1716 was not included in this experiment. HSV-1 strain 17^+ and 1716R were used as positive controls with a known LD_{50} value. 17^+ and 1716R (the rescuant of 1716) had the same LD_{50} of <3.2 pfu/mouse. The 1622 LD_{50} was $1.8x10^2$ pfu/mouse. 1622 showed a significant difference (60-fold) in LD_{50} value following intracranial inoculation.

In the second experiment, 1716 and an additional control virus, gD17+, were used. The mutant virus gD17+ was a control for an insertion in the UL43 locus having the *lacZ* gene inserted in the UL43 *NsiI* site. 1716 was included as a negative control. The LD_{50} for 1622 remained $1.8x10^2$ pfu/mouse. In this experiment, the LD_{50} of 17^+ was $5.6x10^1$ pfu/mouse and the LD_{50} of gD17+ was $1x10^3$ pfu/mouse. The LD_{50} of 17^+ was raised by a factor of 20. The LD_{50} of gD17+, $1x10^3$ pfu/mouse, was 50-fold higher than 17^+ and 5-fold higher than 1622. For 1716 no animals died when infected with $1x10^5$ pfu/mouse and one mouse out of four died from the 1716 infective dose $1x10^6$ pfu/mouse. This is not statistically significant and is consistent with the previously published LD_{50} for 1716 of $>1x10^6$ pfu/mouse.

4.2.7. *In vivo* Replication Kinetics from 1622 Infection

One of the main biological properties of HSV-1 infection is that following infection at a peripheral site the virus replicates and travels by axonal transport to the neuronal cell bodies in the sensory ganglia where the virus establishes and maintains a latent state. The snouts of male Swiss Webster mice aged 4-7 weeks old were abraded and used as the site

Table 10. Neurovirulence of 1622 following inoculation of BALB/c mice infected with varying doses and calculated as LD_{50}.

Experiment 1							
	10^1	10^2	10^3	10^4	10^5	10^6	LD_{50}
17+	4/4*	4/4	ND	ND	ND	ND	<3.2**
1716R	4/4	4/4	ND	ND	ND	ND	<3.2
1716	ND	ND	ND	ND	ND	ND	
1622	0/4	1/4	4/4	ND	ND	ND	$1.8x10^2$

Experiment 2							
	10^1	10^2	10^3	10^4	10^5	10^6	LD_{50}
17+	1/4	2/4	ND	ND	ND	ND	$5.6x10^1$
gD17+	ND	1/4	1/4	ND	ND	ND	$1x10^3$
1716	ND	ND	ND	ND	0/4	1/4	$1.8x10^6$
1622	0/4	1/4	4/4	4/4	4/4	ND	$1.8x10^2$

* Number of deaths per number of mice infected.
** pfu/mouse
ND = Not Done

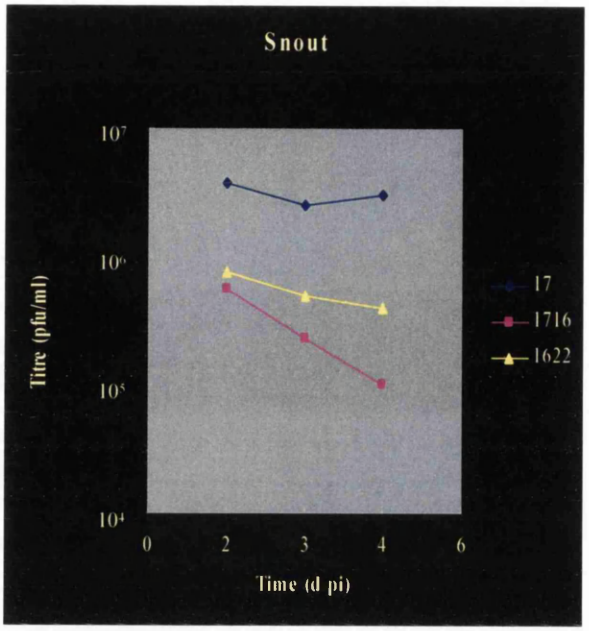
of initial infection of $5x10^5$ pfu/ml of either 17^+, 1716 or 1622 (Fig. 37a; Table 11). On the day of infection the input virus was serially diluted and titrated in triplicate on rabbit skin cell monolayers which confirmed equal input titres (data not shown). On days 2,3 and 4 pi three mice per virus were sacrificed, their snouts and TG dissected, homogenized and titrated on rabbit skin cell monolayers in triplicate as described in Section 3.42. Maximum titres of 17^+, 1622 and 1716 were seen on day 2 pi (Fig. 37a). There was a significant decrease in replication in the snout infected with 1622 and to a greater extent 1716 (Fig. 37a). This difference was more pronounced in the TG where the peak titre on day 3 pi of 17^+ was $1.5x10^4$ pfu/ml (Fig. 37b; Table 11). The 1622 TG titre also peaked on day 3 pi but with a titre of $1.2x10^2$ pfu/ml, a difference of more than 10 times. The peak titre of 1716 infected TG was $9.0x10^1$ pfu/ml on day 2 pi (10-fold less than 1622) with no evidence of viral replication.

4.2.8. Immunohistochemistry of 1622 Infected TG

Based on the replication kinetics experiments carried out in snouts and TG demonstrating that 1622 does not replicate with the same efficiency as 17^+ immunohistochemistry was used to confirm and extend this finding. Seven week old male Swiss Webster mice were infected with $5x10^5$ pfu/ml of 17^+, 1716 or 1622. Based on the peak titres from the *in vivo* replication experiments we looked at days 3 and 4 pi. At days 3 and 4 pi, three mice per day per virus were transcardially perfused and TG dissected. TG were prepared for IHC as described in Section 3.38.2.1. On day 3 pi there was no staining in mock infected (Fig. 38a) or 1716 infected TG (Fig 38b). There were a few positively staining isolated neurones in TG infected with 1622 (Fig. 38c). In contrast, several clusters of neurones stained positively from TG infected with 17^+ (Fig. 38d). A similar pattern of viral staining was seen on day 4 pi (Fig. 39). The positive neurones from 1622 and 17^+ on day 4 pi were magnified 100x and shown in Figure 40. The number of positive neurones per field was calculated per virus and is presented in Table 12. On day 3 pi the average from two separate sections of TG infected with 17^+ was 41%, 1716 was 0%, and 1622 was 1.9%. By day 4 post infection the average of two TG sections from 17+ infected animals was 56.65%, from 1716 was 0%, and from 1622 was 1.4%

To confirm the identity of the viruses used in this experiment a Southern blot was carried out using DNA extracted from infected TG. The DNA was digested with *Bam*HI and analysed using a 1% (w/v) agarose gel and Southern blotted with a radiolabelled

a.



b.

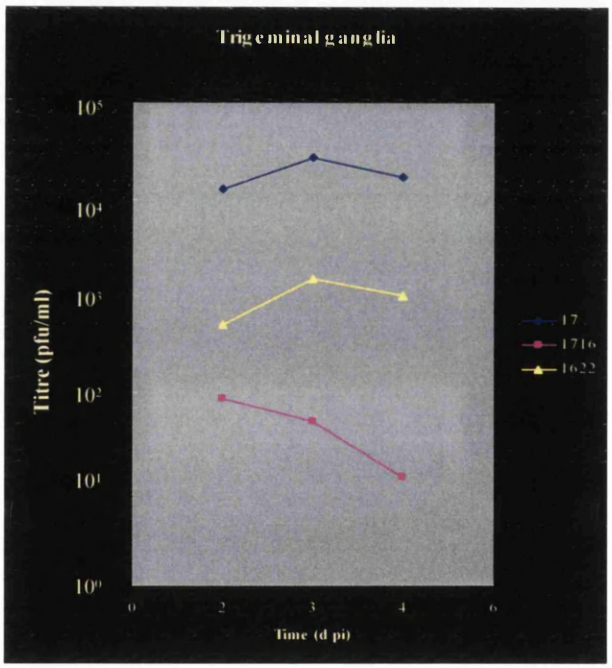


Figure 37. *In vivo* replication from Swiss Webster mice infected with 17^+, 1622 or 1716.

Viral replication in snouts and TG was determined by titrating homogenates of tissues harvested from three mice from each virus time point. Tissues were homogenized in 1 ml of medium and the data are given in pfu/ml of tissue homogenate. **a**. All mice were inoculated with an input dose of $5x10^5$ pfu/ml. All viruses replicated in snouts with differing kinetics. 17^+ (◆) replicated with the highest kinetics. 1622 (▲) and 1716 (■) replicated with reduced kinetics. **b**. In the TG there was a significant difference in peak titres. 1622 (▲) was slightly lower than 17^+ (◆) $1.5x10^4$ pfu/ml with a peak titre of $1.2x10^3$ pfu/ml. 1716 (■) did not appear to replicate in the TG.

Table 11. *In vivo* replication titres of snout and trigeminal ganglia.

17^+			
Snout Day	2	3	4
M1	$7.0x10^4$	$1.0x10^5$	$3.0x10^6$
M2	$4.0x10^3$	$6.0x10^4$	$1.0x10^6$
M3	$6.0x10^3$	$6.0x10^4$	$1.0x10^6$
Mean	$2.7x10^1$	$7.3x10^4$	$1.7x10^6$
Standard deviation	5.7	1.3	1.9

1716			
Snout Day	2	3	4
M1	$1.0x10^4$	$1.1x10^5$	$5.0x10^5$
M2	$1.0x10^3$	$8.0x10^4$	$3.0x10^5$
M3	$9.0x10^3$	$6.0x10^4$	$1.0x10^5$
Mean	$6.7x10^3$	$8.3x10^4$	$3.0x10^5$
Standard deviation	3.9	1.3	56

1622			
Snout Day	2	3	4
M1	$1.4x10^4$	$2.3x10^5$	$1.0x10^5$
M2	$1.9x10^4$	$2.0x10^5$	$1.0x10^5$
M3	$1.8x10^4$	$1.8x10^5$	$1.0x10^5$
Mean	$1.7x10^4$	$2.0x10^5$	$1.0x10^5$
Standard deviation	1.3	1.7	0

17^+			
Trigeminal ganglia/Day	2	3	4
M1	$1.0x10^4$	$3.0x10^4$	$1.3x10^4$
M2	$4.0x10^4$	$5.0x10^4$	$1.0x10^4$
M3	$4.0x10^4$	$2.1x10^4$	$1.1x10^4$
Mean	$3.0x10^4$	$3.4x10^4$	$1.1x10^4$
Standard deviation	2.3	23	1.2

1716			
Trigeminal ganglia/Day	2	3	4
M1	0	$1.0x10^1$	0
M2	$2.0x10^2$	$5.0x10^1$	$1.0x10^1$
M3	$2.0x10^2$	$4.0x10^1$	$1.0x10^1$
Mean	$1.3x10^2$	$3.0x10^1$	7
Standard deviation	1.6	2.4	1.6

1622			
Trigeminal ganglia/Day	2	3	4
M1	$6.0x10^2$	$1.2x10^3$	$1.7x10^2$
M2	$2.0x10^2$	$9.0x10^2$	$8.0x10^1$
M3	$5.0x10^2$	$1.0x10^3$	$1.0x10^2$
Mean	$4.3x10^2$	$1.0x10^3$	$6.7x10^2$
Standard deviation	1.8	1.2	8.9

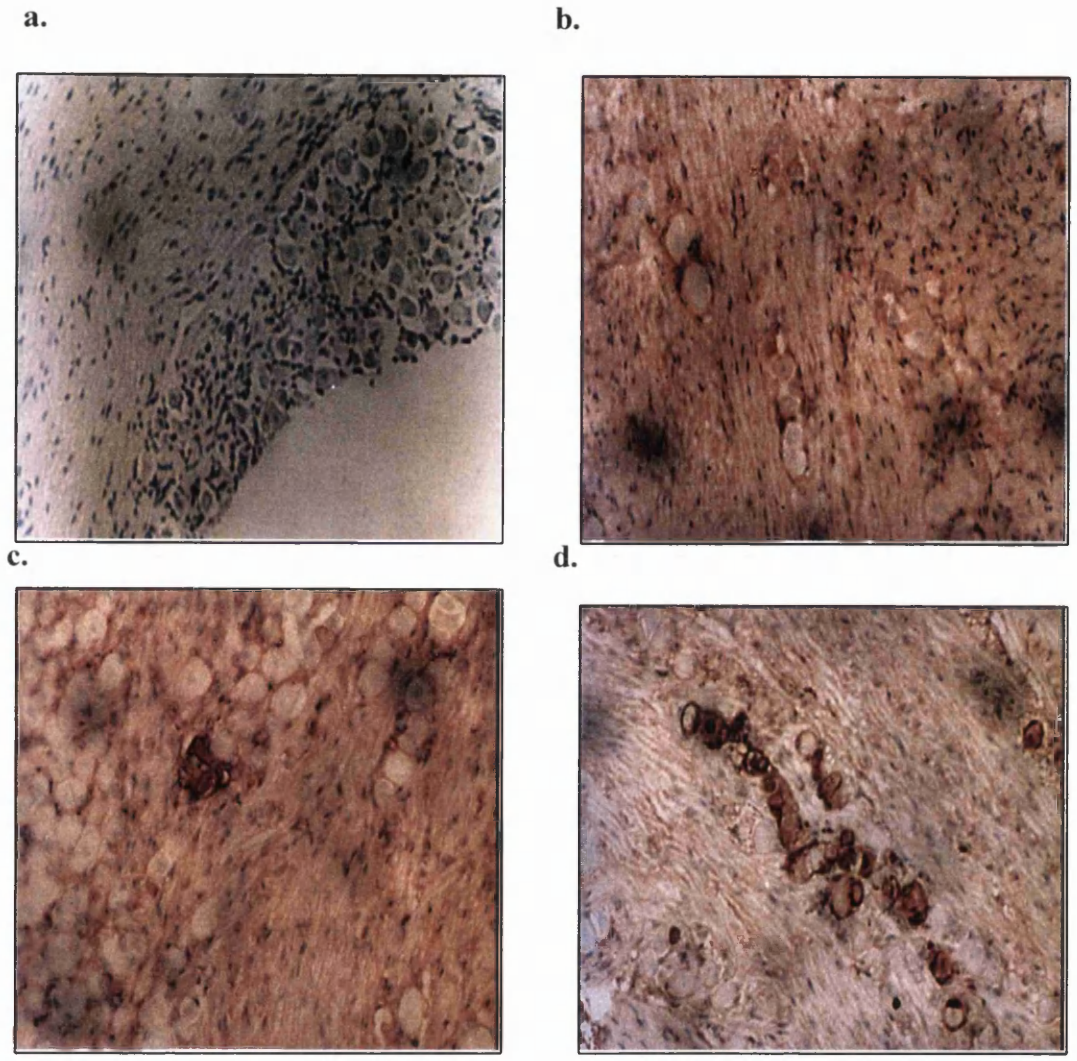


Figure 38. IHC detection of HSV antigen expression in infected Swiss Webster mouse TG 3 days post infection.

Seven week old mice were inoculated with $5x10^5$ pfu/ml of 17^+, 1716 and 1622. On day 3 pi the mice were transcardially perfused and sacrificed. TG were sectioned on a rotary microtone and stained for the presence of HSV antigen (see Section 3.38). **a**. TG of mouse mock infected. **b**. TG of mouse infected with 1716. **c**. TG of mouse infected with 1622. **d**. TG of mouse infected with 17^+. These photo-micrographs were taken at 20x magnification.

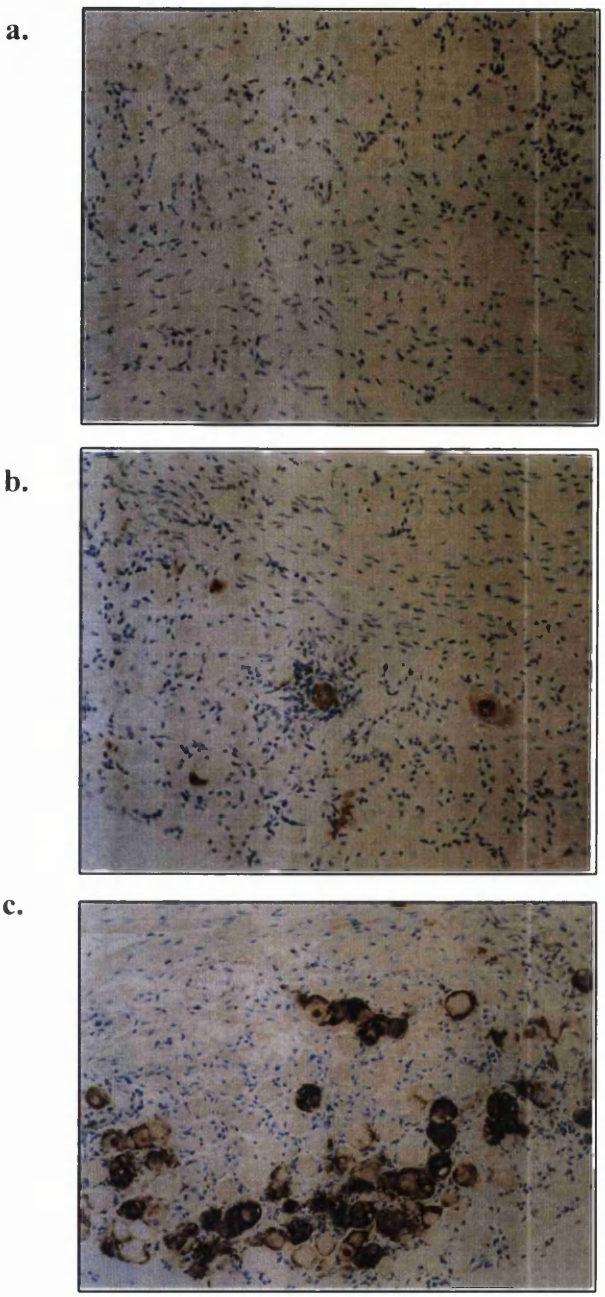


Figure 39. IHC detection of HSV antigen expression in infected Swiss Webster mouse TG 4 days post infection.

Seven week old mice were inoculated with $5x10^5$ pfu/ml of 17^+, 1716 and 1622. On day 4 pi the mice were transcardially perfused and sacrificed. TG were sectioned on a rotary microtone and stained for the presence of HSV antigen (see Section 3.38). **a**. TG of mouse infected with 1716. **b**. TG of mouse infected with 1622. **c**. TG of mouse infected with 17^+. These photo-micrographs were taken at 20x magnification. Photomicrographs were taken by Dr. N. Sawtell.

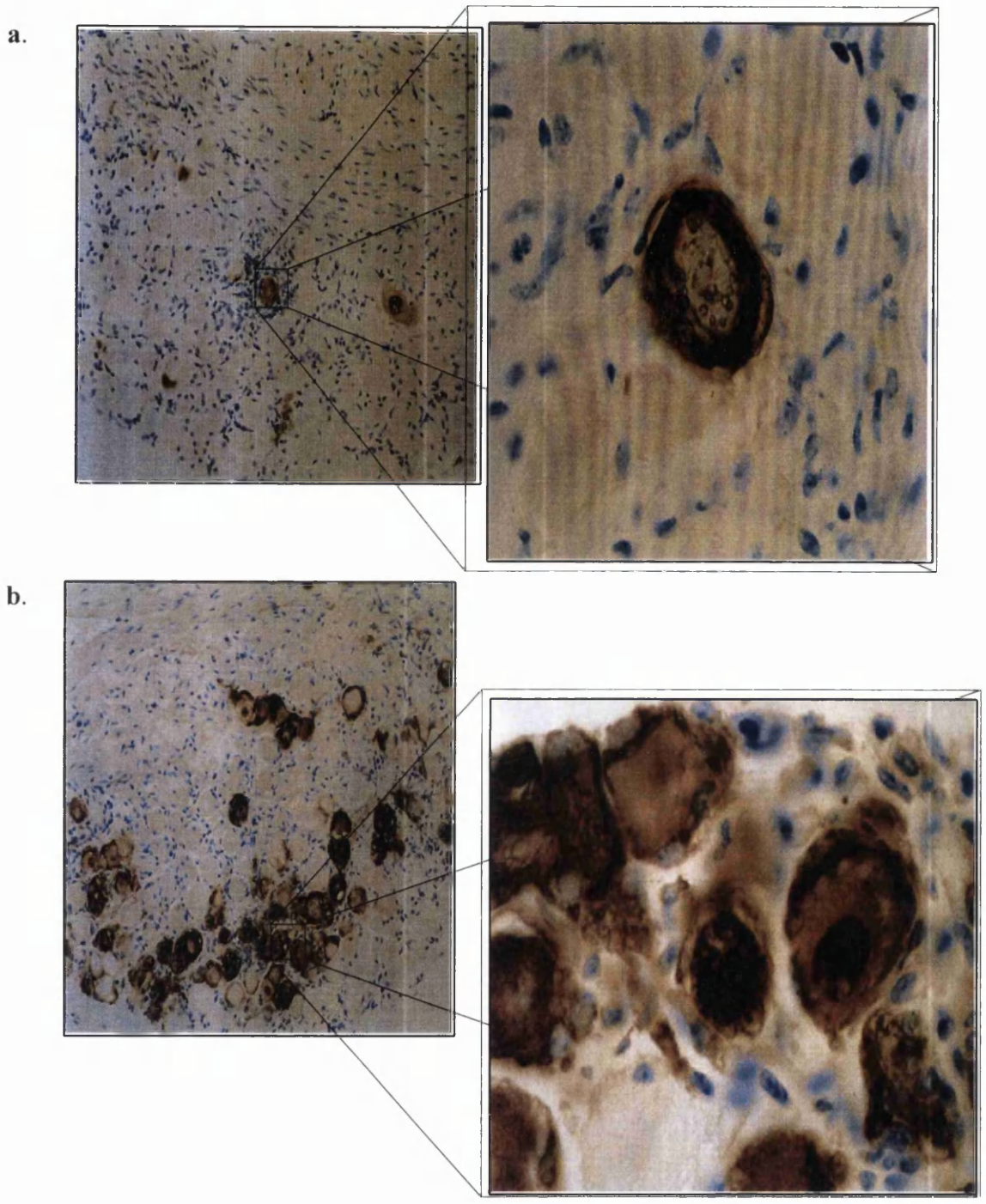


Figure 40. Photomicrograph of single cells positively stained for HSV antigen.

A higher magnification from sections in Figure 41. TG were counterstained with cresal violet to emphasize the cellular components and NovaRed® HSV antigen. The ganglion section is comprised of neurones (large cells) and many small satellite/support cells. **a**. TG infected with 1622 showing one HSV antigen positive neurone. **b**. TG infected with 17^{+} shows widespread HSV antigen positive neurones. Magnification of the left photos are 20x and the right are 100x. Photomicrographs were taken by Dr. N. Sawtell.

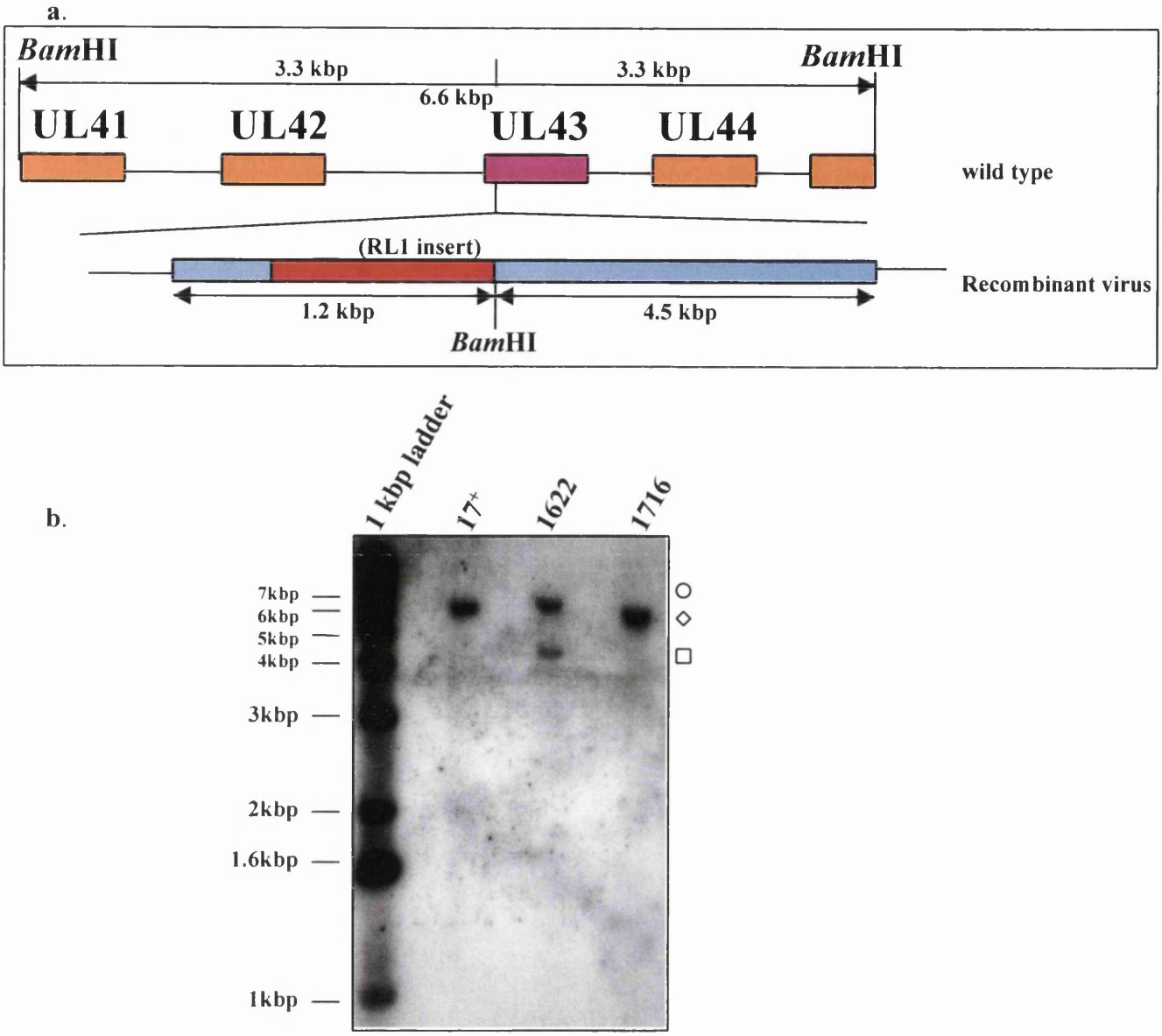


Figure 41. Southern blot of isolated genomic profiles from TG.

a. Schematic diagram of the *Bam*HI fragment with the RL1 insert spanning the UL43 region. **b**. Viral DNA was isolated from TG and digested with *Bam*HI. Samples were analysed using a 1% (w/v) agarose gel and Southern blotted using radiolabelled p35/PacI probe. The blot was exposed to an autoradiograph for 5 h at -70°C. 17^{+} and 1716 show a single band at 6.6 kbp (◇). This is the wild type *Bam*HI fragment spanning UL41 through UL44. 1622 contains an extra *Bam*HI site in which RL1 in the UL43 divides this region yielding two bands, 7.8 kbp(○) and 4.5 kbp (□).

p35(PacI) probe. The blot was exposed to an autoradiograph for 5 h at -70°C (Fig. 41). In the recombinant virus, the insert has an extra *Bam*HI site in UL43 and this digests into two fragments (of 4.6 and 7.8 kbp) as previously described (Section 4.1). In 17^+ and 1716 there is a single *Bam*HI fragment of 6.6 kbp.

4.2.9. Latency and Reactivation by Co-cultivation

The ability of 1622 to reactivate from latency was examined. Groups of 4 week old, female, BALB/c mice were infected with 17^+, 1716, 1716R or 1622 via the footpad model described by Clements and Subak-Sharpe (1983, 1988). Virus was injected into the right rear footpad of each mouse with different viruses and input doses. Mice were monitored twice daily for signs of illness or death. Forty-one days pi, mice were sacrificed and the 10 ipsilateral DRG which innervate the footpad were assayed for the release of virus as described in Section 3.37.1.

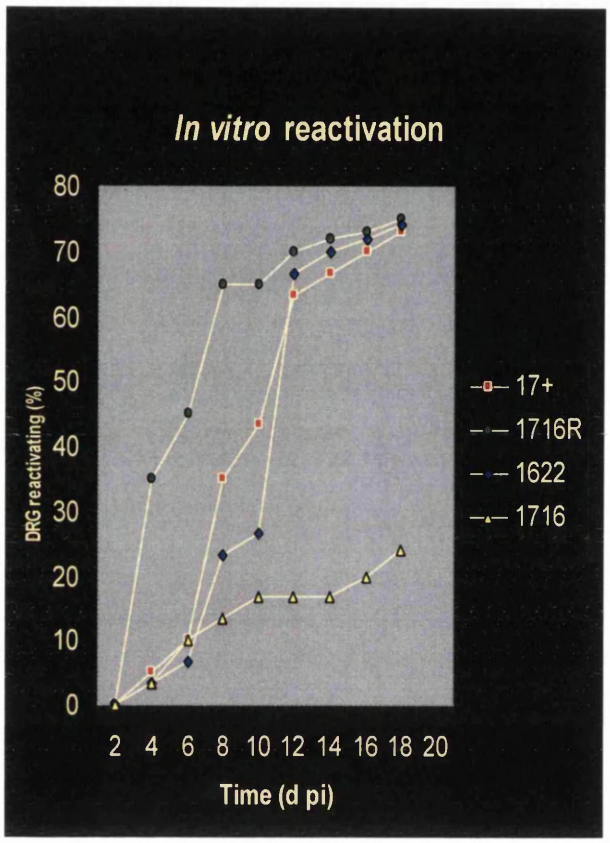
An input dose of $5x10^5$ pfu/mouse showed that HSV-1 strain 17^+, 1716R and 1622 were equally efficient at reactivating from latency with a maximum frequency of ~75% explanted ganglia reactivating by day 18 post explantation (Fig. 42a; Table 13). The percentage reactivation of 1716 was ~22% explanted ganglia by day 18 post explant. In a separate experiment, using an increased input dose of $5x10^6$ pfu/mouse, 1622 was still able to reactivate better than 1716 (Fig. 42b; Table 13) but frequency of reactivating virus from 1716 was better at this higher dose. The maximum reactivation from 1716 infected DRG was ~52%. The maximum frequency of reactivating virus from 1622 $5x10^6$ pfu/mouse infected ganglia was ~78% which is slightly better than the frequency of reactivating virus from 1622 input dose of $5x10^5$ pfu/mouse.

Table 12. Quantitative analysis of HSV antigen positive neurones per section on day 3 and day 4 post infection.

Day 3 post infection						
		17+				
	Positive neurones/Total number of neurones				Percent postive	
Section 1		55/260✦			21.00%■	
Section 2		110/180			61.10%	
Average					41%	
		1716				
	Positive neurones/Total number of neurones					
Section 1		0/130			0%	
Section 2		0/200			0%	
Average					0%	
		1622				
	Positive neurones/Total number of neurones					
Section 1		3/120			2.50%	
Section 2		2/140			1.40%	
Average					1.90%	
Day 4 post infection						
		17+				
	Positive neurones/Total number of neurones				Percent postive	
Section 1		87/120			72.50%	
Section 2		49/180			40.80%	
Average					56.65%	
		1716				
	Positive neurones/Total number of neurones					
Section 1		0/200			0%	
Section 2		0/1800			0%	
Average					0%	
		1622				
	Positive neurones/Total number of neurones					
Section 1		0/200			0%	
Section 2		5/180			2.80%	
Average					1.40%	

✦ **Number of ganglia counted.**
■ **Percent HSV positive ganglia.**

a.



b.

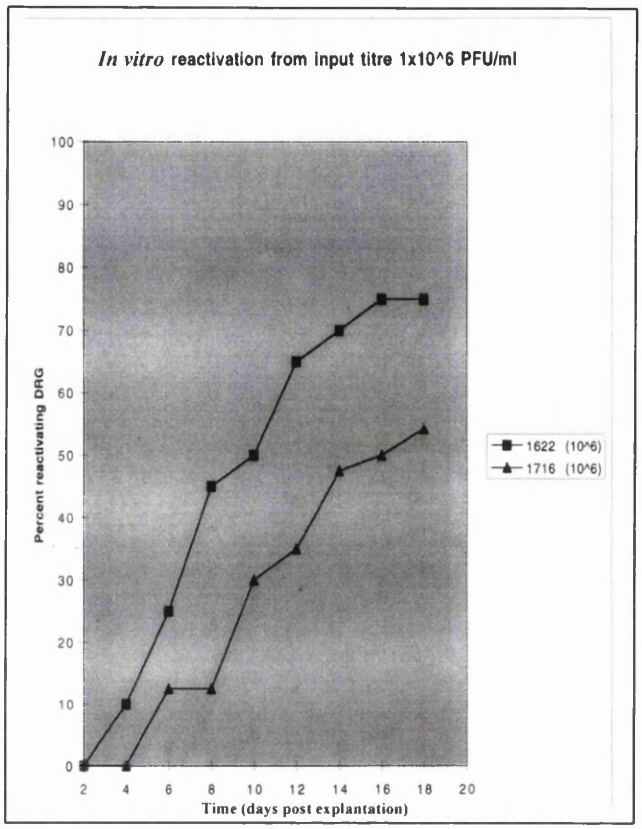


Figure 42. ***In vitro*** **reactivation characteristics of 1622.**

a. BALB/c mice were inoculated with an input dose of $5x10^5$ pfu/mouse and mice were left for 41 days to establish latency. DRG were dissected, placed into microtitre plates and media replated every two days onto fresh BHK monolayers which were examined for cpe. The frequency of DRG reactivating at each time point was calculated as a percentage of the total number of ganglia explanted, for each virus at each dilution. In general each time point represents four mice per group.
b. BALB/c mice were inoculated with 1622 and 1716 of $5x10^6$ pfu/mouse and left for 41 days to establish latency. Each time point represents, on average, four mice per group.

Table 13. Co-cultivation reactivation percentages.

Input dose of $5x10^5$ pfu/mouse									
Days post explant	2	4	6	8	10	12	14	16	18
17+	0	5	10	35	43.3	63.3	66.7	70	73
1716R	0	35	45	65	65	70	72	73	75
1622	0	3.3	6.6	23.3	26.7	66.7	70	72	74
1716	0	3.3	10	13.3	16.7	16.7	16.7	20	23

Input dose of $5x10^5$ pfu/mouse									
Days post explant	2	4	6	8	10	12	14	16	18
1622	0	10	25	45	50	65	70	75	75
1716	0	3.3	12.5	12.3	30	35	47.5	50	54.3

4.2.10. Discussion

In this section the recombinant virus 1622 was characterized. 1622 only expresses RL1 and not ORF P. Initially ICP34.5 expression was analysed by Western blotting. By placing the RL1 ORF under the gD promoter in the UL43 locus ICP34.5 is expressed approximately 8 times more than in wild type 17^+ by 16 h pi. Immunoprecipitation using 137 antiserum from 1622 infected BHK and 3T6 cells confirmed that ICP34.5 is overexpressed. Western blots of time course experiments show that ICP34.5 in strain 17^+ and 1622 is expressed by 2 h pi. Two additional viral induced proteins of 40 kDa and 70 kDa are recognized by 137 and R1 (Brown *et al.*, 1997). It is not known what these protein are but some theories will be described in the final discussion (Section 7).

It was believed since ICP34.5 was expressed in 1622 at least as well as wild type levels, that replication in non-permissive cells *in vitro* would be restored. This was confirmed in 3T6, Vero, and HeLa cells. 1716 had not been previously studied in Vero or HeLa cells. The reduction from 1716 replication in 3T6 cells has been morphologically examined by Brown *et al* (1997). Using an electron microscope they showed that 1716 is kept in the lamellae of 3T6 cells and unable to be released or fully packaged to spread from cell to cell. The mechanism for this phenomenon has yet to be determined. 1716 did not have wild type kinetics in Vero cells. One explanation for this might be based on the Vero cells being a primate cell line. In HeLa cells, 1716 failed to replicate. This was supported by the host protein synthesis shutoff experiments that confirmed published data by Chou and Roizman (1992) in HSV-1 strain F and the RL1 deleted mutant, R3616. 1622 had restored the ICP34.5 function of maintaining host and viral protein synthesis in SK-N-SH cells. Infection of 1716 and R3616 in SK-N-SH cells shuts off protein synthesis by a mechanism which involves ICP34.5 binding to protein phosphatase 1α to dephosphorylate a translation initiation factor eIF-2α.

Interestingly, a cell specificity was found in rabbit skin cells. Viruses generated and grown in rabbit skin cells were able to replicate to a higher titre in rabbit skin cells than viruses generated and grown in BHK cells, but the reverse was not true for growth in BHK cells.

In vivo experiments showed a difference in replication and virulence between 1622 and 17^+. 1622 had a slightly lower LD_{50} upon intracranial inoculation (between 3 and 60 fold). The defect in replication kinetics was even more pronounced following peripheral inoculation via the snout and replication in the TG. Immunohistochemistry showed vastly fewer HSV positive antigen in 1622 than in 17^+. No HSV antigen staining was seen in TG

from mice infected with 1716. This indicates that 1622 is able to replicate *in vivo* in the TG but not as well as 17^+. One hypothesis for why this may be occurring is due to the ICP34.5 level of expression. Overexpression may block an integral function or interaction of ICP34.5 *in vivo*. A second hypothesis may be that the initial time ICP34.5 is expressed differs between wild type 17^+ and 1622. Here we give new data showing *in vitro* ICP34.5 is expressed early in HSV infection. The expression of ICP34.5 from 1622 may be delayed compared to 17^+ because it is promoted by the gD promoter. The ability to detect ICP34.5 so far has been dependent upon Western blotting and it is probable that there may be a low level of expression before 2 h pi which may be determined with different immunological detection methods. Further studies to look for transcripts at early time than analysed here could elucidate this. Additionally, the kinetics and strength of the gD promoter may not give the same activity *in vivo* as the RL1 promoter.

A theory to explain why the reactivation frequency is reduced from 1716 and slightly from 1622 infected mice is that the amount of virus getting to the ganglia to establish latency is low. As seen by the immunohistochemistry experiments the very low of HSV positive antigen in the ganglia of 1716 represents a lack of virus in the ganglia. Therefore, an *in situ* experiment looking at RNA levels of 1716 and 1622 in ganglia during acute replication could elucidate a more quantitative answer to just how little 1622 replicates in the murine ganglia.

4.3. CHARACTERIZATION OF 1623

4.3.1. Expression of ICP34.5

The HSV-1 RL1 promoter is located within the *a* sequence. ICP34.5 was originally published to be a late (γ_1) protein, expressed at 16 h pi from HSV-1 strain F. This laboratory has shown expression of ICP34.5 from HSV-1 strain 17^+ as early as 2 h pi by Western blotting (Section 4.2). In order to test if ICP34.5 could be expressed with the RL1 5' leader sequence and possible transcription initiation site downstream of the HSV-1 gD promoter a second recombinant virus, 1623, was constructed. 1623 has the RL1 ORF with 134 bp of additional upstream sequences. Chou and Roizman (1986) considered this 134 bp, which is outside the *a* sequence, sufficient to promote expression of RL1. This RL1 fragment is 918 bp and has *Dra*I at the 5' terminus and *Bam*HI (from pGEM34.5) at the 3' terminus. The 918 bp, *DraI/Bam*HI fragment was sub-cloned downstream of the gD promoter as described in Section 4.1. 1623 was used to compare the times of ICP34.5 expression without the anti-sense gene, ORF P. We hypothesised that the two recombinant viruses, 1622 and 1623, would express ICP34.5 at the same time with similar levels as wild type, 17^+. We also hypothesized that ICP34.5 expression from 1623 *in vitro* would restore growth and virulence *in vivo* of 1716.

BHK cells were infected at a m.o.i. of 10 pfu/cell and samples harvested at different times post infection (2 to 12 h), and analysed by SDS-PAGE and Western blotting using the HSV-1 ICP34.5 antiserum 137. No ICP34.5 expression from 1623 was detected up to 12 h pi (data not shown). ICP34.5 expression was detected on a 5-12.5% gradient SDS-PAGE by 16 h pi from 1623 infected BHK cell extracts (Fig. 43). The level of ICP34.5 detection was considerably less than 17^+. There is no ICP34.5 expression from 1716. The Western blot shown in Figure 43 demonstrates that there is a pre-domeninant HSV induced cellular protein migrating close to ICP34.5, at approximately 40 kDa. This gives the appearance of a protein in the adjacent lane containing a mock infected extract. There is also strong recognition of two virus induced proteins whose apparent M_r are 25 kDa and 21 kDa.

A serial 2-fold dilution of the 17^+ infected BHK cell extracts was made to compare the level of ICP34.5 with that of 1623. In Figure 44, a Western blot shows that a 1 in 8 dilution of 17^+ infected BHK cell extract is closest to the neat 1623 infected BHK extract: thus 1623 expresses ICP34.5 at only 12.5% the level of 17^+.

To test if 1623 was expressing wild type levels of other viral protein a Western blot using an antiserum to the UL42 HSV protein was carried out (Fig. 45). Infected cell

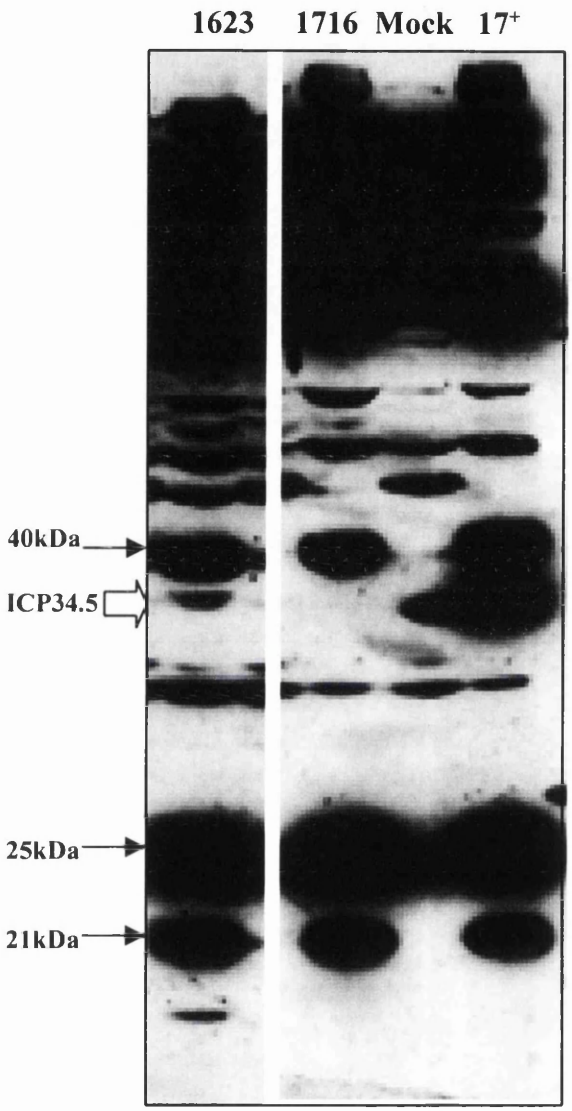


Figure 43. Western blot of 1623 ICP34.5 expression using 137.

Infected BHK cells were harvested at 16 h pi analysed on a 5-12.5% gradient SDS-PAGE and Western blotted using 137 antiserum. Recognition of the 25 kDa is so strong that it gives the appearance of being present in the mock infected lanes.

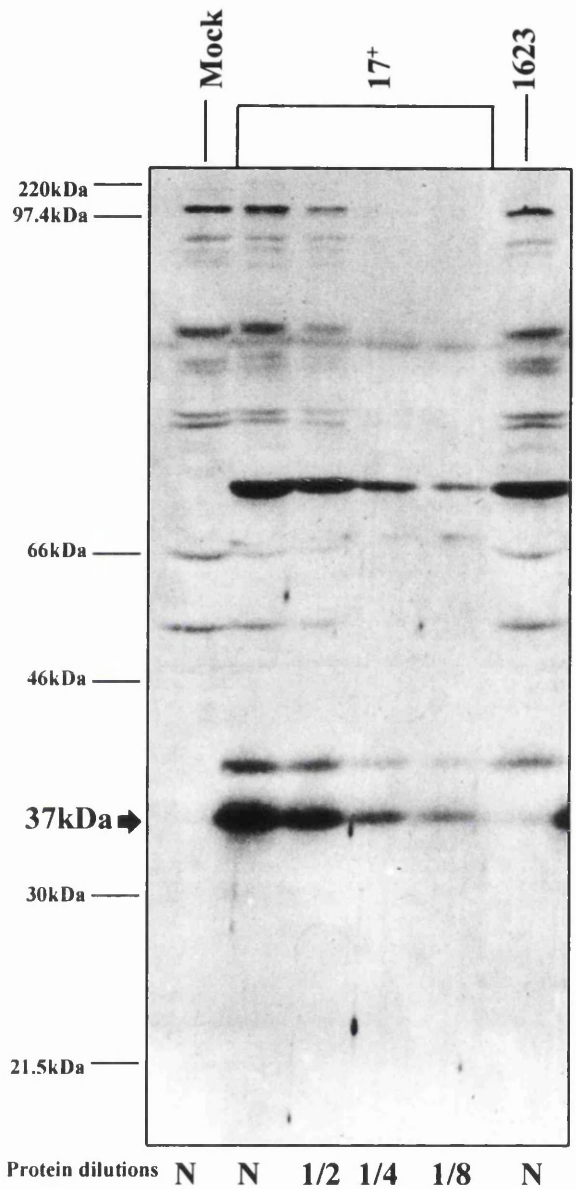


Figure 44. Semi-quantitative comparison of ICP34.5 expression from 1623

A serial 2-fold dilution was made of 17^+ extracts in lysis buffer. Infected BHK cells 16 h pi were analysed by 10% SDS-PAGE,Western blotted using 137 antiserum, Protein A-HRP and ECL. The dilution showing the closest level of ICP34.5 from the neat 1623 extract (N) is the 1 in 8 dilution of 17+ (1/8).

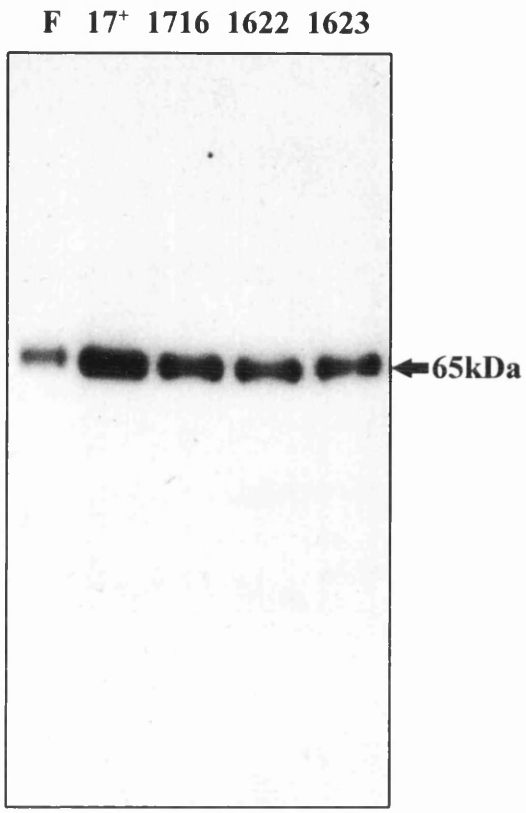


Figure 45. Western blot of BHK infected whole cell extracts using anti-UL42 serum.

A monoclonal antibody against the UL42 65 kDa DNA binding protein was used in a Western blot. These were the same neat extracts used in Figure 44.

extracts on this gel are the same extracts that were used in the Western blot of ICP34.5 in the previous figures (Fig. 43 and 44). The level of UL42 expression is slightly higher in 17^+ compared to 1716 and 1622 and strain F, as explained in Section 4.2. The Western blot shows that 1623 is capable of synthesising other viral proteins efficiently. It also shows that the level of another viral protein is similar between recombinant viruses.

4.3.2. Host Protein Synthesis from 1623 Infection

An experiment was carried out to determine whether 1623 infected cells expressing a reduced level of ICP34.5 could prevent preclusion of protein synthesis. BHK, SK-N-SH and HeLa cells were infected with 17^+, 1716 and 1623 at a m.o.i. of 10 pfu/cell. Infected cells were pulse labelled with ^{35}[S]methionine for 2 h from 14 to 16 h pi and protein synthesis was visualised by autoradiography of a 10% SDS-PAGE gel (Fig. 46). Although the expression of ICP34.5 is much less than in 17^+, the function of maintaining protein synthesis is restored in SK-N-SH cells. 1623 infection was not as good as 17^+ and 1622 as seen in the high number of mock cellular bands, however, several viral bands are present, particularly visible from infected HeLa cell extracts (Fig. 46c). A second protein synthesis experiment was carried out using BHK and 3T6 cells (Fig. 47). BHK and 3T6 cells were infected at a m.o.i. of 10 pfu/cell and radiolabelled with [^{35}S]methionine for 2 h (14-16 h pi). At 16 h pi cells were harvested in SDS lysis buffer and analysed using 10% SDS-PAGE. Viral induced proteins were visible, indicative of productive infection in all viruses (17^+, 1716, 1622 and 1623; Fig. 47). There was no overall difference in protein synthesis between wild type 17^+, 1716, 1622, or 1623 (Fig. 47).

4.3.3. *In vitro* Replication Kinetics from 1623 Infection

We wanted to determine if an 8-fold reduction in ICP34.5 expression from 1623 would affect replication in non-permissive cells as the level was sufficient to restore the function of maintaining host cell and viral protein synthesis. Single-cycle and multi-cycle replication kinetics were examined *in vitro*.

4.3.4. Single-cycle Replication Kinetics

One step growth experiments using a m.o.i. of 10 pfu/cell were carried out in permissive BHK and non-permissive 3T6 cells at 37°C (see Section 3.25.). The virus yield, at regular time intervals pi, was determined by titration on BHK monolayers at 37°C.

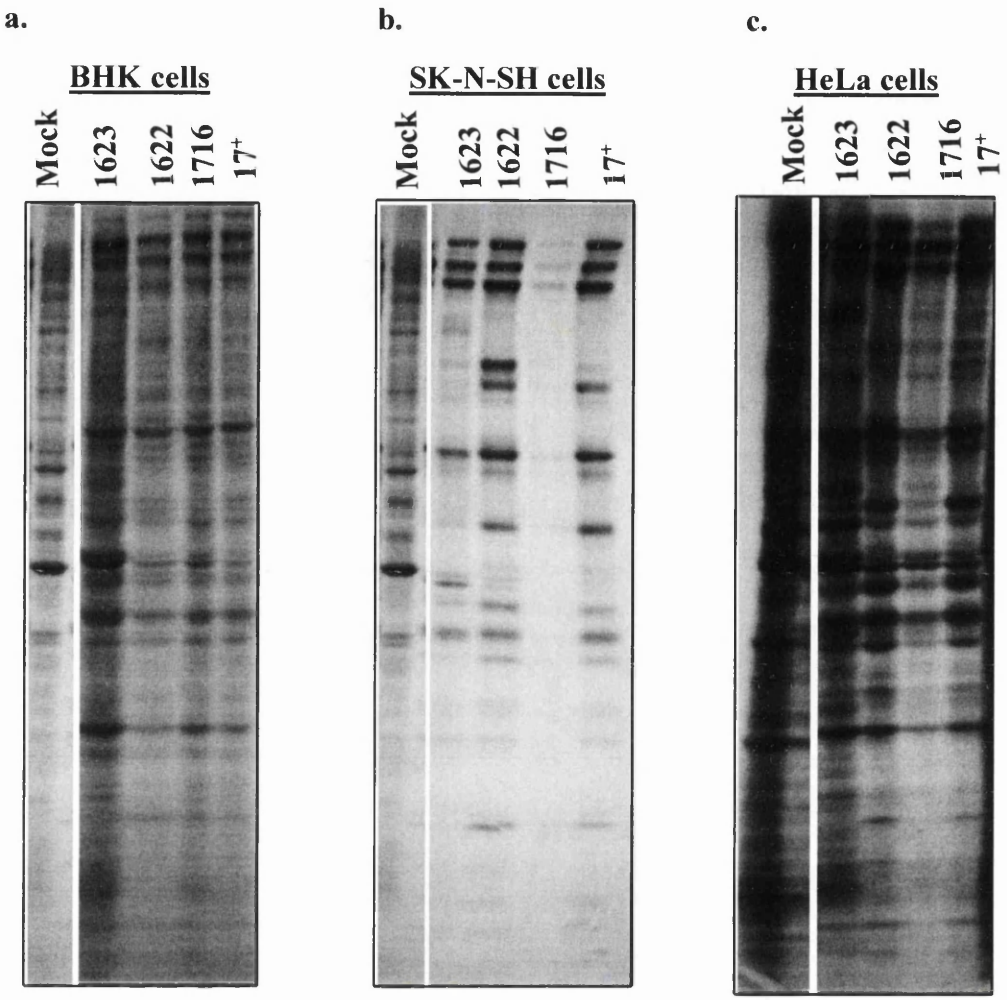


Figure 46. Host cell protein synthesis shut-off in BHK, SK-N-SH and HeLa cells.

BHK, SK-N-SH and HeLa cells were infected with a m.o.i. of 10 pfu/cell and at 14 h pi pulse labelled with ^{35}[S] methionine until 16 h pi. The infected cells were harvested at 16 h pi and analysed by 10% SDS-PAGE. The dried gel was exposed to an autoradiograph o/n at -70°C. **a**. In all BHK cell extracts, there was no difference in overall protein synthesis between viral and mock infections. **b**. In SK-N-SH cell infections, there is protein synthesis shut-off from 1716. But no shut-off in protein synthesis from 1623 and 17$^+$. **c**. Infection in HeLa cells shows protein synthesis shut-off from 1716 only.

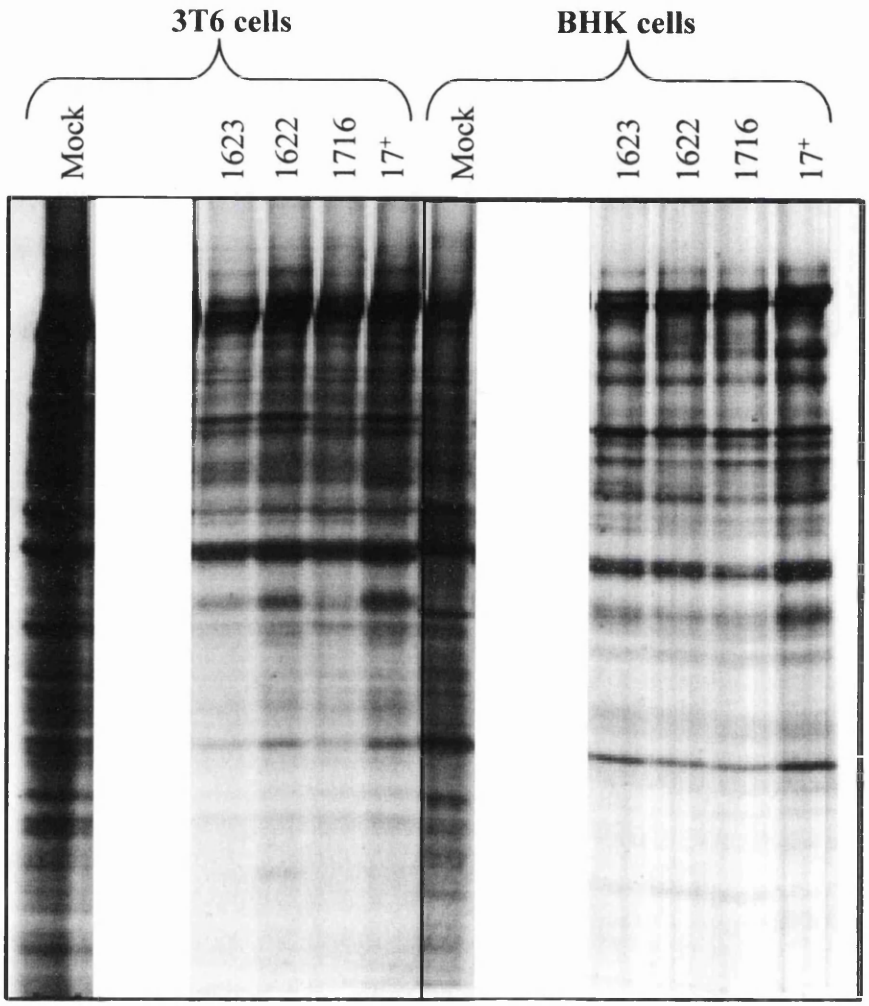


Figure 47. Host cell protein synthesis in BHK and 3T6 cells.

Infected BHK and 3T6 cells were radiolabelled with [^{35}S]methionine for 2 h (14 to 16 h pi) and harvested at 16 h pi. Samples were electrophoresed through a 10% SDS-PAGE. There is no difference in protein synthesis between BHK infected cells and 3T6 infected cells among different viral infections.

There was a slight difference between 17^+, 1716, 1622 and 1623 replication kinetics in BHK cells (Fig. 48a; Table 14).

In 3T6 cells, 1716 and 1623 did not replicate as well as 17^+ and 1622 (Fig. 48b; Table 14). 1716 replicated with reduced kinetics producing a maximum titre at 36 h pi of $1.2x10^6$ pfu/cell, 50-fold less than 17^+ or 1622 ($>5x10^7$ pfu/cell). 1623 failed to replicate in 3T6 cells having a final titre, which was the same as the input titre of $1.2x10^5$ pfu/cell, 10-fold less than 1716. This unexpected result will be discussed at the end of this section.

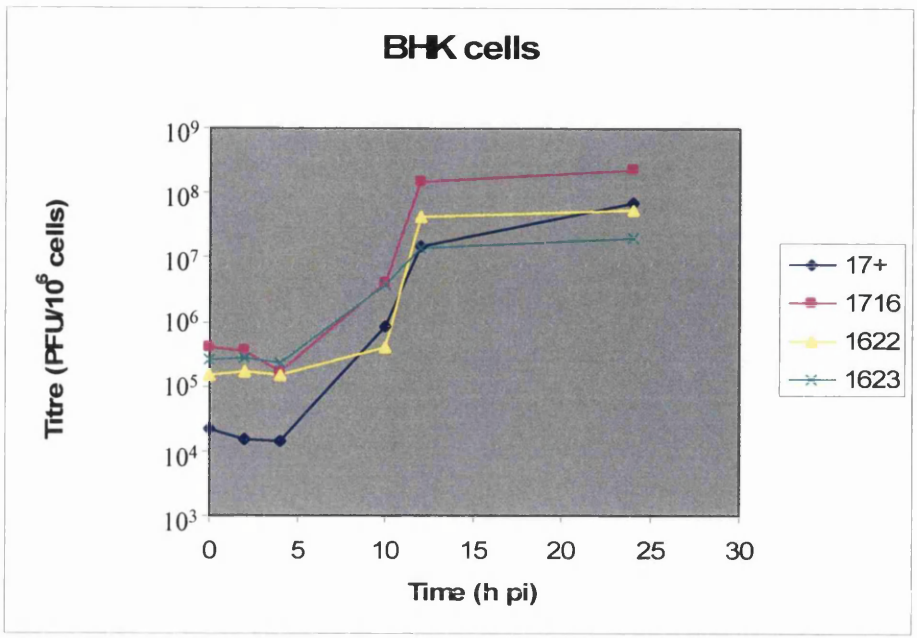
4.3.5. Multi-cycle Replication Kinetics

Multi-cycle replication kinetics using a m.o.i. of 0.001 pfu/cell mimicked the one step growth kinetics in 3T6 cells in that 1623 and 1716 grew poorly but 1622 grew as well as 17^+ (data not shown).

Using a m.o.i. of 0.001 pfu/cell to infect human and primate cells, replication of viruses was examined. In Figure 49a, Vero cells infected with 1716 and 1623 showed a marked reduction in replication. The titre from both 1716 and 1623 by 75 h pi was $1.2x10^5$ pfu/10^6 cells and $1.0x10^5$ pfu/10^6 cells, respectively (Table 15). The titre of 17^+ at 75 h was $1.1x10^7$ pfu/cell and from 1622 was $1.5x10^6$ pfu/10^6 cells. The maximum titre of 1623 was approximately 1 log less than 17^+ and 1622 at 95 h post infection and 1 log greater than 1716.

In HeLa cells (Fig. 49b; Table 15) there was a marked difference between replication of different viruses. 1716 and 1623 did not replicate in this cell line demonstrating a classic "death curve". 17^+ and 1622 replicated to reach $1.5x10^5$ pfu/10^6 cells at 75 h pi. At 130 h pi there was a difference in kinetics between 17^+ and 1622, this observed artefact was due to the age of the cells and not a true depiction of virus replication.

a.



b.

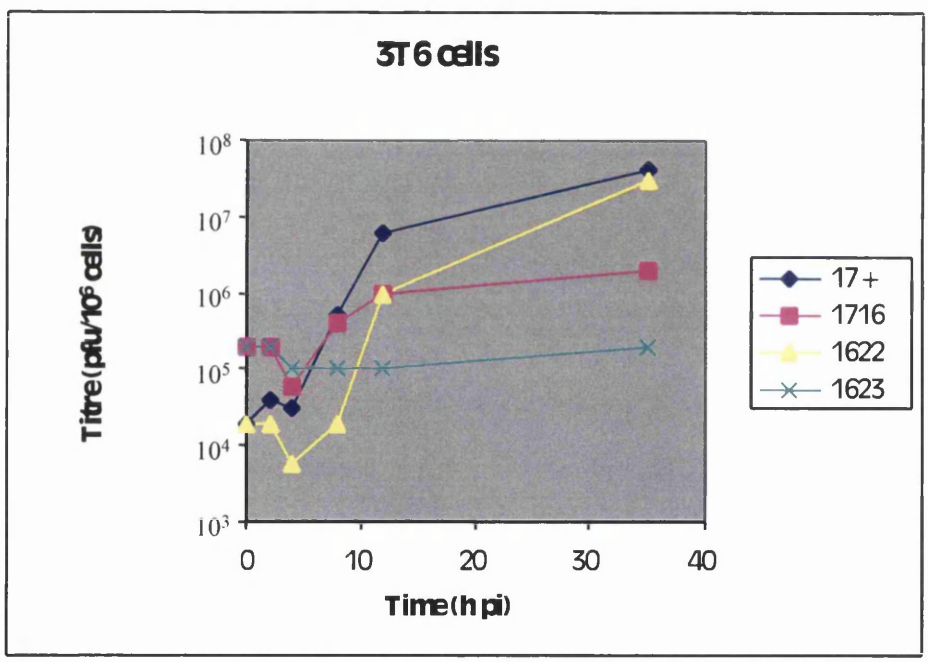


Figure 48. Single cycle replication kinetics in different animal cells.

BHK and 3T6 cells were infected at a m.o.i. of 10 pfu/cell. At regular time intervals, cells were scraped into the medium, sonicated and titrated onto a BHK monolayer. Titre (pfu/10^6 cells) is plotted on the y-axis and time (h pi) is plotted on the x-axis. **a**. All viruses grow with similar kinetics 17+ (◆), 1716 (■), 1622 (▲) and1623 (×). **b**. In 3T6 cells 1716 (■) does not replicate as well as 17+ (◆) and 1622 (▲), 1623 (×) fails to replicate.

Table 14. BHK and 3T6 titres for single cycle growth kinetics.

BHK cells				
	17^+	1716	1622	1623
0	$2.2x10^4$	$4.0x10^5$	$1.5x10^5$	$2.7x10^5$
2	$1.5x10^4$	$3.5x10^5$	$1.7x10^5$	$2.8x10^5$
4	$1.4x10^4$	$1.7x10^5$	$1.5x10^5$	$2.3x10^5$
10	$8.7x10^5$	$4.0x10^6$	$4.0x10^5$	$3.9x10^5$
12	$1.5x10^7$	$1.5x10^8$	$4.2x10^7$	$1.4x10^5$
24	$6.9x10^7$	$2.3x10^8$	$5.3x10^7$	$2.0x10^5$

3T6 cells				
	17^+	1716	1622	1623
0	$2.0x10^4$	$2.0x10^5$	$2.0x10^4$	$2.0x10^5$
2	$4.0x10^4$	$2.0x10^5$	$2.0x10^4$	$2.0x10^5$
4	$3.0x10^4$	$6.0x10^4$	$6.0x10^3$	$1.0x10^5$
8	$5.0x10^5$	$4.0x10^5$	$2.0x10^4$	$1.0x10^5$
12	$6.0x10^6$	$1.0x10^6$	$1.0x10^6$	$1.0x10^5$
35	$4.0x10^7$	$2.0x10^6$	$3.0x10^7$	$2.0x10^5$

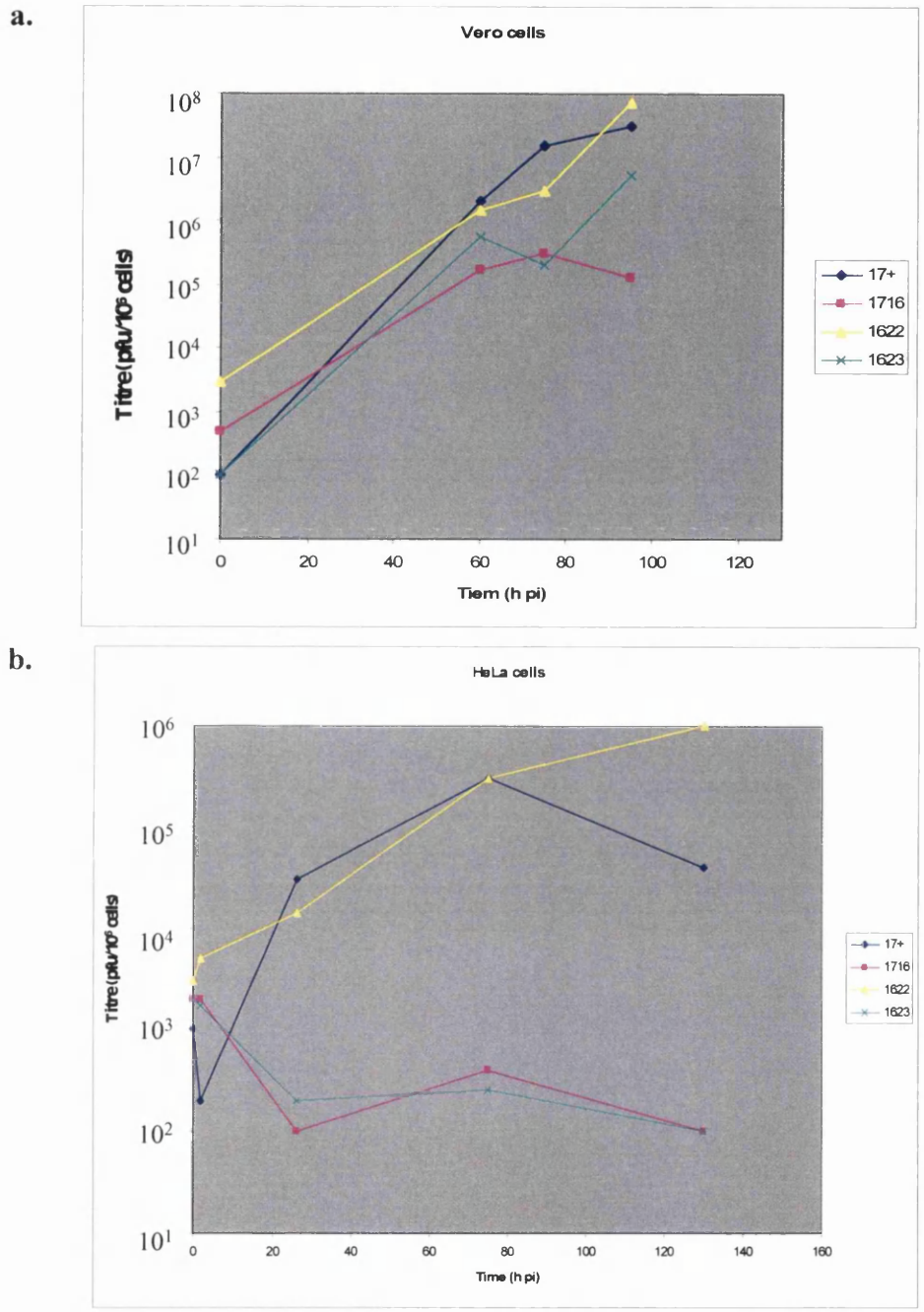


Figure 49. Multi cycle replication kinetics in primate and human cell lines.

HeLa and Vero cells were infected at a m.o.i. of 10 pfu/cell. At regular time intervals, cells were scraped into the medium, sonicated and titrated onto a BHK monolayer. **a**. 1623 replication kinetics in Vero cells was similar between 17^+ and 1622. **b**. HeLa cells infected with 17^+ and 1622 show simliar kinetics but 1716 and 1623 fail to replicate in HeLa cells.

Table 15. Vero and HeLa titres for single cycle growth kinetics.

Vero cells				
	17^+	1716	1622	1623
0	$1.0x10^2$	$5.0x10^2$	$3.0x10^3$	$1.0x10^2$
60	$2.0x10^6$	$1.7x10^5$	$1.5x10^6$	$5.7x10^5$
75	$1.5x10^7$	$3.0x10^5$	$3.0x10^6$	$2.0x10^5$
95	$3.0x10^7$	$1.3x10^5$	$7.3x10^7$	$5.0x10^6$

HeLa cells				
	17^+	1716	1622	1623
0	$1.0x10^3$	$2.0x10^3$	$3.0x10^3$	$2.0x10^3$
2	$2.0x10^2$	$2.0x10^3$	$5.0x10^3$	$1.7x10^3$
26	$1.8x10^4$	$1.0x10^2$	$1.4x10^4$	$1.0x10^2$
75	$3.0x10^5$	$1.2x10^2$	$3.0x10^5$	$1.0x10^2$
130	$5.0x10^4$	$1.0x10^2$	$1.0x10^6$	$1.0x10^2$

4.3.6. Discussion

The purpose of constructing the virus 1623 was to ensure RL1 expression from the recombinant virus system. 1623 was being constructed at the same time as 1622 (Section 4.2) and cloning was carried out before protein expression analysis could be made. It was believed that the additional 134 bp might help in the expression of the full length ICP34.5 protein. 1623, like 1622, had the feature of singly expressing RL1 without the ORF P gene expression.

By Western blotting we determined that 1623 expresses approximately eight times less ICP34.5 than wild type 17^{+}. This level of ICP34.5 expression from 1623 is able to maintain protein synthesis in neuronal SK-N-SH cells and HeLa cells at late times (16 h) post infection. The protein synthesis from 1623 is substantiated by results published by Chou and Roizman (1994) showing a mutant virus with lower ICP34.5 expression in HSV-1 strain F but which maintains protein synthesis in SK-N-SH cells.

The replication kinetics of 1623 were slightly poorer than 17^{+} in BHK cells, but the phenotypic similarity between 17^{+} and 1623 stopped there. In 3T6 cells, the input dose for 1623, 1716 and 17^{+} was the same but 1623 replicated more poorly than 1623. This was unexpected as the RL1 negative virus 1716 replicates poorly in 3T6 cells. The lack of viral replication from 1623 in 3T6 cells and HeLa cells indicates that there may be another mutation in 1623.

4.4. CONSTRUCTION OF 1624

4.4.1. Introduction

In 1994, Lagunoff and Roizman observed 16 potential ORFs which were predicted to encode 50 or more codons within the largest reported unspliced 8.3 kbp LAT. They examined expression of five of these ORFs by in-frame insertions of an epitope reacting with a monoclonal antibody against a human cytomegalovirus protein, gB. Lagunoff and Roizman (1994) demonstrated expression of two open reading frames, O and P. In HSV-1 strain KOS, a family of transcripts spanning the L-S junction have been identified (Yeh and Schaffer, 1993). However, these transcripts were only synthesized in cells infected with a virus with a mutation in ICP4. This prevented ICP4 from binding to the consensus sequence, ATCGTC, upstream of the ORF P transcription initiation site, and hence prevented transcription.

More recent studies (Lagunoff and Roizman 1994; Lagunoff *et al.,* 1996) demonstrated that ORF P is not expressed during productive infection in Vero cells by HSV-1 strain F at the permissive temperature of 37°C. However, ORF P is detectable following infection of F at the non-permissive temperature of 39.5°C as ICP4 is temperature sensitive in this strain (Lagunoff and Roizman, 1995). ORF P has also been detected following infection with a variant of strain F with the ICP4 binding site in the ORF P promoter mutagenised.

Here we wanted to examine the role of ORF P expression in a lytic HSV-1 strain 17^+ infection. Using the ORF P and RL1 deleted virus, 1716, the ORF P gene was re-introduced under the gD promoter in a novel location in the UL43 locus as described in Section 4.1. ORF P should be expressed with the early/late kinetics of gD since the ICP4 binding sequence is not present. The purpose of constructing this recombinant virus was to examine the effect of expression of only ORF P and not RL1 or any synergistic effect of ORF P and RL1 and elucidate the functional dichotomy of ORF P and RL1.

4.4.2. Isolation of the ORF P Gene

The ORF P gene fragment was first isolated using pGEM34.5 as template DNA in a PCR reaction. The oligonucleotide primers consisted of HSV-1 sequences flanking ORF P and additional *Sma*I and *Bam*HI restriction enzyme sites at the ends for ease in sub-cloning into pFJ14H. The primer sequences are shown in Figure 50b. Initial PCR reactions consisted of varying primer and template DNA concentrations (Table 16).

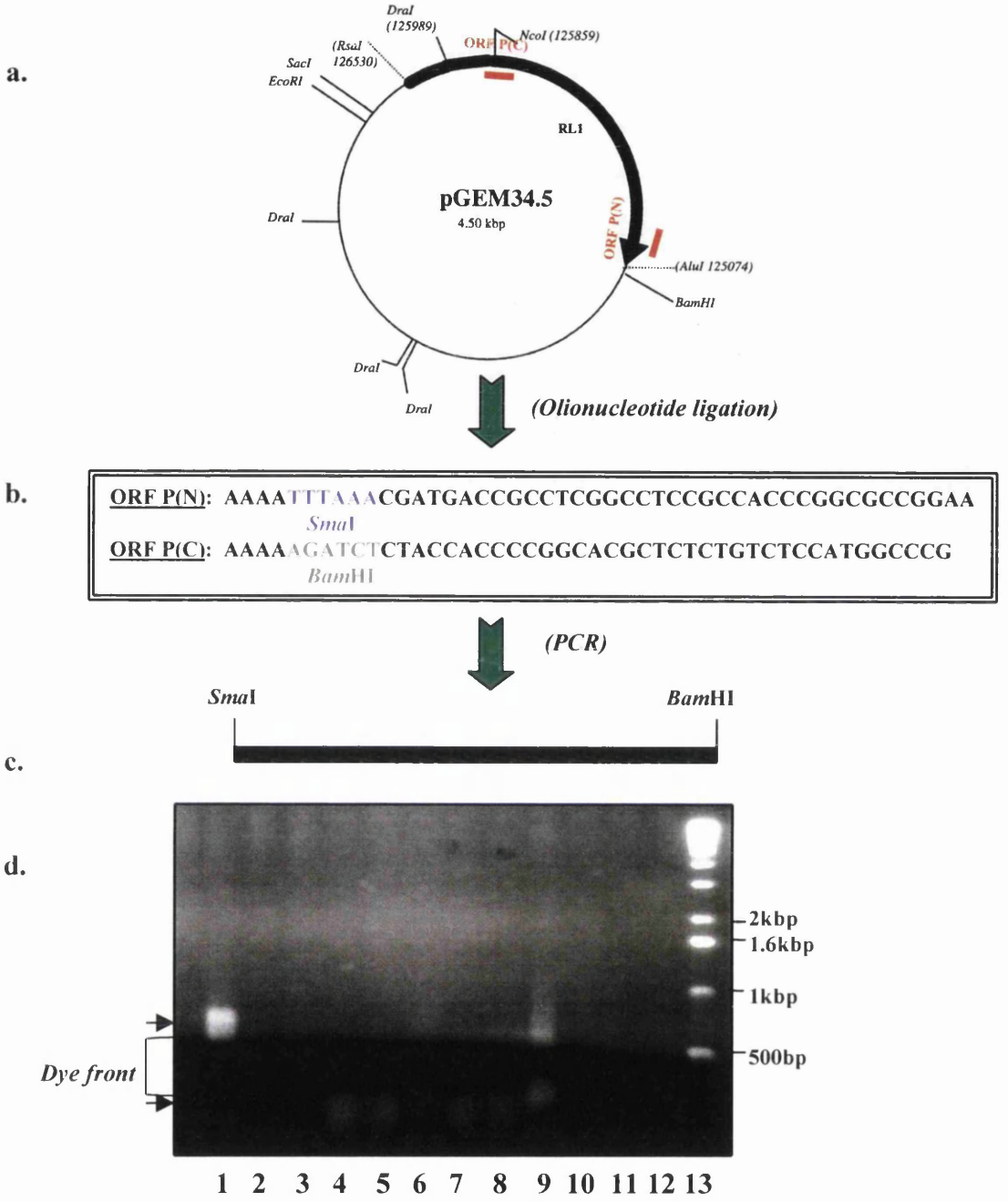


Figure 50. ORF P gene isolation from plasmid pGEM34.5.

PCR was carried out on pGEM34.5 (**a**.) using primers ORF P(N) and ORF P(C) whose sequence is shown **b**. **c**. PCR generated a 705 bp fragment containing the entire ORF P gene depicted in this cartoon. **d**. There were several controls used in the PCR reaction (see Table 3 for exact details). Only one condition generated the ORF P gene (lane 1) which was isolated on a 1% agarose gel and purified for further cloning into the plasmid pFJ14H. The 1 kbp DNA ladder (lane 13) shows the 705 bp ORF P gene. Lanes 4 through 9 show excess primers. Lane 9 also shows a partial PCR product which was not used.

The optimised concentration of primers (75pM each) and template DNA (0.5 ng) generated a large amount of 705 bp ORF P product which was analysed by electrophoresis through a 1.0% (w/v) agarose gel (Fig. 50). There was a partial product from conditions of 0.5 ng template DNA and 100pM primer that resulted in smearing (Fig. 50; lane 9).

Unfortunately, this ORF P product continued to be lost during the gel isolation experiments and consequently not enough DNA was isolated and successfully ligated into pFJ14H. The next strategy used to isolate the ORF P gene alone was to use a plasmid cloned by Mr. R. Reid (unpublished). Reid performed PCR on pGEM34.5 using different parameters and ligated the ORF P product into a pGEX vector. The plasmid containing ORF P was named pGEX/ORF P. This plasmid fused ORF P in frame with GST and the protein was used to generate the ORF P antisera used in this study. Using pGEX/ORF P the ORF P gene was isolated by *Sma*I and *Bam*HI digestion (data not shown). This 704 bp fragment was previously sequenced by Reid (personal communication). Subsequently, it was sub-cloned into pFJ14H.

4.4.3. Sub-cloning ORF P under the gD Promoter

The ORF P fragment was sub-cloned into pFJ14H using compatible restriction enzyme sites. Ligation of ORF P into pFJ14H positioned it immediately downstream of the HSV-1 gD promoter and directly upstream of the HSV-2 immediately early IE5 polyadenylation sequences. pFJ14H contains *lacZ* under the control of a SV40 promoter with its own SV40 polyadenylated sequence (Fig. 51). The *lacZ* gene is in the opposite orientation to the ORF P gene. pFJ14H derived plasmid was digested with *Xba*I and the ORF P/*lacZ* cassette isolated from pFJ14H.

4.4.4. Sub-cloning pFJ14H ORF P Cassette into p35(PacI)

The ORF P/*lacZ* cassette from pFJ14H was sub-cloned into p35(PacI) by methods similar to those described in Section 4.1. p35(PacI) was digested with *Spe*I (cleavage site in the linker) which is compatible with *Xba*I which enabled the ORF P/*lacZ* cassettes to be ligated and generated plasmid pHH3 (Fig. 51).

4.4.5. Generation of 1624

Cloning of the ORF P gene was difficult and subsequently isolation of the recombinant virus 1624 was delayed. Unlike 1622 and 1623, 1624 was never plaque

Table 16. The conditions for obtaining ORF P from PCR reaction.

Lane Number (Corresponds to Figure 50)	pGEM34.5 DNA (ng concentration)	Primer DNA (molar concentration for each primer)
1*	0.5*	75pM*
2	0.5	200pM
3	5.0	75pM
4	5.0	200nM
5	0.05	100pM
6	0.05	50pM
7	0.05	10pM
8	0.05	5pM
9	0.5	100pM
10	0.5	50pM
11	0.5	10pM
12	0.5	5pM

Overall reaction mix:	
Template DNA	X
Primer (ORF P (N))	X
Primer (ORF P (C))	X
H_2O	*up to 100µl*
Buffer (10x)	10.0
dNTP [25pM/µl]	4.0
Vent DNA polymerase [1U/µl]	0.5

* Corresponds to the ORF P PCR product.

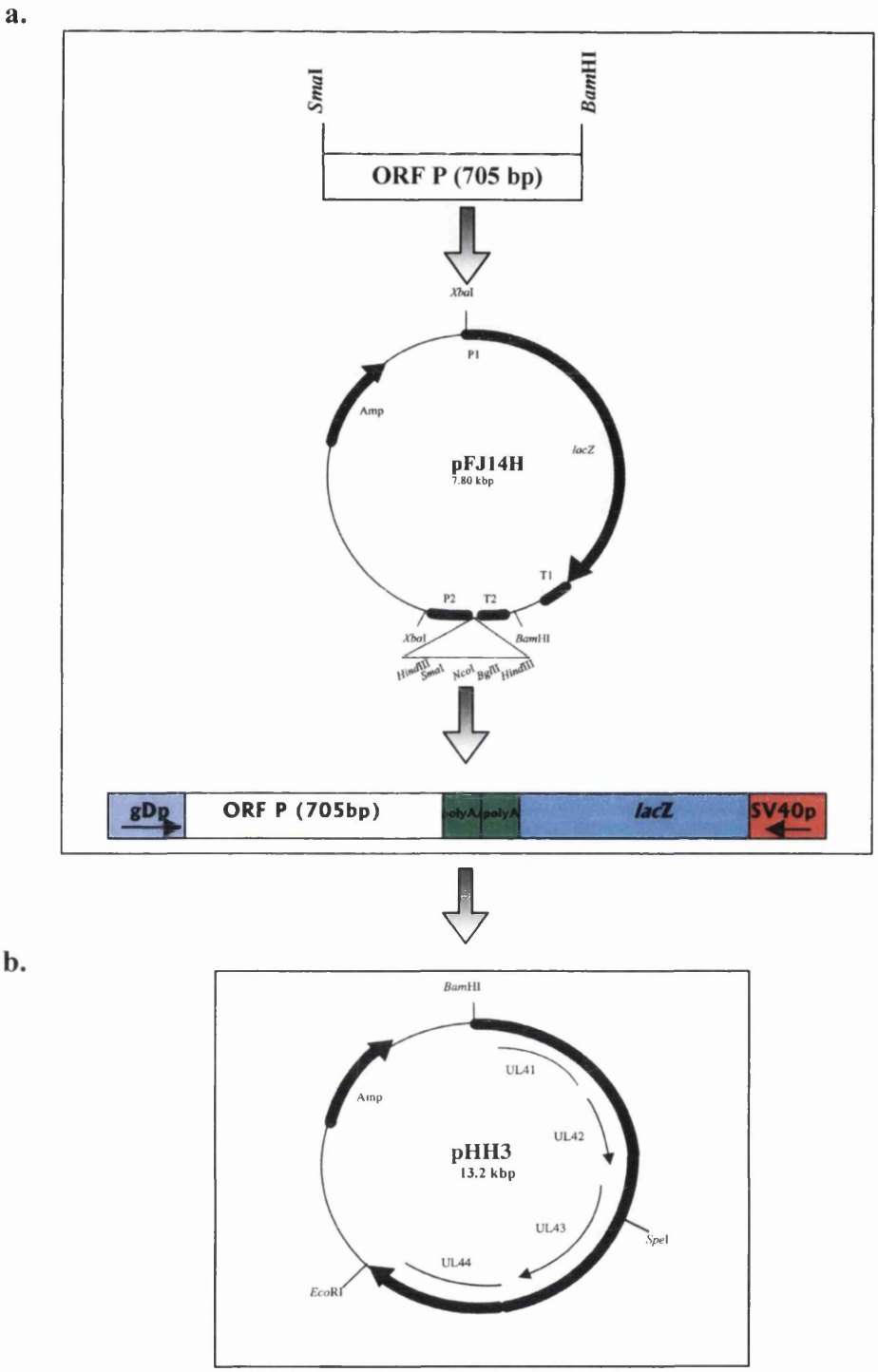


Figure 51. Diagram of ORF P sub-cloning.

a. The fragment containing the gene of interest, ORF P 705 bp was digested from pFJ14H with *Xba*I where it had been placed under the gD promoter with the HSV-2 IE5 polyadenylation and *lacZ* under the SV40 promoter in the opposite direction. This *Xba*I fragment was gel purified and ligated into the *Spe*I site of p35(PacI).
b. p35(PacI) containing the *Xba*I insert was named pHH3.

purified as described in Section 4.1. A diagrammatic representation of the recombinant virus is shown in Figure 52.

4.4.6. Expression of ORF P

Two antisera (129 and 130) against a GST/ORF P fusion protein were previously generated in this laboratory (Reid, unpublished data). Initial findings using the 129 antiserum in Western blot analysis of several viruses grown at different temperatures found that 17+ expresses ORF P during wild type infection (McKie and Reid, unpublished data). I repeated these findings using 129 in a Western blot with BHK infected cells at 37°C for 16 h (Fig. 53). Different bands were detected migrating around the size of the expected ORF P protein. In 17^{+}, a band of 32 kDa was detected (Fig 53 and 54), but in tsK (ICP4 temperature sensitive at 39.5°C) a band of 25 kDa was detected. 1621 was the only other virus that had detectable ORF P expression. 1621 is a recombinant virus that I constructed from 17^{+} which has the intact RL1 gene with a 34 bp epitope tag at the initiating methionine. 1621 shows two ORF P bands at 26 kDa and 33 kDa (Fig. 53 and 54). It is not known why there are apparent M_r differences in ORF P proteins between 1621 and tsK, and wild type. In 1716 and other HSV-1 and HSV-2 infected cell extracts no ORF P bands were detected. For a complete listing of the various mutant viruses used in Figure 53, see Table 17. In 1716, the ORF P gene has been deleted and hence we do not see any ORF P protein expression. There was no ORF P protein detection from any of the HSV-2 viruses.

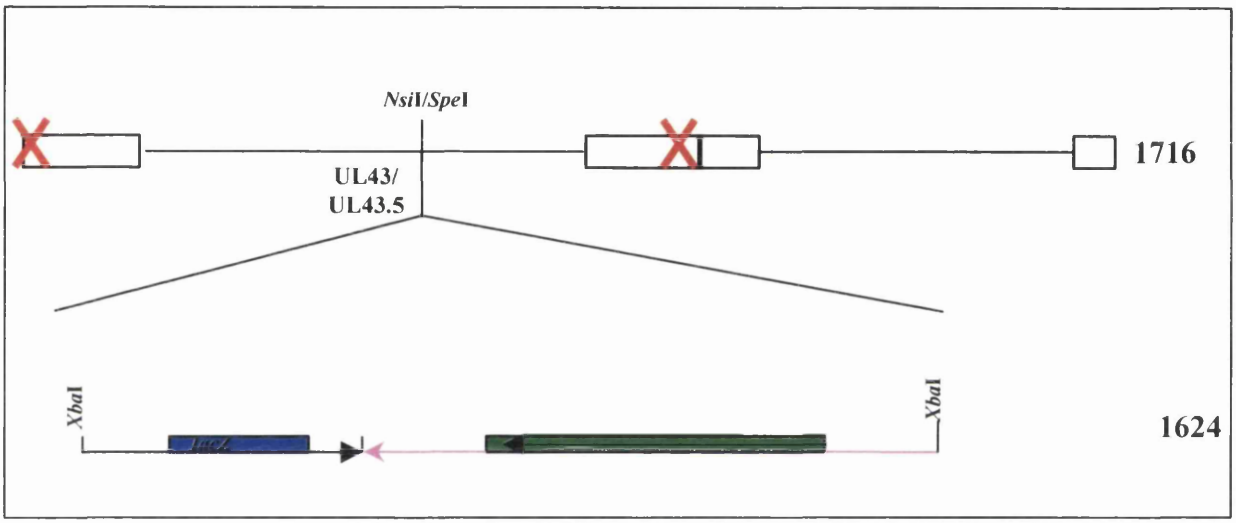


Figure 52. Schematic representation of recombinant virus 1624.

The HSV-1 strain 17^+ RL1 mutant virus 1716 was used as the parental strain for the recombinant virus. 1624 has the ORF P open reading frame inserted downstream of the HSV-1 gD promoter and upstream of the HSV-2 IE5 polyadenylation sequence. It also has *lacZ* in the opposite orientation for detection of recombinant viruses upon X-gal staining. X Denotes the RL1 deletion in 1716.

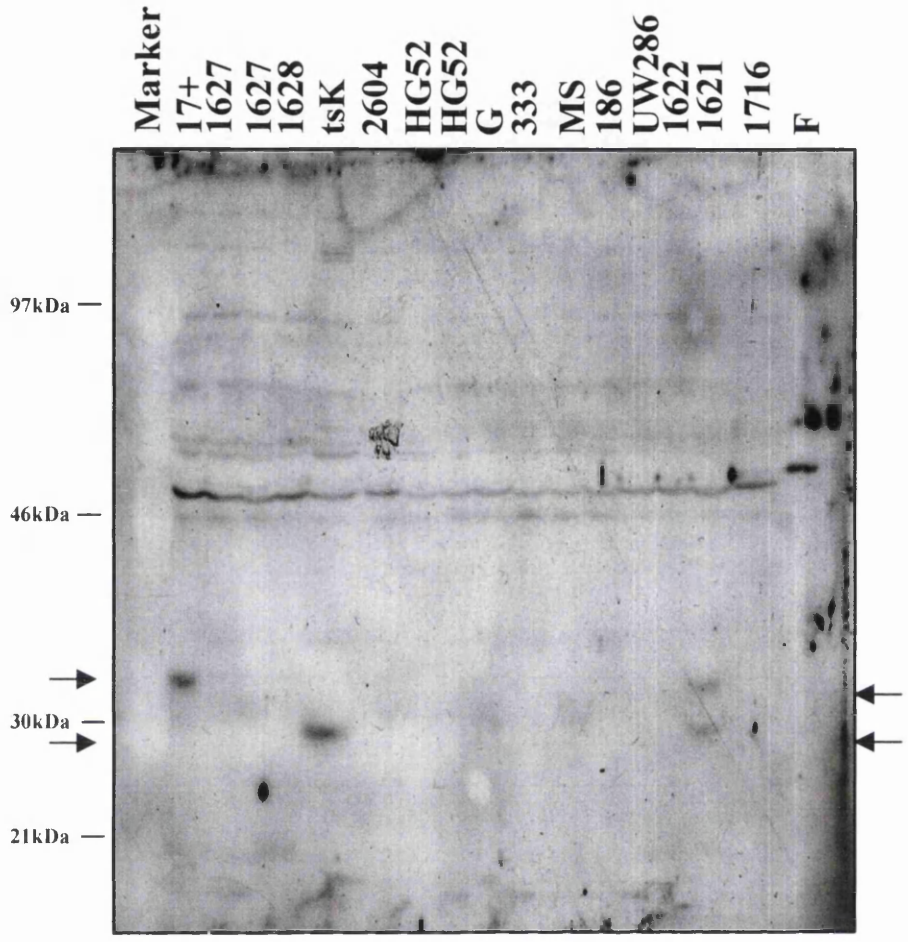


Figure 53. Western blot of ORF P expression using 129.

Infected BHK cells were harvested 16 h pi and a Western blot of a 10% SDS-PAGE with 129 is shown here. In 17^{+} the 32 kDa ORF P is detected and denoted by (◄). In tsK (ICP4 null mutant) only the 25 kDa ORF P protein is detected. In a recombinant virus 1621 which has RL1 with an epitope tag, two bands at 26 kDa (◄) and 33 kDa (◄) are detected.

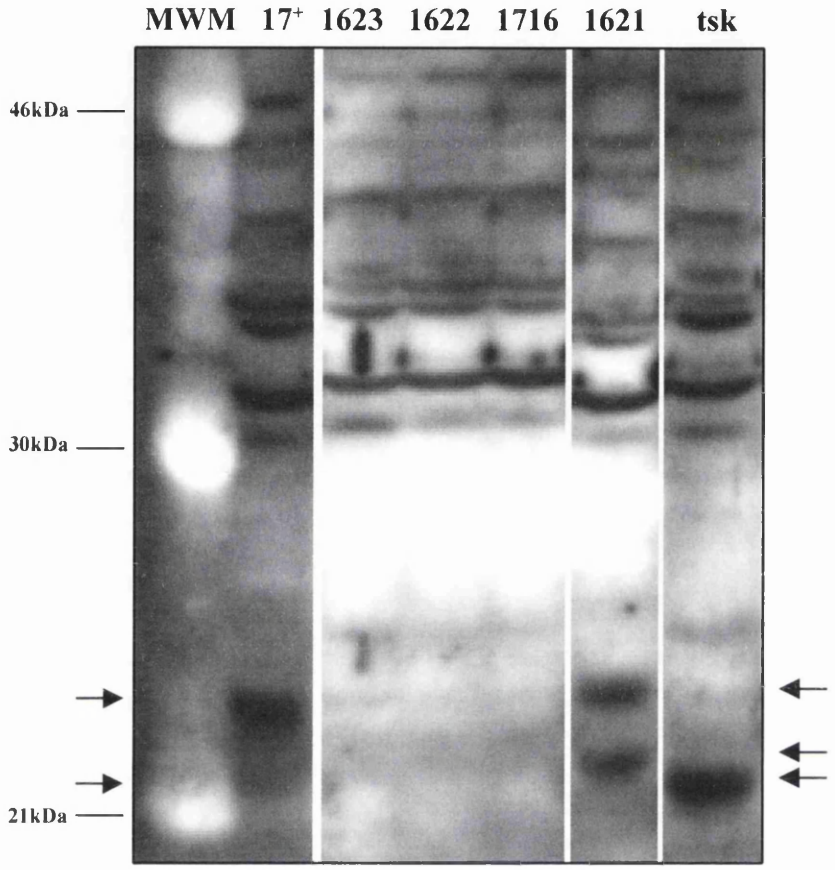


Figure 54. Western blot of ORF P expression using 129.

A Western blot of a gradient SDS-PAGE using 129 demonstrated a better separation of the different ORF P forms. Using a different set of BHK infected extracts 16 h pi, the 25 kDa ORF P protein is detected in 17^+ (◀) but not in tsK. The tsK mutant has a 22 kDa ORF P protein detected (◀). 1621 has ORF P proteins migrating at 23 kDa and 25 kDa, as above.

Table 17. Names and brief description of HSV-1 and HSV-2 viruses.

HSV-1	
17^+	**wild type HSV-1 strain**
1716	**RL1 deleted 17+ mutant**
tsK	**ICP4 temperature sensitive 17+ mutant**
1621	**Epitope tag RL1 17+ mutant**
1622*	**Recombinant RL1 overexpressed in 1716**
1627*	**Intertypic HSV-2 RL1 with intron in 1716**
1628*	**Intertypic HSV-2 RL1 without intron in 1716**
HSV-2	
HG52	**wild type HSV-2 strain**
2604	**RL1 deleted HG52 mutant**
G	**wild type HSV-2 strain**
333	**wild type HSV-2 strain**
UW286	**wild type HSV-2 strain**
MS	**wild type HSV-2 strain**

*** = Characterized in this text.**

4.4.7. Discussion

Isolating the ORF P gene was not straight forward, the first strategy used in obtaining ORF P was PCR. Although PCR did generate an ORF P product the frequency was very low (1 in 20). The second strategy, using a plasmid containing the ORF P gene cloned by Reid, was more successful. The ORF P gene digested from this plasmid was sub-cloned into pFJ14H. The ORF P/*lacZ* cassette was then sub-cloned into p35(PacI) yielding the new plasmid pHH3. 1716 DNA and pHH3 were co-transfected to generate the recombinant virus 1624. The plaque purification of 1624 was not completed since cloning difficulties delayed transfection of this recombinant virus. The aim of generating 1624 was to directly compare it with 1622, or another recombinant virus which only expresses ICP34.5. The future of 1624 will hopefully determine the phenotype attributed to ORF P *in vitro* and *in vivo* in strain 17^+.

A novel result found in this study using other mutant viruses and the antisera made by Reid is that strain 17^+ can express ORF P during a lytic infection at 37°C. This contradicts published work using HSV-1 strains F and KOS which demonstrated that ORF P was repressed by a strong ICP4 binding site in the promoter. Another mutant virus, 1621, also showed expression of different forms of ORF P. 1621 was a mutant virus generated to locate at ICP34.5 by immunofluorescence with a 34 bp epitope at the initiating methionine. The 11 amino acid tag at the carboxy terminus of ORF P in 1621 probably contributes to the increased size of ORF P proteins by SDS-PAGE. HSV-2 viruses had no detectable ORF P expression by Western blotting.

5. HSV-2 RESULTS AND DISCUSSION

5.1. CONSTRUCTION OF A HSV-2 ICP34.5 /GST FUSION PROTEIN

5.1.1. Introduction

Due to the inability of HSV-1 ICP34.5 antisera to recognize HSV-2 ICP34.5, we generated antisera against ICP34.5 of HSV-2 strain HG52. In order to do this, a HSV-2 ICP34.5/GST fusion protein was constructed. The relevance of these findings will be discussed in the following sections and in the Final Discussion (Section 7).

5.1.2. Cloning of pGEX-2T(N+1) and HSV-2 RL1

The 782 bp PCR fragment generated from pSB2 (Section 5.2) which contains the complete ICP34.5 ORF, including the initiating methionine but lacking the intron was digested with *Hin*dIII and ligated in frame to the 3'end of GST at the *Hin*dIII site of the vector pGEX-2T(N+1) (Fig. 55, 56; Merideth *et al.*, 1994). Recombinant colonies were isolated and their DNA structure determined (data not shown).

AGA	ATT	CGG	**AAG CTT**	**CGC**	**CGA**	**GCC**	**CAG**	**CCG**	**CCC**	**GCC**	**(ATG)**
R	I	R	**K**	**L**	**R**	**R**	**A**	**E**	**P**	**P**	**A**	**(M)**

Figure 55. Amino acids at the junction of the GST and ICP34.5 fusion protein. Sequence at the junction between the 3' end of GST in pGEX2T(N+1), and the RL1 PCR product from pSB1. The encoded amino acids are indicated below the DNA sequence. The RL1 PCR product, ending in the initiating ATG, is in bold text. The ATG is bracketed to indicate it was not in the primer used to generate the PCR product. The *Hin*dIII site used for cloning into pGEX-2T(N+1) is underlined.

5.1.3. Protein Purification

To express the GST/ICP34.5 fusion protein, plasmids were transformed into *E. coli* strain BL21. Following induction with IPTG, the 26 kDa GST band seen with pGEX-2T(N+1) was replaced by several bands, the largest of which corresponded to the expected 55 kDa size of the fusion protein, based on the number of amino acids of ICP34.5 (261) and the apparent M_r of the HSV-1 ICP34.5/GST fusion protein (Brown *et al.*, 1997).

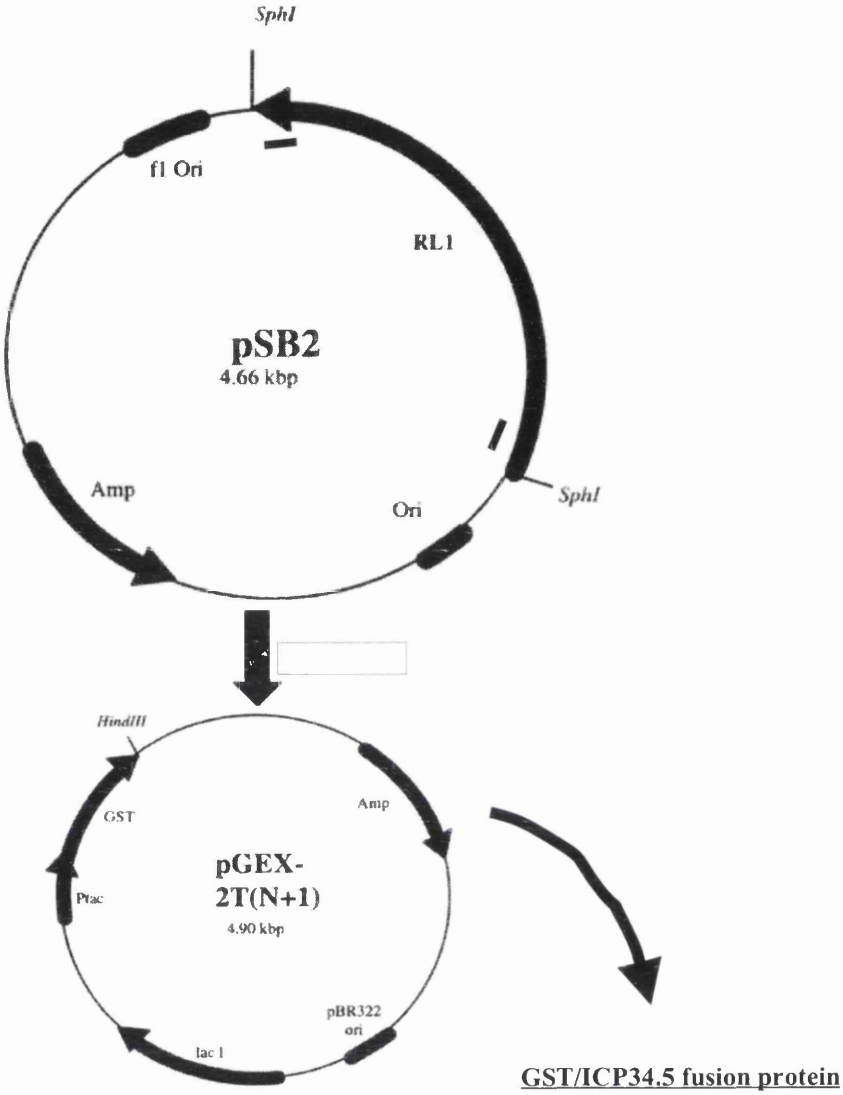


Figure 56. Diagrammatic representation of cloning HSV-2 ICP34.5/GST fusion.

The HSV-2 RL1 PCR product from pSB2 which contained the RL1 gene was sub-cloned into pGEX-2T(N+1). The position of the primers are marked by blocks. This generated a fusion between glutathione S-transferase and ICP34.5.

Despite the presence of protease inhibitors, there was an abundance of lower M_r products, which were believed to be the result of proteolytic degradation. All of these proteins bound efficiently to the glutathione agarose beads and were readily eluted with reduced glutathione. These bands were apparent both on Coomassie staining (Fig. 57a) and Western blotting with an anti-GST antibody (Fig. 57b).

5.1.4. Screening of Antisera 596 and 597

A sufficient quantity of fusion protein was generated by bulk growth, to immunise two New Zealand White rabbits (596 and 597) as described in Section 3.32. Test bleeds were taken every seven weeks, and a final bleed taken 20 weeks post immunization. Antisera were screened against HSV-2 infected cells by Western blotting. Extracts were prepared from mock infected cells and cells infected with HSV-2 strain HG52 and the RL1 deletion mutant 2616. Both antisera (596 and 597) specifically detected ICP34.5 in HG52 extracts up to a dilution of 1:800 (Section 5.3).

5.1.5. Interaction of HSV-2 ICP34.5 with PCNA

Previously, Brown *et al* (1997) demonstrated that ICP34.5 of HSV-1 interacts with the 36 kDa proliferating cell nuclear antigen (PCNA) through the 63 amino acid conserved domain at the carboxy terminus. To determine if HSV-2 ICP34.5 had a similar interaction a pull-down experiment was performed using the GST/HSV-2 ICP34.5 protein. The pulled down proteins were analysed by Western blot using a monoclonal antibody against PCNA. This monoclonal antibody specifically recognised a 36 kDa protein present in BHK cell extracts (Fig. 58). This protein was pulled down by HSV-2 ICP34.5/GST but not by GST alone demonstrating that ICP34.5 of HSV-2 also interacts with PCNA.

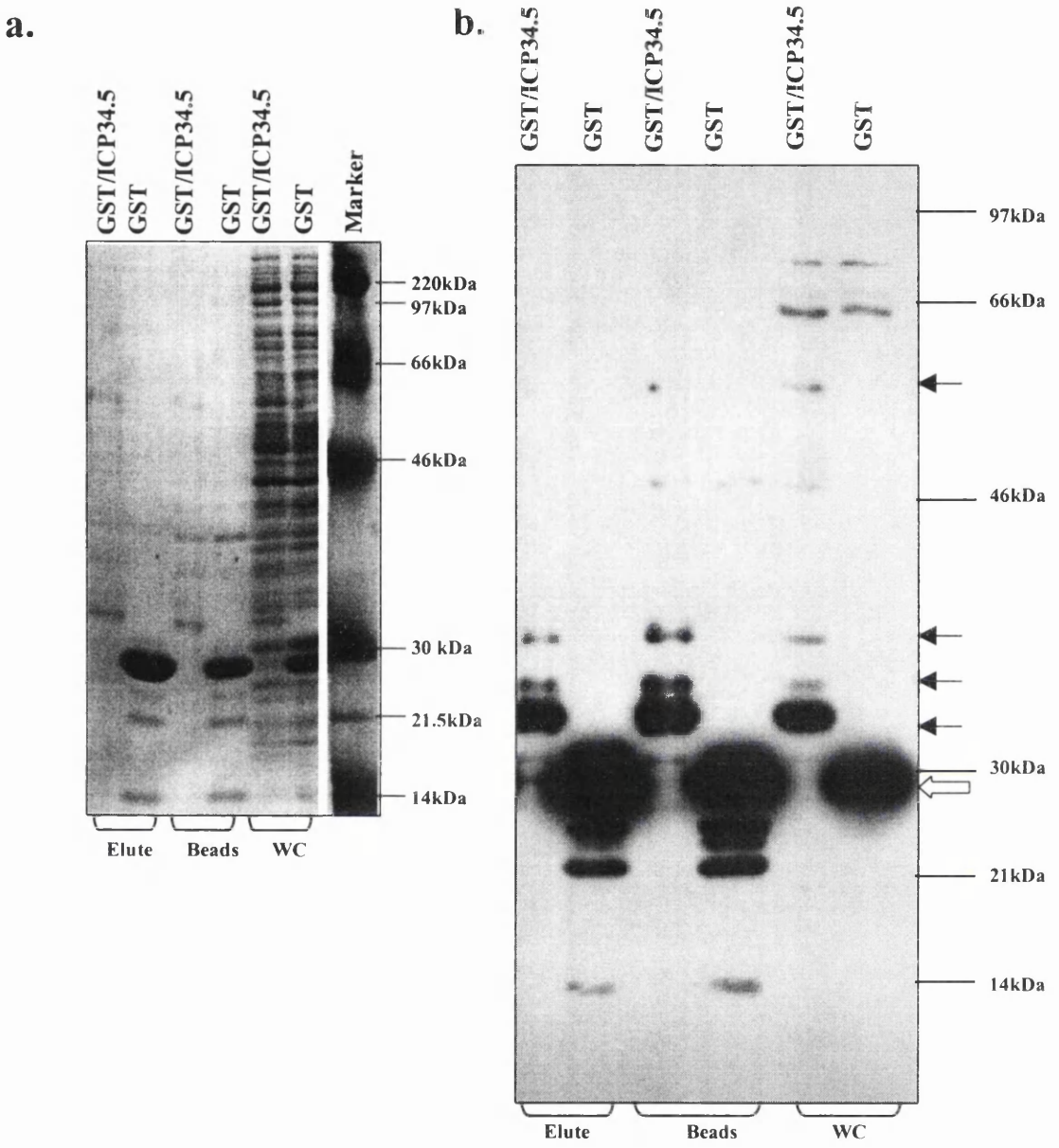


Figure 57. Induction of GST/ICP34.5 fusion protein.

a. Coomassie stained gel of GST and GST/ICP34.5 fusion protein with Markers shown.
b. A Western blot of GST or GST/ICP34.5, analysed by 10% SDS-PAGE,
Western blotted and reacted with a GST monoclonal antibody. Samples eluted from beads (Elute), samples bound to glutathione agarose beads (Beads) whole cell extracts (WC). A novel GST/ICP34.5 fusion protein migrates at 55 kDa with predominant degradation products at 45 kDa, 40 kDa and 36 kDa . GST is also indicated by an open arrow.

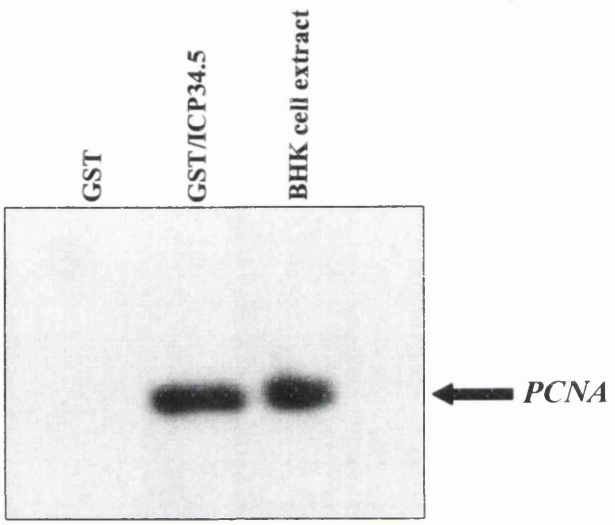


Figure 58. Western blot of a pull-down of PCNA by GST/ICP34.5.

A GST pull-down using the GST/ICP34.5 fusion protein (with GST as a control) was performed on BHK cell extracts, analysed by 10% SDS-PAGE, Western blotted and reacted with a monoclonal antibody to PCNA. The 36 kDa PCNA protein is indicated.

5.2. CONSTRUCTION OF HSV-2 RL1 RECOMBINANT VIRUSES

5.2.1. Introduction

The protein, ICP34.5, encoded by the RL1 gene was first detected in HSV-1 (Chou and Roizman, 1986, 1990; McKie *et al.*, 1994). The RL1 gene has also been demonstrated in HSV-2 strain HG52 (McGeoch *et al.*, 1991; Dolan *et al.*, 1998). The HSV-2 RL1 gene has a high level of homology especially at the carboxy terminus with HSV-1 RL1, although there is a 154 nucleotide intron present in the HSV-2 gene which is not present in HSV-1 (Harland *et al.*, 1996).

The functional similarities of HSV-1 and HSV-2 RL1 have been studied through deletion mutants in both viruses. Deletion of the RL1 ORF causes replication defects in certain cell types and renders the virus non-pathogenic *in vivo*. In HSV-1, ICP34.5 has been detected by Western blots with polyclonal antisera raised against the HSV-1 RL1 ORF. These antisera do not recognise HSV-2 ICP34.5. In order to identify ICP34.5 in HSV-2 a 2-fold strategy was devised. First, recombinant viruses expressing HSV-2 RL1 under a strong promoter in an HSV-1 RL1 deleted backbone were constructed. Second, antisera to a HSV-2 RL1/GST fusion protein were raised (Section 5.1).

Recombinant viruses were studied to determine if there is a difference in ICP34.5 expression in the presence or absence of the intron. Cassettes expressing ICP34.5 were inserted into the nonessential UL43 locus in the RL1 deletion mutant 1716. The basic cassette was composed of *lac*Z downstream of the SV40 promoter in one orientation and RL1 in the other orientation downstream of the HSV-1 gD promoter. These cassettes will be described briefly since they follow the same experimental procedures as for the mutant viruses 1622, 1623 and 1624 described in detail in previous sections (Sections 4.2-4.4).

5.2.2. pSB1 and pSB2

The HSV-2 RL1 gene was originally cloned from the *Bam*HI *g* fragment (Fig. 59; Davison, 1981). pSB1 contains the intact RL1 gene from HSV-2. pSB2 was constructed by site directed mutagenesis to specifically delete the intron within RL1 (Bdour, 1995) (Fig. 60c). PCR was performed on pSB1 and pSB2 to isolate RL1 with and without the intron (Fig. 60b and d, respectively). PCR products varied in length due to the intron. The RL1 PCR product from pSB1 which included the intron was 1015 bp and was correspondingly 154 bp longer than the PCR product from pSB2 which is 961 bp. The oligonucleotide primers were designed to have *Hin*dIII ends for compatibility of sub-

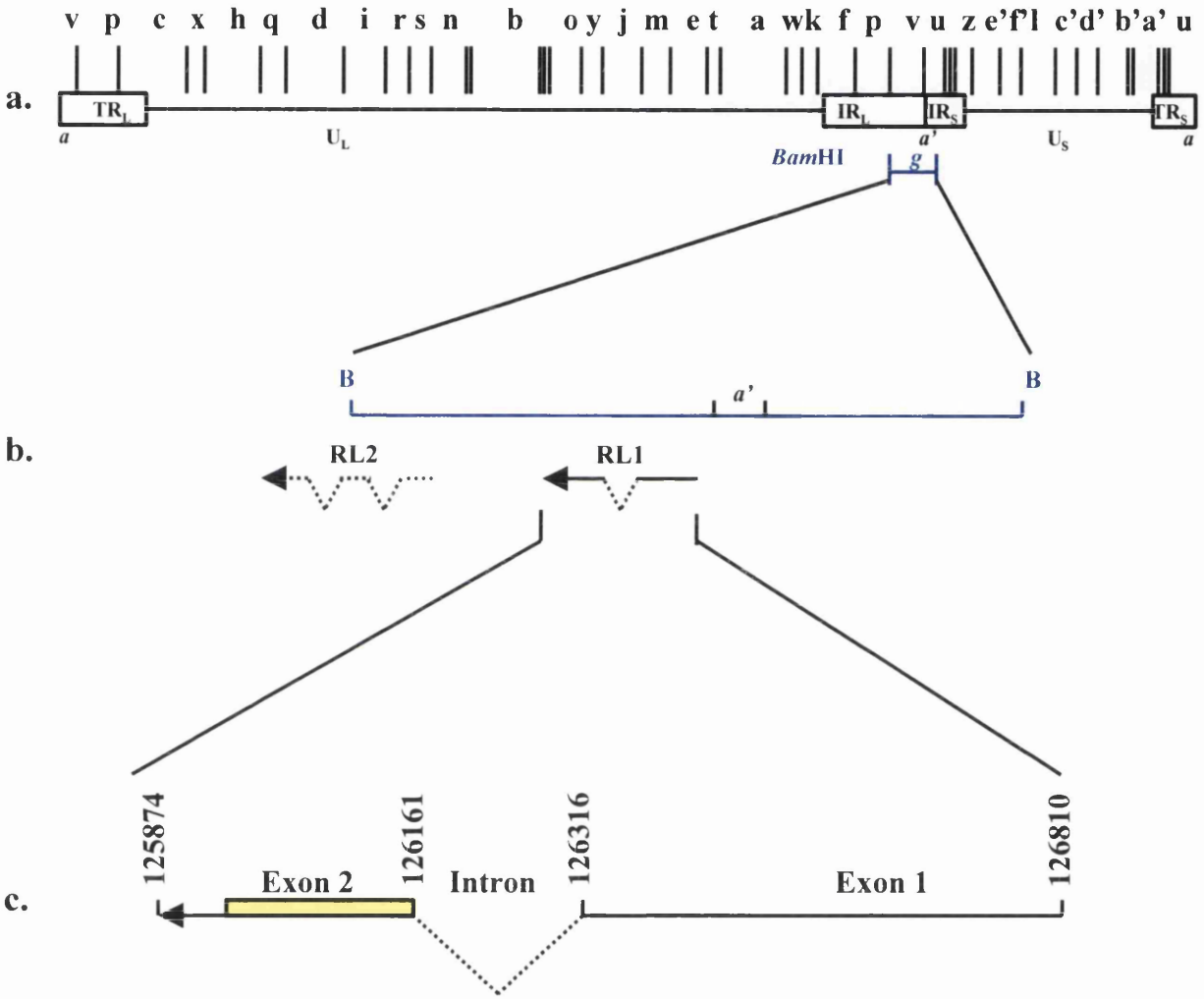


Figure 59. Schematic representation of *Bam*HI sites in the HSV-2 genome and RL1 diagrammatic location.

a. HSV-2 strain HG52 genome with *Bam*HI sites indicated by a through f'. The *Bam*HI fragment, *g*, the L/S junction fragment consists of the *v* and *u* sequences. **b**. The *g* fragment expanded to show the position of the RL1 and RL2 genes and the *a*' sequence. **c**. The RL1 gene including n.p. positions of the IR_L copy of the gene and intron. The consensus sequence found in Exon 2 is indicated by the yellow box.

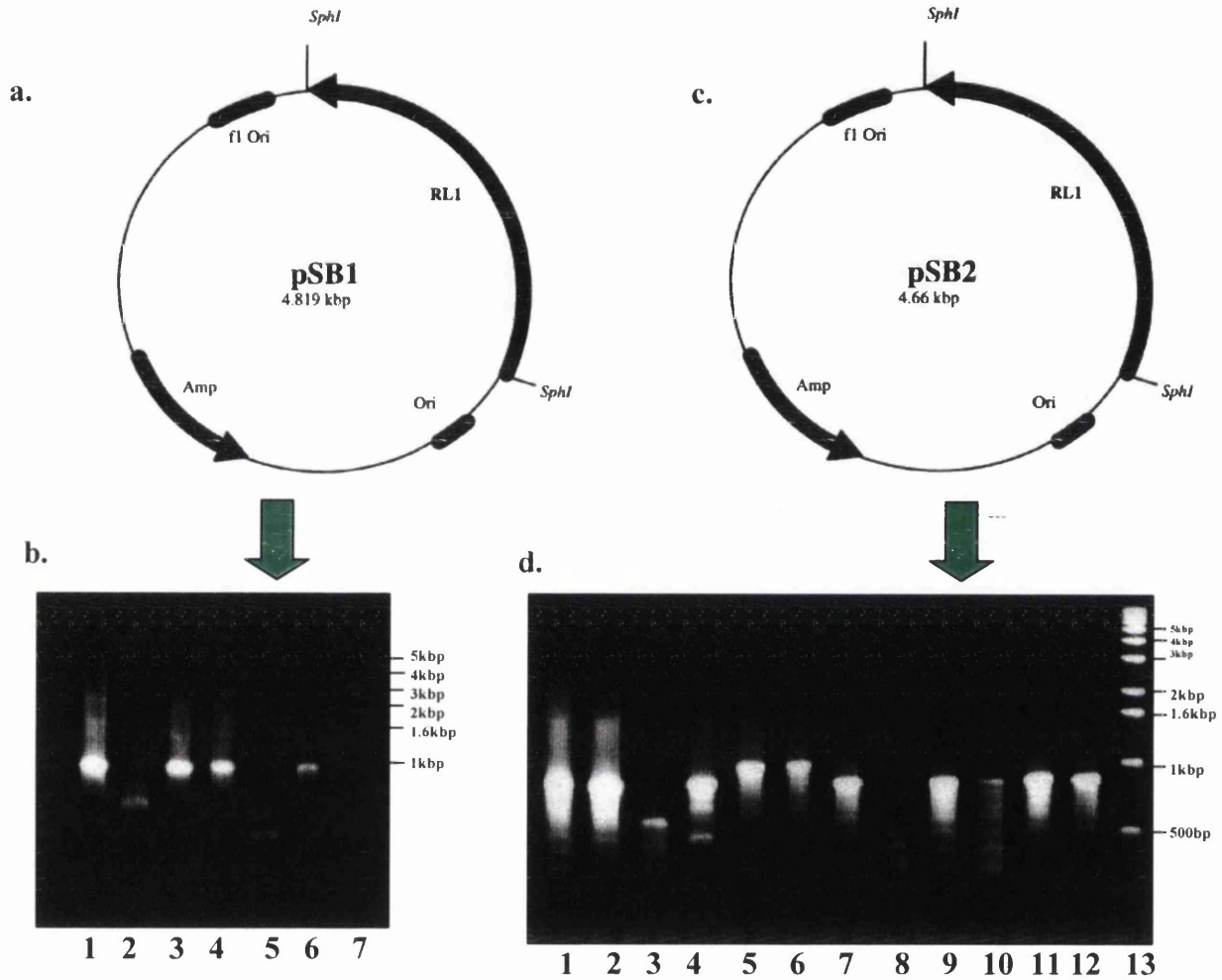


Figure 60. pSB1 and pSB2 PCR products containing HSV-2 RL1.

a. and **c**. are maps of the HSV-2 RL1 containing plasmids.
b. and **d**. 2% agarose gels of PCR products from the pSB1 or pSB2. **b**. Lanes 1, 3, 4, and 6 contain the correct size PCR product, 1015 bp from RL1. **d**. Lanes 1,2, 4, 7, 9, 11, and 12 contain the correct size PCR product, 961 bp from pSB2

cloning into pFJ14H.

5.2.3. pFJ14H/I$^+$, pFJ14H/I$^-$ and p35(PacI)

The HSV-2 RL1 PCR products were sub-cloned into pFJ14H placing the gene downstream of the gD promoter (Fig. 61a,b). Thus two new plasmids, pFJ14H/I$^+$ and pFJ14H/I$^-$, were constructed. The orientation of the PCR product was determined to ensure RL1 was in the correct orientation with respect to the gD promoter (data not shown). These two plasmids were digested with *Xba*I to isolate the RL1 and *lacZ* genes in one cassette which was sub-cloned into plasmid p35(PacI) (Fig. 61c,d). These plasmids were separately co-transfected with 1716 DNA to construct recombinant viruses.

To confirm the gene structure integrity, the RL1 PCR fragments were sub-cloned into the plasmid pGEM3zf(-) and sequenced with forward and reverse primers with an ABI 370A automated DNA sequencer at the Institute of Virology, Glasgow. These sequences were placed in the GCG program 'best-fit' to confirm sequence identity.

5.2.5. Construction of 1627 and 1628

Co-transfections of recombinant plasmid p35(PacI), containing HSV-2 RL1 with or without the intron, with 1716 DNA were carried out on BHK cells to generate new recombinant viruses, 1627 and 1628 respectively, as described in Section 3.19 (Fig 62). Several individual plaques were isolated and taken through 2 to 3 rounds of plaque purification and detection by X-gal staining (Section 3.15.1.). DNA from each plaque isolate was screened by digestion with the restriction enzymes *Bam*HI and *Hin*dIII and Southern blotting with radiolabelled p35(PacI) (Fig. 63). 1627 and 1628 contain one extra *Bam*HI and two extra *Hin*dIII sites within the UL41-UL44 region due to the inserted RL1/*lacZ* genes. The *Hin*dIII sites flank the HSV-2 RL1 and promoter giving fragments of 4.1 kbp and 12.3 kbp (Fig 63a,b). There was also a fragment of 1 kbp containing RL1 which was not detected by the p35(PacI) probe used. The *Bam*HI site is located between the RL1 and *lacZ* genes giving two fragments of 4.5 kbp and 7.8 kbp (Fig 63a,b).

Once plaque purification of 1627 and 1628 was complete viral stocks were made on BHK cells (Section 3.13). To confirm the DNA structure and purity of the recombinant viruses after stock growth, DNA was extracted, purified and digested with *Bam*HI. Wild type, 17$^+$, and 1716 were used as controls. 17$^+$ and 1716 have one *Bam*HI fragment of 6.6

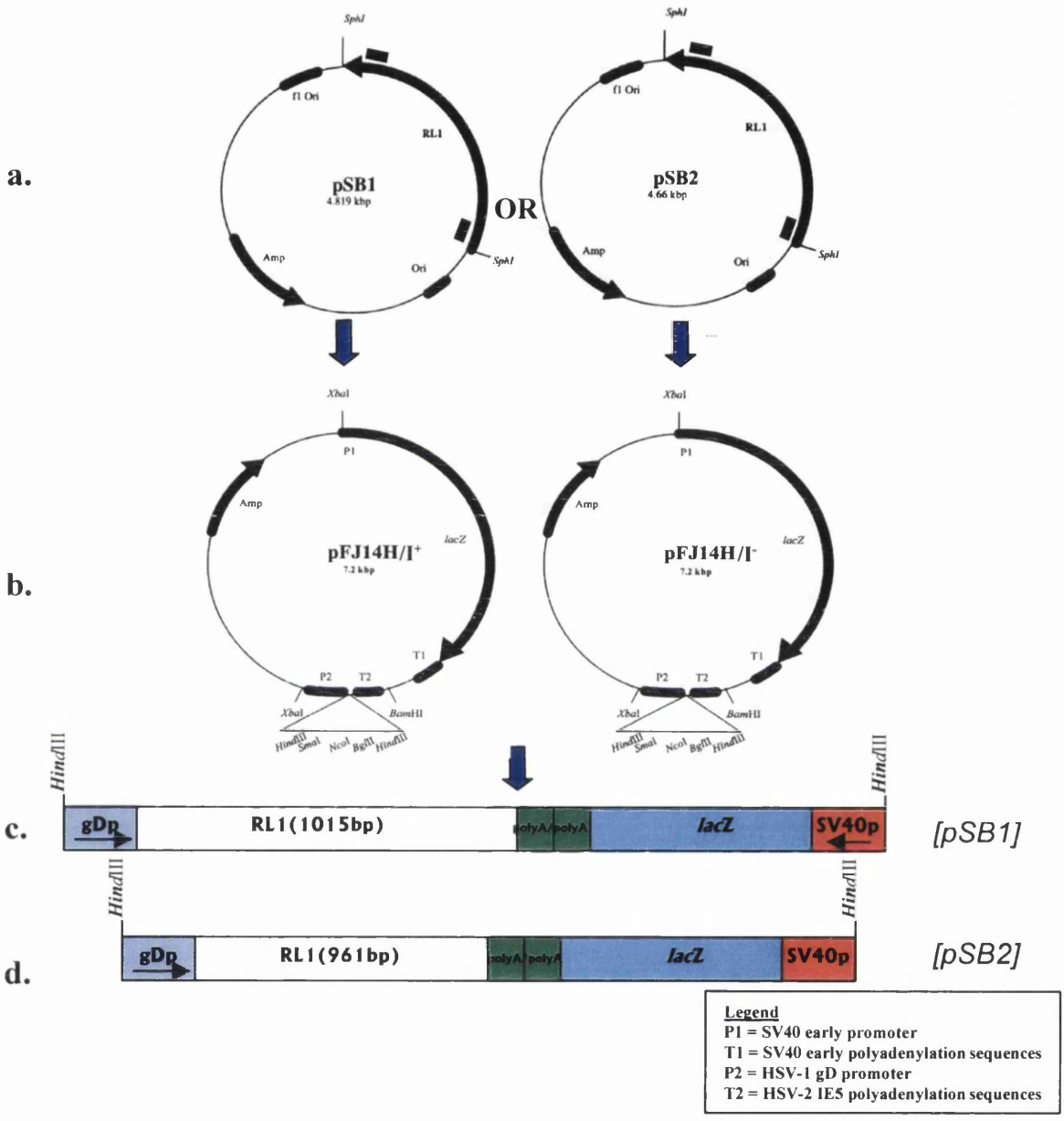


Figure 61. Plasmids used to sub-clone HSV-2 RL1 into 1716 UL43.

a. PCR was performed on pSB1 and pSB2, the PCR products were separated by agarose gel electrophoresis and purified (Fig. 58). **b.** PCR fragments from pSB1 and pSB2 were individually sub-cloned into pFJ14H using compatible restriction enzyme sites. **c.** and **d.** The pFJ14H fragments containing HSV-2 RL1 from pSB1 or pSB2 were isolated by *Xba*I digestion, 1% (w/v) agarose gel electrophoresis and purification (not shown). A schematic representation of the cassettes isolated from pFJ14H is shown here. The cassettes depicted were individually sub-cloned into p35(PacI). The individual clones were co-transfected with 1716 DNA by $CaPO_4$ method.

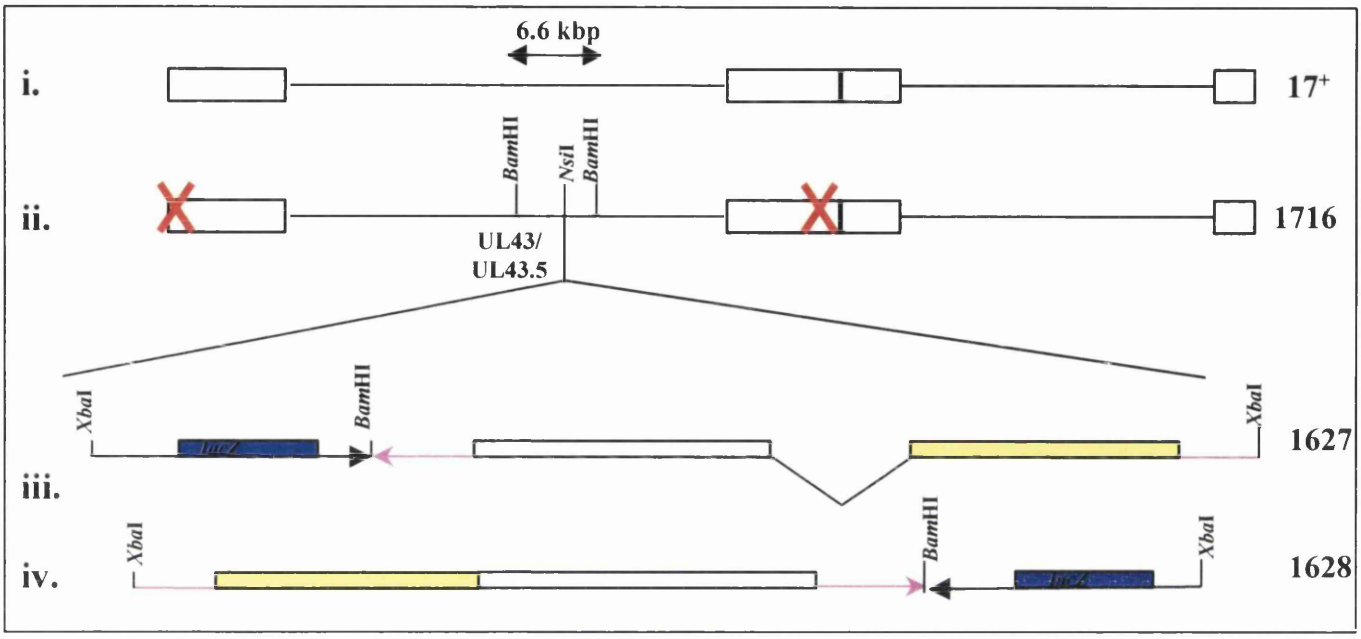


Figure 62. Schematic representation of the recombinant viruses.

i. A diagrammatic representation of the wild type HSV-1 strain 17^+ genome with the size of *Bam*HI fragment spanning UL43. **ii.** 1716 which has both copies of RL1 deleted which is depicted by X. **iii. and iv.** 1627 and 1628 are new intertypic recombinant viruses containing the HSV-2 RL1 under the HSV-1 gD promoter and a *lacZ* gene under a SV40 promoter in the UL43 gene of 1716. **iii.** 1627 has the HSV-2 RL1 gene with the intron. **iv.** 1628 contains the HSV-2 RL1 excluding the intron.

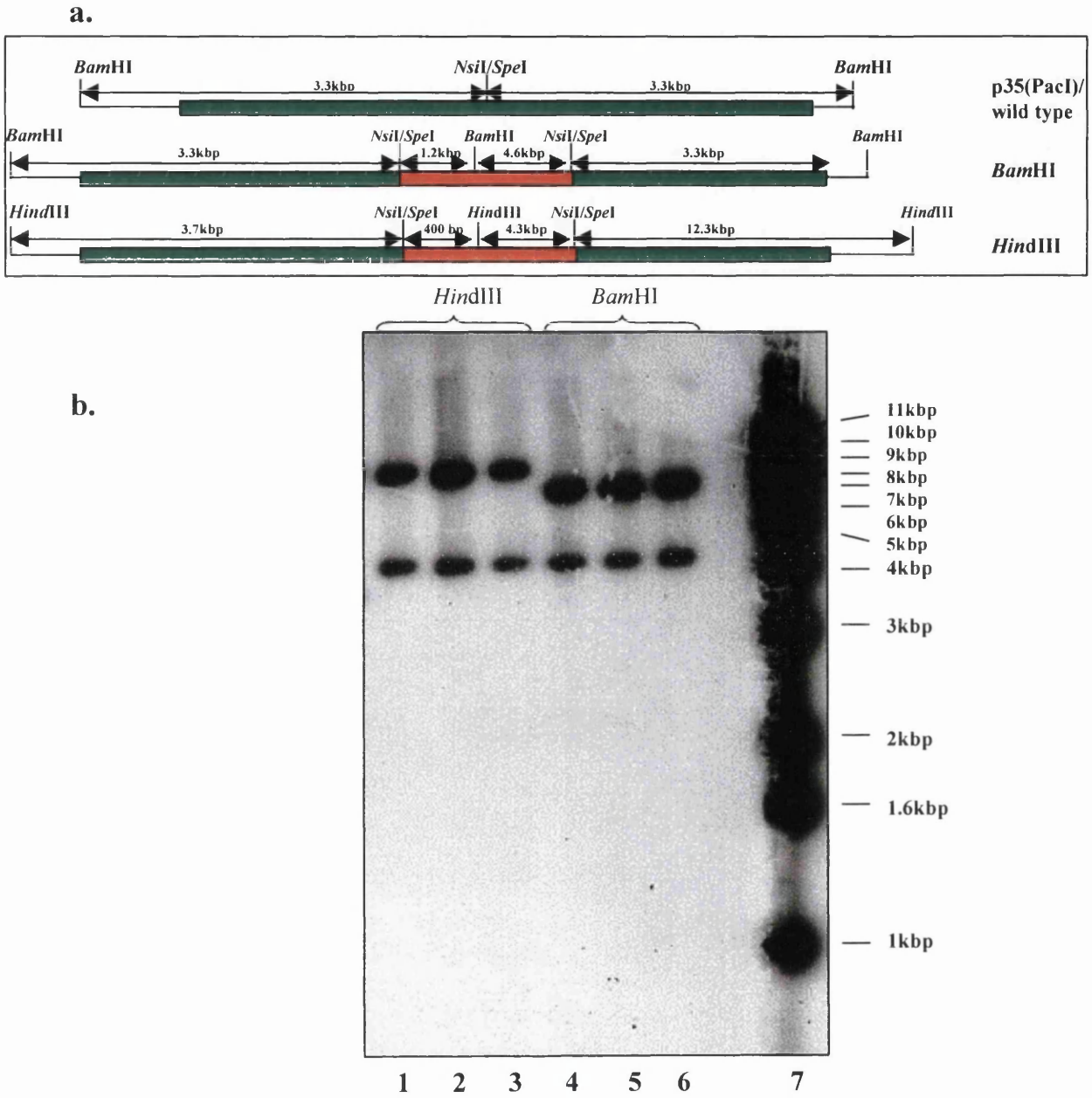


Figure 63. Southern blots of 1627 and 1628 plaque purification.

a. Schematic diagram of a *Hin*dIII and *Bam*HI digestion in the UL43 sequence of 17^+ and 1627/1628. **b**. After each round of plaque purification recombinant, different virus plaques were selected for Southern blotting. This Southern blot shows 2 plaques from 1627 (lanes 1-2; 5-6) and 1 from 1628 (lanes 3 and 6). Viral DNA was extracted and digested with *Hin*dIII or *Bam*HI and electrophoresed through a 1% (w/v) agarose gel with a 1 kbp DNA ladder (lane 7) for size comparison and Southern blotted with p35(PacI). The *Hin*dIII digest gives bands of 4.1 kbp and 12.3 kbp. *Bam*HI digestion gives bands of 4.5 kbp and 7.8 kbp.

kbp spanning the UL41-UL44 region when digested with *Bam*HI and hybridised with radiolabelled p35(PacI) (Fig. 64). The recombinant viruses contain an extra *Bam*HI site in the RL1 cassette which divides the fragments into two bands of 4.5 kbp and 7.8 kbp (Fig. 64).

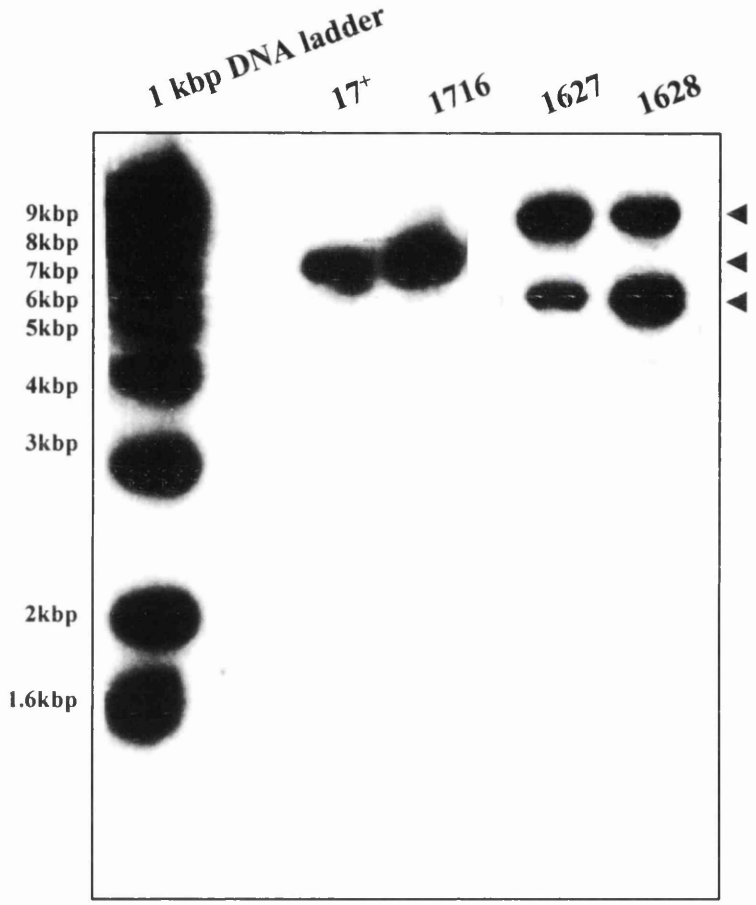


Figure 64. Southern blot of viral stocks.

HSV DNA was digested with *Bam*HI, analysed on a 1% (w/v) agarose gel and Southern blot. The membrane was probed with [^{32}P] labelled p35(PacI). There is a single 6.6 kbp band in 17^+ and 1716. 1627 and 1628 have two bands of 7.8 kbp and 4.5 kbp.

5.2.5. Discussion

Several queries about HSV-1/2 ICP34.5 and virulence led us to address if HSV-2 RL1 could complement HSV-1 RL1. We also wanted to determine if HSV-2 ICP34.5 was expressed. Western blots using antisera against HSV-1 ICP34.5 did not detect protein from wild type HSV-2 strain HG52, 1627 or 1628. The HSV-2 RL1 gene was placed under a strong promoter (HSV-1 gD) which was believed would aid in the detection of HSV-2 ICP34.5. Two recombinant viruses were constructed based on the HSV-1 ICP34.5 negative mutant, 1716. These recombinant viruses contain the HSV-2 RL1 gene with and without the 154 bp intron. Detection of ICP34.5 was assessed in the subsequent sections (Sections 5.2 and 5.3).

5.3. CHARACTERIZATION OF 1627 AND 1628

5.3.1. Introduction

Initial studies using 1627 and 1628 in Western blots using the HSV-1 ICP34.5 antisera did not lead to the recognition of HSV-2 ICP34.5. Using the new antisera (596 and 597) raised against the HSV-2 RL1/GST fusion protein, Western blots were carried out.

5.3.2. HSV-2 ICP34.5 Expression

Two bands of 37 kDa and 27 kDa were specifically detected in HG52 infected extracts (Fig. 65a,b). The 37 kDa band corresponds to the size of the HSV-1 protein (Fig. 66a) which migrates approximately 10 kDa slower than its predicted M_r. The 27 kDa protein is close to the predicted size of 27.9 kDa. To help determine which protein corresponded to ICP34.5 and to see if there was a relationship between the two proteins, expression was analysed from a number of different viruses: 2624, a HSV-2 mutant lacking the RL1 intron; and the two HSV-1 1716 based recombinants described in Section 4.6: 1627 (expressing HSV-2 ICP34.5 containing the intron) and 1628 (expressing HSV-2 ICP34.5 lacking the intron). HG52 and 1627 gave similar profiles with both the 37 kDa and 27 kDa proteins present with the 27 kDa protein being the predominant form (Fig. 65a,b). In contrast, 2624 and 1628 only expressed the 37 kDa protein (Fig. 65a,b).

In order to demonstrate ICP34.5 protein specificity, 17^+, HG52, 1627, and 2616 infected BHK extracts were harvested at 16 h pi. These samples were analysed by 10% SDS-PAGE and Western blotting using 137 and 597 (Fig. 66a,b). ICP34.5 from 17^+ co-migrated with ICP34.5 from HG52 by comparison with different antisera. This suggests that the protein expressed from the predicted HSV-2 RL1 ORF is the 37 kDa protein. The antisera raised against HSV-2 ICP34.5 did not recognize the HSV-1 homologue (Fig. 66b). A number of other virus induced bands were detected by Western blot (especially with antiserum 596; Fig. 65a). One particular band to note is a 36 kDa protein migrating faster than the 37 kDa ICP34.5. To test other viral protein synthesis and ascertain a Western blot using an HSV-1 US11 antiserum was carried out. Infected cell extracts used in Figure 65 were used to look for expression of the 21 kDa US11 protein (Fig. 67). This confirms that another HSV-1 protein is expressed in 1627 and 1628 with the same kinetics as 17^+ and 1716.

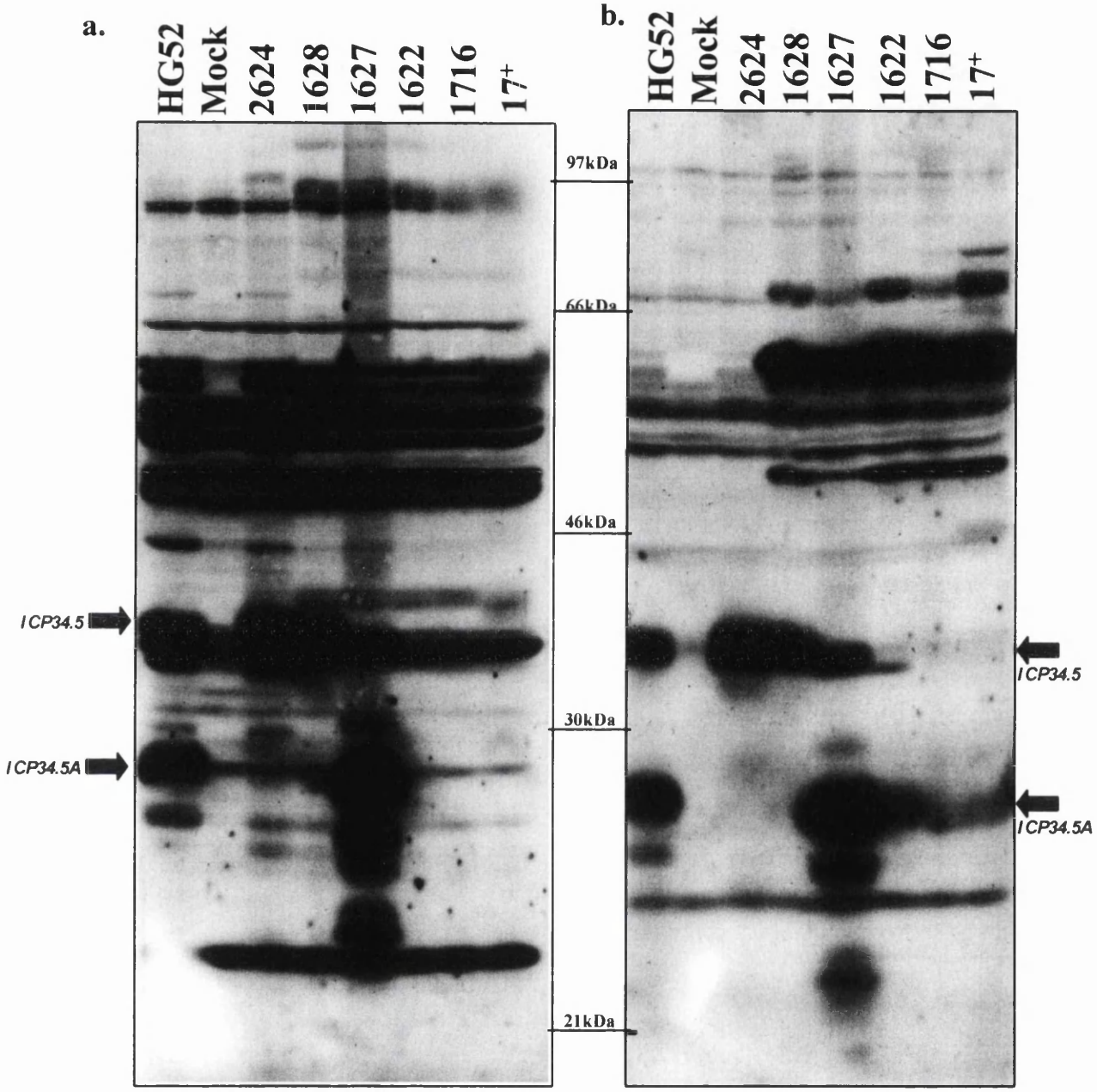


Figure 65. Western blots with anti-ICP34.5 sera.

BHK cells were infected with a m.o.i. of 20 pfu/cell for 16 hours. The infected cells were harvested, analysed on a 10% SDS-polyacrylamide gel and Western blotted with antisera from the GST/HSV-2 ICP34.5 fusion protein, 596 and 597, using a 1:800 dilution of each **a**. and **b**, respectively. Both antisera 596 and 597 recognize two proteins of 27 kDa and at 37 kDa, in HG52 and 1627. The antisera recognise only one protein of 37 kDa in 2624 and 1628. HSV-1 strain 17^+ ICP34.5 is not recognized by either 596 or 597. Molecular weight markers are indicated.

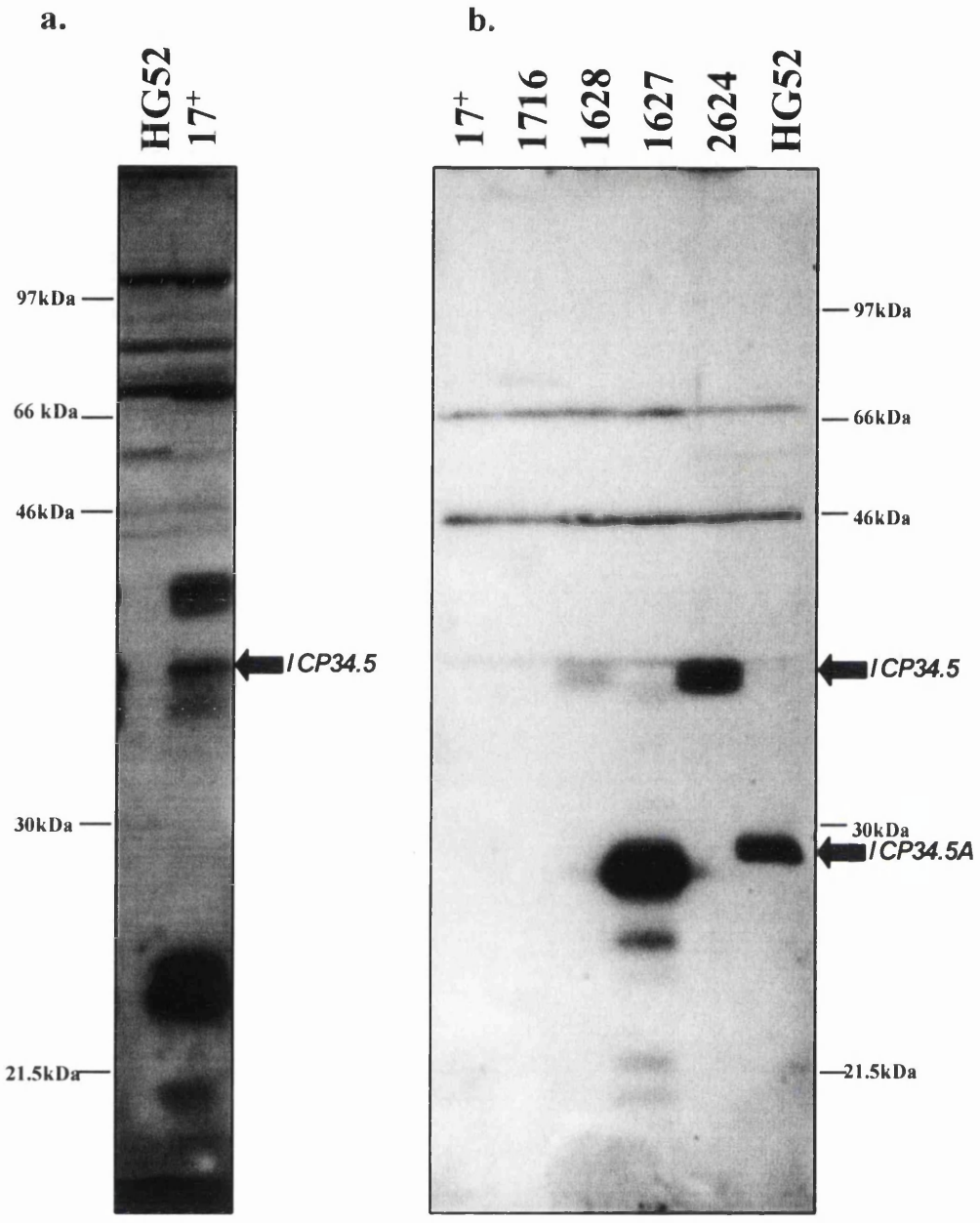


Figure 66. Western blots with anti-ICP34.5 sera.

BHK cells were infected with a m.o.i. of 20 pfu/cell for 16 hours. The infected cells were harvested, analysed on a 10% SDS-PAGE and Western blotted with 137, a GST/HSV-1 ICP34.5 fusion protein, or with antiserum 597 against GST/HSV-2 ICP34.5 fusion protein. **a**. As previously observed antiserum 137 recognizes the 37 kDa ICP34.5 in HSV-1 strain 17^+ infected cell extracts, but fails to recognise any specific bands in HSV-2 strain HG52 extracts. **b**. A Western blot with antiserum 597 shows specific recognition of two proteins 37 kDa and 27 kDa in HG52 and 1627, but only 37kDa in 2624 and 1628. Molecular weight markers are given by the side of gel.

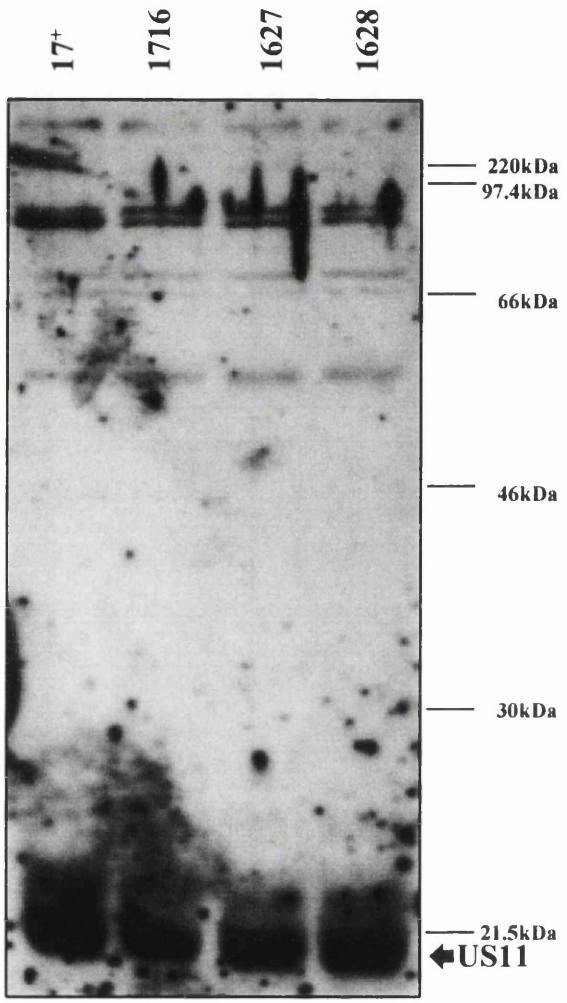


Figure 67. Western blot of US11 protein.

BHK cells were infected with a mulitplicity of 20 pfu/cell. Infected cells were harvested 16 h pi and analysed by 10% SDS-PAGE and Western blotted with anti-US11 monoclonal serum. US11 is expressed in 1627 and 1628 at the same level as wild type 17^{+} and 1716.

5.3.3. Fractionation of ICP34.5 in HSV-2

Cell fractionation experiments were performed to determine the location of the two ICP34.5 proteins. Cells infected with HG52, 2624 and the RL1 negative virus 2616 were harvested at 16 h pi (Fig. 68). Samples were separated into nuclear and cytoplasmic fractions by hypotonic buffer at 4°C, analysed by 10% SDS-PAGE and Western blotted using antiserum 596. The 27 kDa and 37 kDa proteins were distributed similarly, predominantly present in the cytoplasm (Fig. 68). This HSV-2 ICP34.5 protein compartmentalization parallels the HSV-1 ICP34.5 cellular distribution.

5.3.4. Immunoprecipitation of HSV-2 ICP34.5

Immunoprecipitation (IP) experiments using both antisera, 596 and 597, were carried out on [^{35}S]methionine labelled extracts prepared from HG52, 2624, 2616, 1627, 1628, and mock infected cells harvested 6 and 16 h pi. There are only 2 methionines in HSV-2 ICP34.5, and incorporation of [^{35}S]methionine is poor. Upon autoradiography no specific bands could be detected (data not shown). The IP samples were Western blotted using 597. When BHK cells were infected at 10 pfu/cell and harvested at 6 h, immunoprecipitated and Western blotted with 597 no ICP34.5 proteins were detected (Fig. 69, lanes 1-6). Infected cells harvested at 16 h pi which were immunoprecipitated and Western blotted with 597 showed an unusual ICP34.5 protein profile (Fig. 69, lanes 7-12). The ICP34.5 protein with an apparent M_r of 37 kDa was barely detected in HG52 (lane 7). The ICP34.5 protein of M_r 37 kDa fluctuated by 1–2 kDa between 2624 (lane 9), 1628 (lane 12) and 1627 (lane 11). The faster migrating protein named ICP34.5A with an apparent M_r of 27 kDa was detected in HG52 (lane 7) and 1627 (lane 11). ICP34.5A of 27 kDa from 1627 was detected better than from HG52. There was an additional strong band in 1627 with a M_r of 23 kDa (lane 11). Two additional bands less strongly recognised in 1627 were 30 kDa and 20 kDa (lane 11).

The HG52 and 1627 ICP34.5A protein of 27 kDa was detected at 6 h pi using 596 antiserum in an IP migrating slightly faster in 1627 (Fig. 69, lane 13,17). No other proteins were detected from other infections by 596 IP at 6 h pi (lanes 14-15,18). At 16 h pi the HG52 IP using 596 shows several bands surrounding the major protein ICP34.5 of 37 kDa (Fig. 69, lane 19). To clarify that these were individual bands a lighter exposure of the autoradiograph was taken and shown in Figure 69b. An unidentified protein migrating

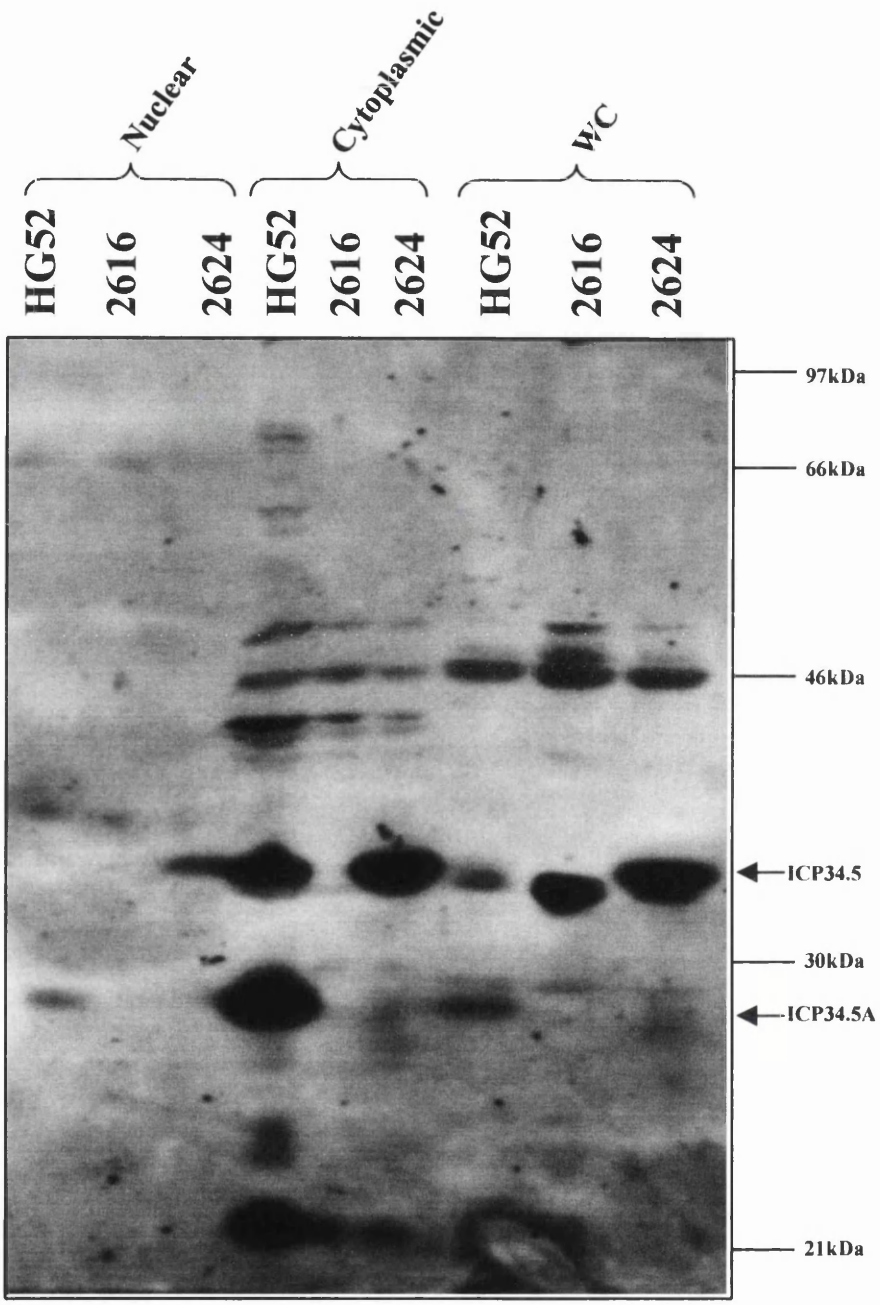


Figure 68. Localisation of ICP34.5 in BHK cells.

BHK cells were infected at a m.o.i. of 20 pfu/cell with HG52, 2616 or 2624 and harvested at 16 hours post infection. A fraction of the whole cell extract (WC) was kept for analysis and the remainder separated into nuclear and cytoplasmic fractions. The samples were analysed by 10% SDS-PAGE and Western blotted with 597 antiserum.

The 37 kDa ICP34.5 and 27 kDa ICP34.5A bands are present in the whole cell extract and predominantly the cytoplasmic fraction. Molecular weight markers are shown.

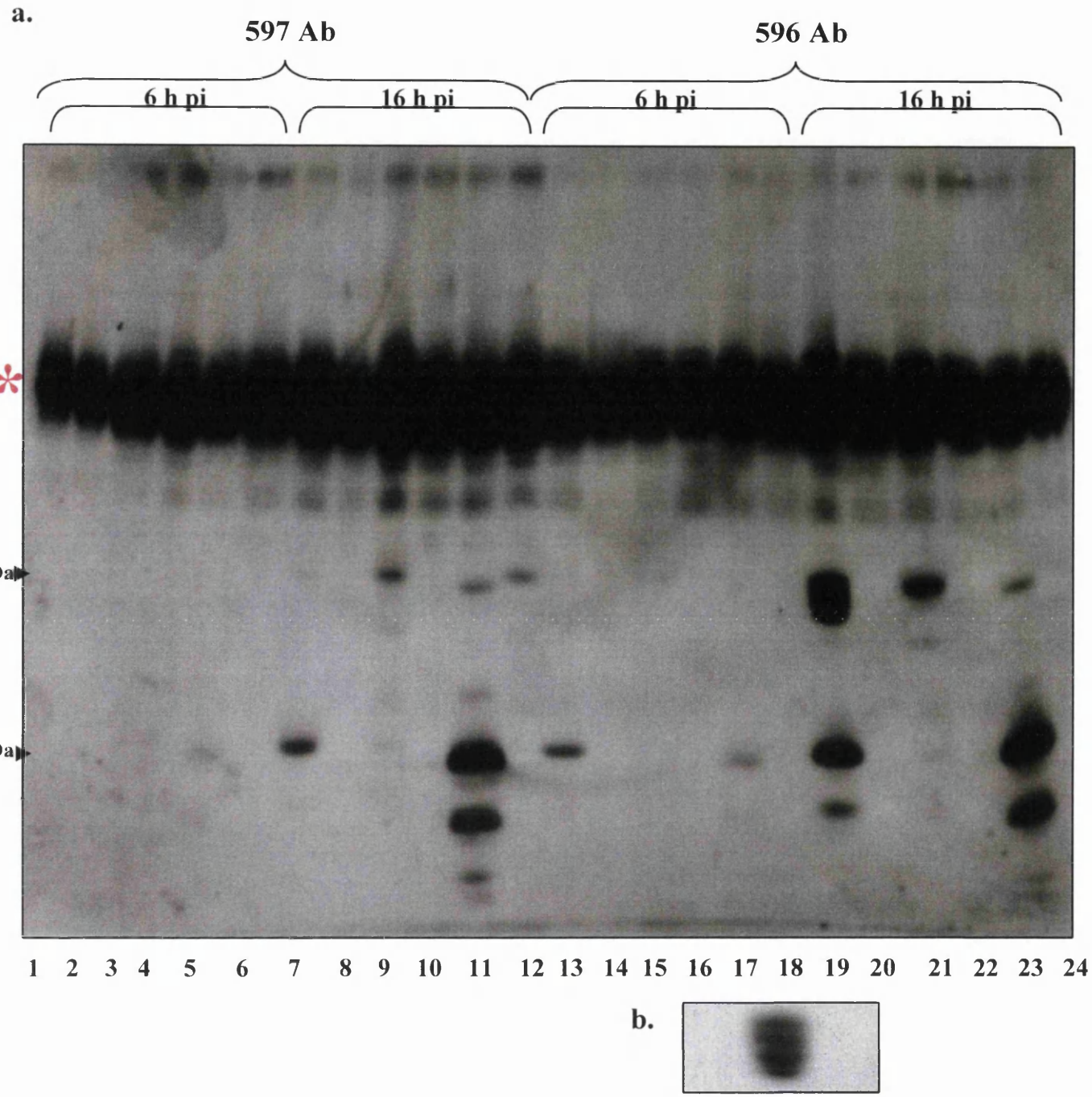


Figure 69. Western blot of HSV-2 ICP34.5 IP.

a. BHK cells infected at a m.o.i. of 10 pfu/cell. Samples were harvested either at 6 h pi (lanes 1-6; 13-18) or 16 h pi (lanes 7-12; 19-24) The * denotes the reacting 50 kDa IgG heavy chain. HG52 (lanes 1,7,13,19), mock infected (2,8,14,20), 2624 (3,9,15,21), 2616 (4,10,16,22), 1627 (5,11,17,23), and 1628 (6,12,18,24).

b. To clarify individual bands seen in HG52 migrating around 37 kDa (lane 19), a lighter exposure was made. There are at least 4 distinct bands.

at 23 kDa was detected from HG52 (lane 19) and 1627 migrating apparently slightly faster (lane 23). This protein of 23 kDa was also detected from 1627 IP using 597 (Fig. 69, lane 11). The 37 kDa ICP34.5 is detected from 2624 (lane 21) and 1627 (lane 23) but not detected from 1628 (lane 24).

The experiment was repeated to examine more closely the ICP34.5 and ICP34.5A migration patterns from infected cell extracts. Infected BHK cells were harvested at 16 h pi (Fig. 70). Only the 27 kDa ICP34.5A protein was detected from HG52 (Fig. 70, lane 5) and 1627 (Fig. 70, lane 3) while 2624 and 1628 showed only detection of the 37 kDa ICP34.5 protein. A faint cellular band migrating at 36 kDa could be detected in all viruses (Fig. 70, lanes 2-6) and mock (Fig 70, lane 1) infected extracts.

Samples were immunoprecipitated and subjected to 10% SDS-PAGE. A Western blot using 597 was performed and shown in Figure 70. The 50 kDa IgG heavy chain is detected in the Western blot due to the interaction with Protein A-HRP.

From the Western blot and IP experiments it appears that the 37 kDa protein is the same size in all four viruses analysed, but from 1627 infection the 27 kDa ICP34.5A protein migrates slightly (1-2 kDa) faster than that from HG52.

5.3.5. *In vitro* Replication from 1627 and 1628 Infected Cells

To determine if expression of HSV-2 ICP34.5 could compensate for loss of HSV-1 ICP34.5 in an HSV-1 background and if the 27 kDa protein performs an essential *in vitro* function, we analysed the growth of several different HSV-1 and HSV-2 viruses in different cells.

In replication experiments, all viruses grew like their wild type parent in BHK cells. 17^+, 1716, 1627 and 1628 grew similarly as did HG52, 2624 and 2616 (data not shown) in the permissive BHK cells (Fig. 71; Table 18).

In additional experiments using 3T6 cells, a cell line that is non-permissive for RL1 deleted viruses, 1627 returned to the wild type growth of HSV-1 strain 17^+ (Fig 72; Table 18). The input titre of 1628 was approximately 1 log lower than 17^+ and 1627, consequently the replication of this virus appears slightly impaired. Alternatively, this might be a consequence of the lower level expression of HSV-2 ICP34.5.

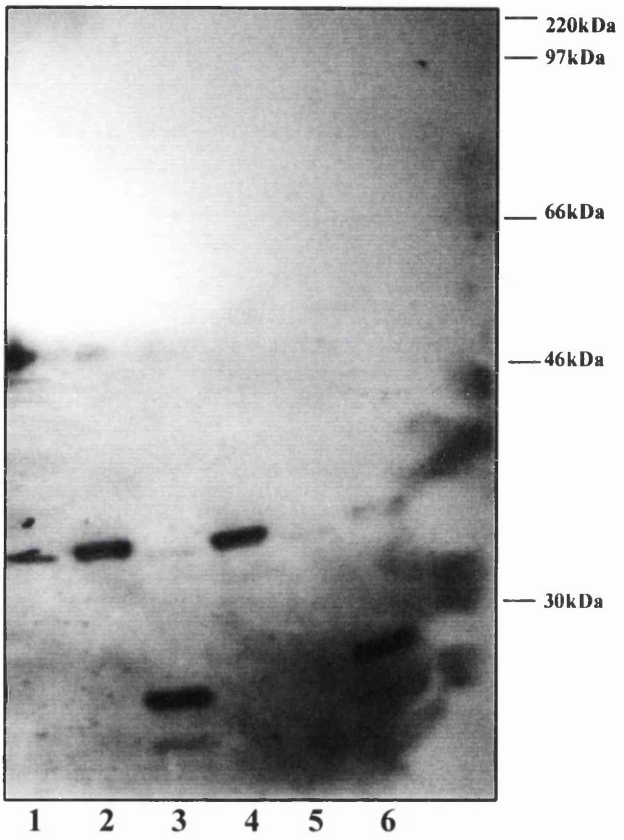


Figure 70. Western blot of immunoprecipitated HSV-2 ICP34.5.

BHK cells were infected at a m.o.i. of 20 pfu/cell with mock (lane 1), 1628 (lane 2), 1627 (lane 3), 2624 (lane 4), 2616 (lane 5), and HG52 (lane 6). Samples were harvested at 16 h pi, immunoprecipitated with antiserum 597, electrophoresed through 10% SDS-PAGE and Western blotted with 597. Infected cell extracts before IP shows detection of the 27 kDa ICP34.5A from 1627 (lane 3) and HG52 (lane (6). The 37 kDa ICP34.5 protein is detected from 1628 (lane 2) and 2624 (lane 4). A cellular protein of apparent M_r 36 kDa is detected in all virus infections.

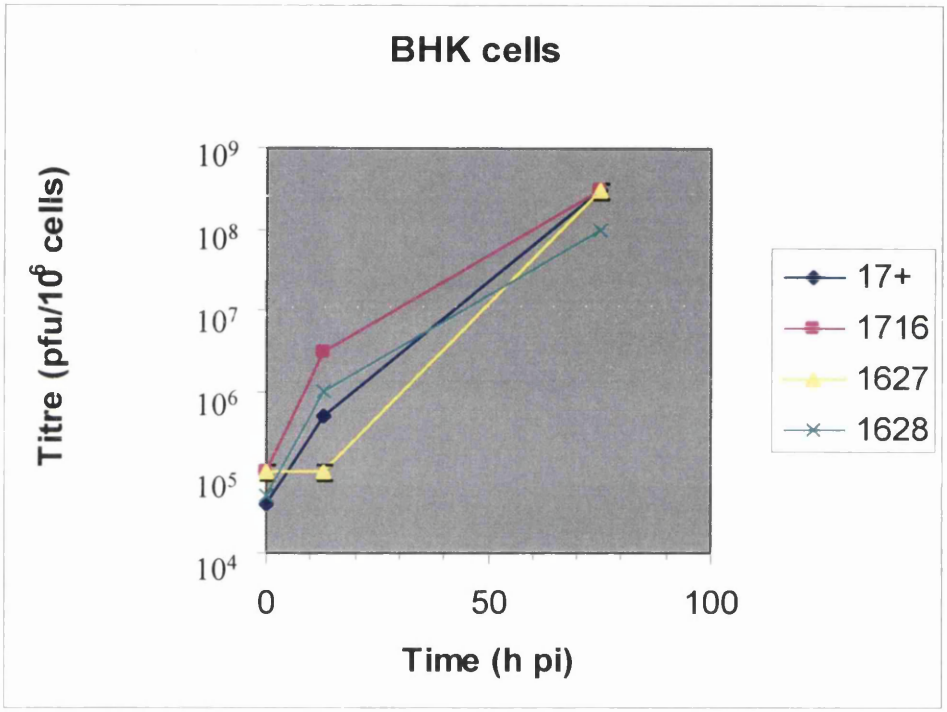


Figure 71. *In vitro* growth of 1627 and 1628 in BHK cells.

BHK cells were infected at a m.o.i. of 0.1 pfu/cell. Infected cells were harvested at 0, 12 and 72 h pi. Infected cells were harvested, sonicated and titrated on BHK cell monolayers.

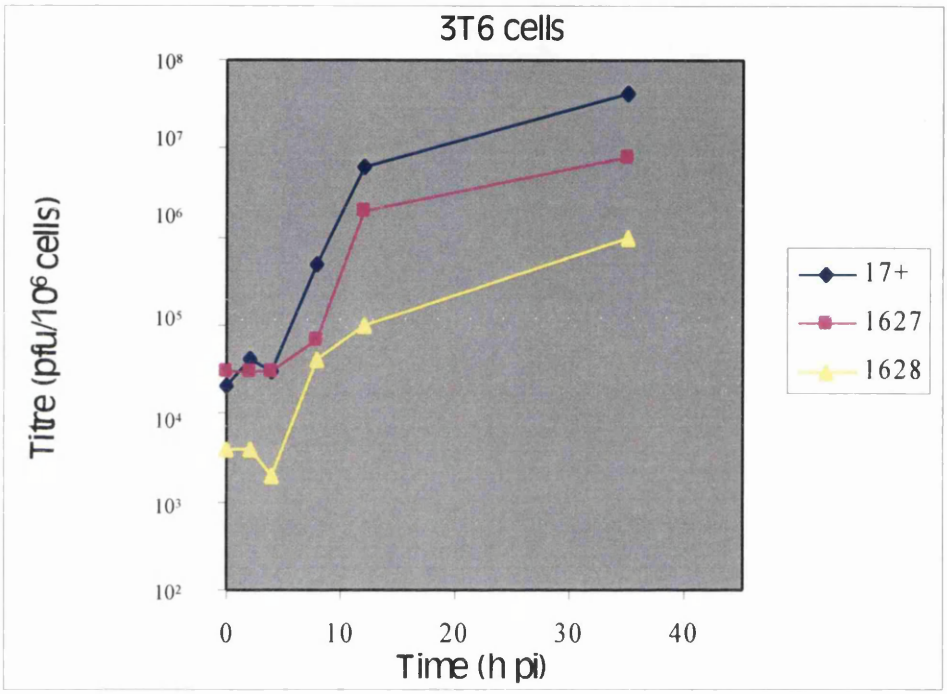


Figure 72. *In vitro* growth of 1627 and 1628 in 3T6 cells.

3T6 cells were infected at a m.o.i. of 0.1 pfu/cell and harvested at 0, 2, 6, 8, 12 and 36 h pi. Infected cells were harvested, sonicated and titrated on BHK cell monolayers.

Table 18. BHK and 3T6 titres for single cycle growth kinetics.

BHK cells				
	17^+	1716	1627	1628
0	$4.0x10^4$	$1.0x10^5$	$1.0x10^5$	$5.0x10^4$
13	$4.8x10^5$	$3.0x10^6$	$1.0x10^5$	$1.0x10^6$
75	$3.0x10^8$	$3.0x10^8$	$3.0x10^8$	$1.0x10^8$

3T6 cells				
	17^+	1716	1627	1628
0	$2.0x10^4$	$2.0x10^5$	$3.0x10^4$	$4.0x10^3$
2	$4.0x10^4$	$2.0x10^5$	$3.0x10^4$	$4.0x10^3$
4	$3.0x10^4$	$6.0x10^4$	$3.0x10^4$	$2.0x10^3$
8	$5.0x10^5$	$4.0x10^5$	$7.0x10^4$	$4.0x10^4$
12	$6.0x10^6$	$1.0x10^6$	$2.0x10^6$	$1.0x10^5$
35	$4.0x10^7$	$2.0x10^6$	$8.0x10^6$	$1.0x10^6$

5.3.6. Host and Viral Protein Synthesis from 1627 and 1628 Infected Cells

The inability of HSV-1 ICP34.5 deleted viruses to prevent host and viral protein synthesis shut-off in SK-N-SH cells has been extensively characterized. However, this effect has been less well studied in HSV-2. To look at the phenotype of HSV-2 ICP34.5 and to determine if the intron plays a role in this function, host protein synthesis shut-off experiments were performed in SK-N-SH neuroblastoma, 3T6, HeLa and BHK cells.

The intertypic recombinants 1627 and 1628 were used in an initial experiment to determine if HSV-2 RL1 could restore protein synthesis in HSV infected SK-N-SH and HeLa cells (Fig. 73). No protein shut-off was observed by any virus infected BHK cell extracts. 1627 and 1628 prevented the preclusion of host shut-off seen with 1716 in SK-N-SH cells. In HeLa cells, the ICP34.5 negative HSV-1 virus 1716 and 1628 had reduced protein synthesis compared to wild type 17^+ and 1627 (Fig. 73). Additional experiments were carried out to analyse HSV-2 RL1 deleted viruses since this had not previously been studied (Fig. 74). 2616 did not shut-off protein synthesis in BHK cells which mimics the HSV-1 RL1 deleted virus, 1716 data (Fig. 73).

There was no observable difference between wild type and the intron containing recombinant virus, 1627 (Fig. 73 and 74). The intronless recombinant virus, 1628, appeared to give a partial shut-off of protein synthesis in SK-N-SH cells (Fig. 73 and 74). In SK-N-SH cells, viruses fell into two groups: 1) those which continued protein synthesis: 17^+, HG52, 2624, 1627 and 1628; and 2) those where protein synthesis was severely reduced: 1716 and 2616 (Fig. 74). 3T6 cells were also examined and show that protein synthesis, as predicted, was maintained in all viruses studied (Fig. 75).

Similarly to the growth kinetics, we can conclude that HSV-2 ICP34.5 complements the function of the HSV-1 homologue in HSV-1 RL1 deleted viruses with this protein conferring the full function of preventing the cessation of protein synthesis in SK-N-SH neuroblastoma cells.

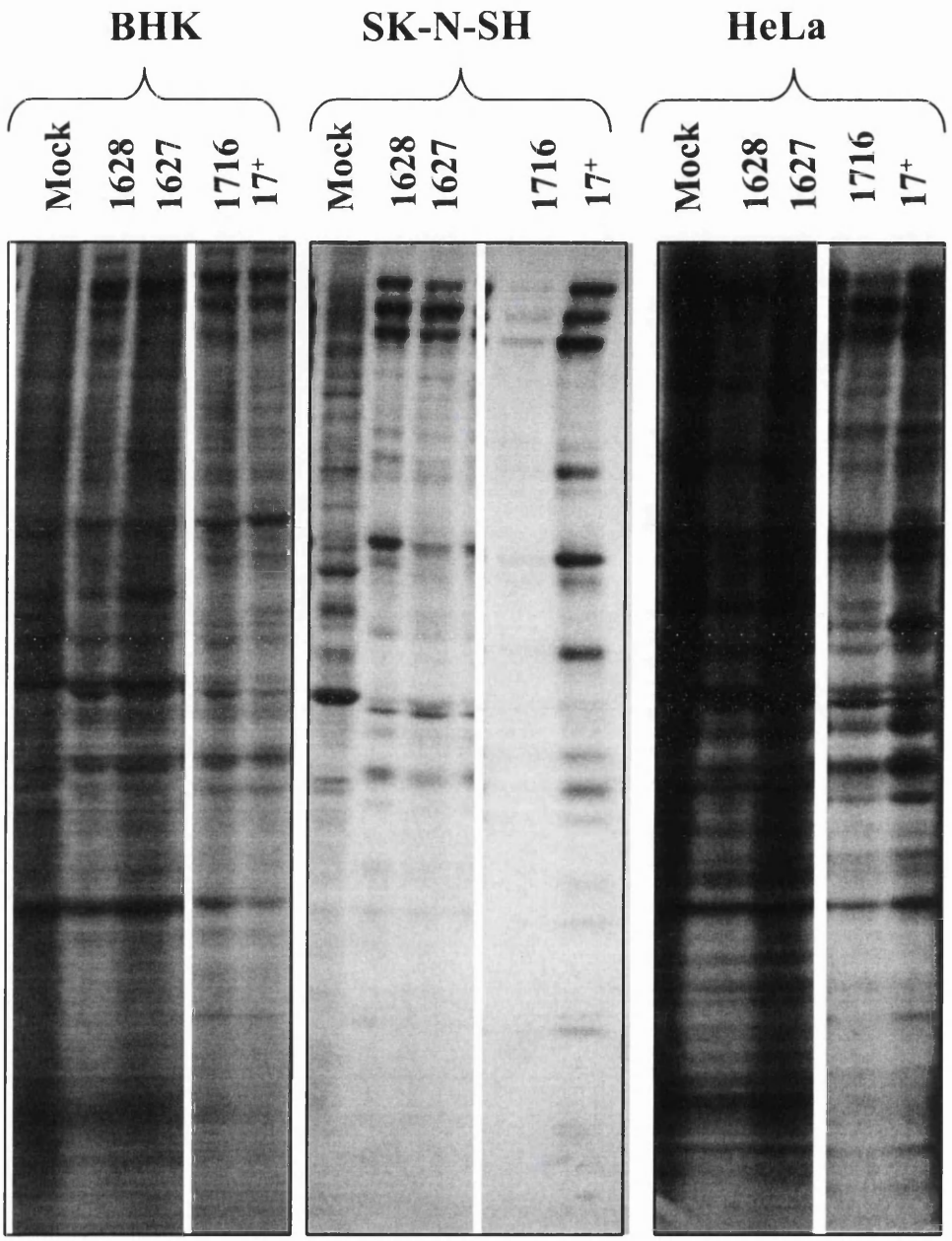


Figure 73. Viral and host protein synthesis in human neuroblastoma cells.

BHK, HeLa, and SK-N-SH cells were infected with a m.o.i. of 20 pfu/cell for 14 h and pulse labelled with [^{35}S]methionine for 2 h. Infected cells were harvested 16 h pi and analysed by 10% SDS-PAGE. HSV-1 viruses, 17^{+}, 1716 and recombinant viruses, 1627 and 1628, synthesized proteins equally well in BHK cells. In SK-N-SH cells 1716 displayed shut-off of protein synthesis. In HeLa cells 1716 and 1628 gave a partial shutoff of protein synthesis.

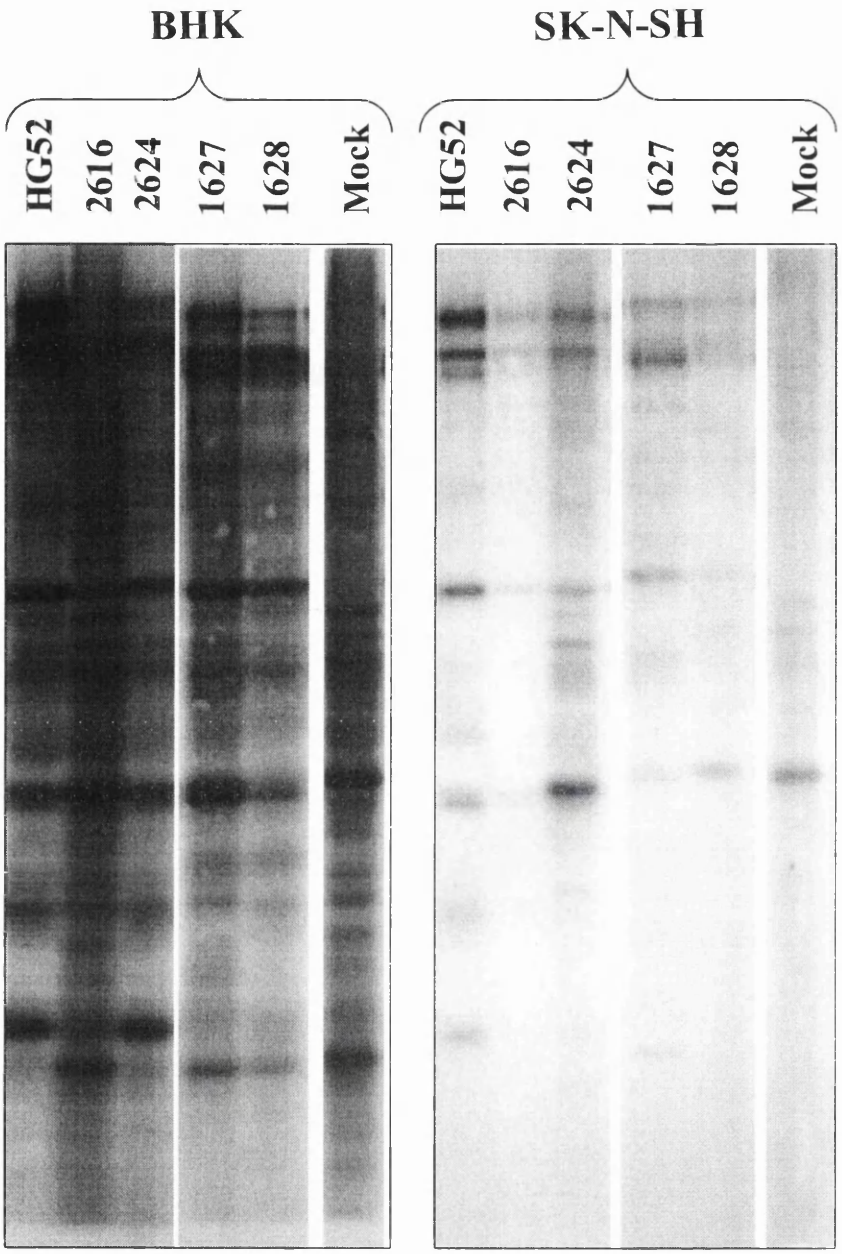


Figure 74. Viral and host protein synthesis in human neuroblastoma cells.

BHK and SK-N-SH cells were infected with a m.o.i. of 20 pfu/cell for 14 h and pulse labelled with [^{35}S]methionine for 2 h. Infected cells were harvested 16 h pi and analysed by 10% SDS-PAGE. HG52, 2616, 2624, and 1716 recombinant viruses, 1627 and 1628, synthesized proteins equally well in BHK cells. In SK-N-SH cells 2616 and 1628 displayed shut-off of protein synthesis.

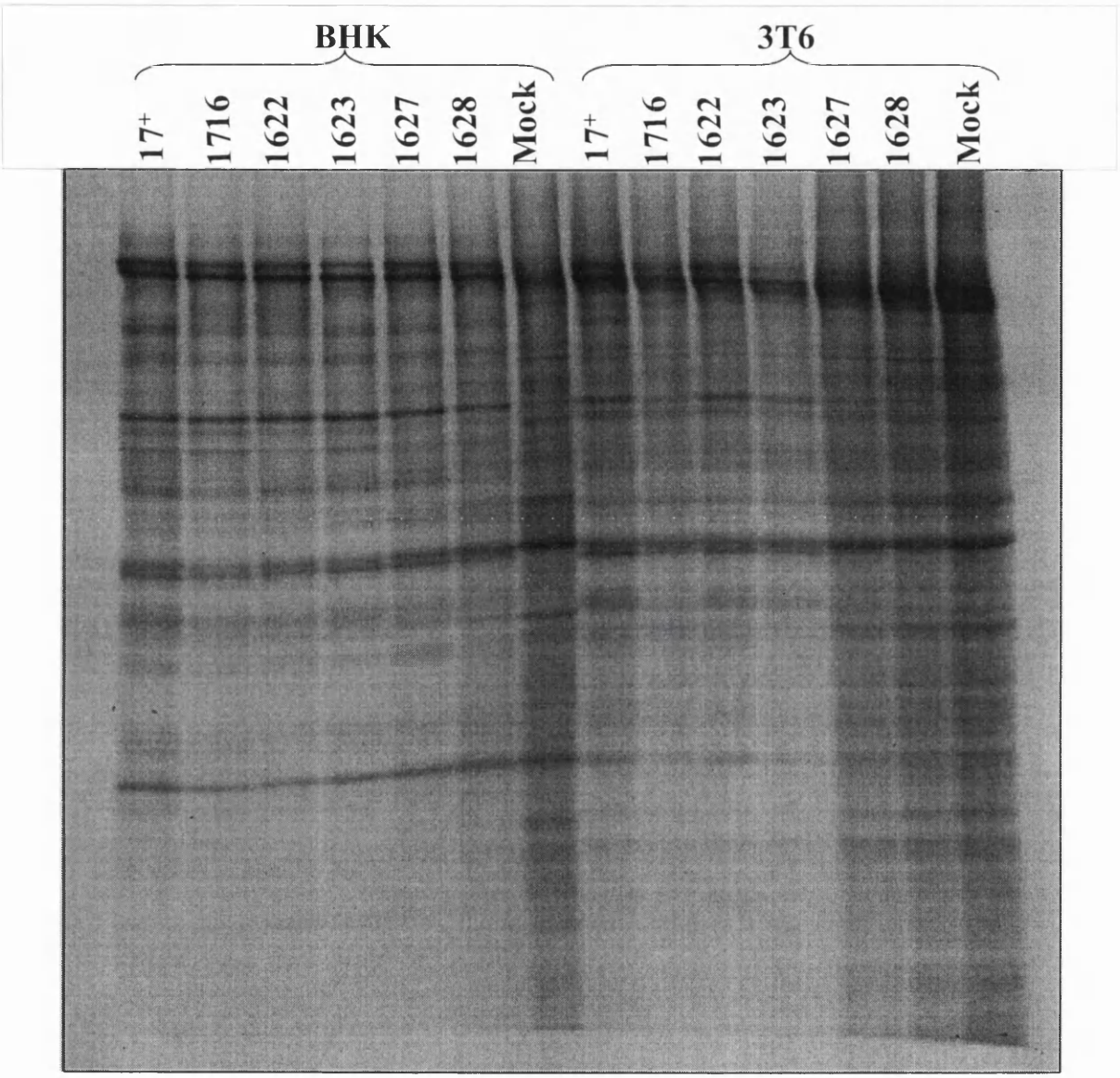


Figure 75. Viral and host protein synthesis in 3T6 cells.

BHK and 3T6 cells were infected with a m.o.i. of 20 pfu/cell for 14 h and pulse labelled with [^{35}]Smethionine for 2 h (14 to 16 h pi). Infected cells were harvested 16 h pi and analysed by 10% SDS-PAGE. All viruses in both cell lines maintained protein synthesis.

5.3.7. Discussion

This work provides the first demonstration that the RL1 gene in HSV-2 strain HG52 expresses a homologue of HSV-1 ICP34.5. This is not surprising as deletion of RL1 in both HSV-1 and 2 produces a similar avirulent phenotype (Taha *et al.*, 1989, MacLean, A., *et al.*, 1991). Antisera raised against the HSV-1 homologue do not cross-react with the putative HSV-2 gene (Brown *et al.*, 1997). Therefore, we constructed a GST fusion protein containing the whole of the HSV-2 RL1 ORF. This was used to raise two rabbit polyclonal antisera. These new antisera specifically recognize two proteins of 27 kDa and 37 kDa from wild type HSV-2 infected cells. Interestingly, infection with viruses expressing RL1 without the intron exclusively express the 37 kDa protein. This implies that the 37 kDa protein is the product of the predicted spliced RL1 gene and that the 27 kDa protein contains sequences from the intron. To indicate its close relationship to ICP34.5, we have named this 27 kDa protein ICP34.5A. The origin of the 27 kDa protein requires further investigation. One possible explanation is translation of a non-spliced transcript, or one terminating before the intron acceptor site, to the first in frame stop codon in the intron. This would produce a 185 amino acid protein containing the first exon of ICP34.5 plus additional 21 amino acids from the intron (Fig. 76), approximately two-thirds of the 261 amino acid length and the M_r of ICP34.5. The theoretical M_r would be 19 kDa compared to that of ICP34.5 of 27.9 kDa (McGeoch *et al.*, 1991). However, like the HSV-1 protein (McKay *et al.*, 1993) the apparent M_r of HSV-2 ICP34.5 by SDS-PAGE is 37 kDa, larger than the predicted M_r of ICP34.5A of 26-27 kDa. The increased apparent M_r of ICP34.5 is a feature in common with many other HSV polypeptides (McGeoch *et al.*, 1988).

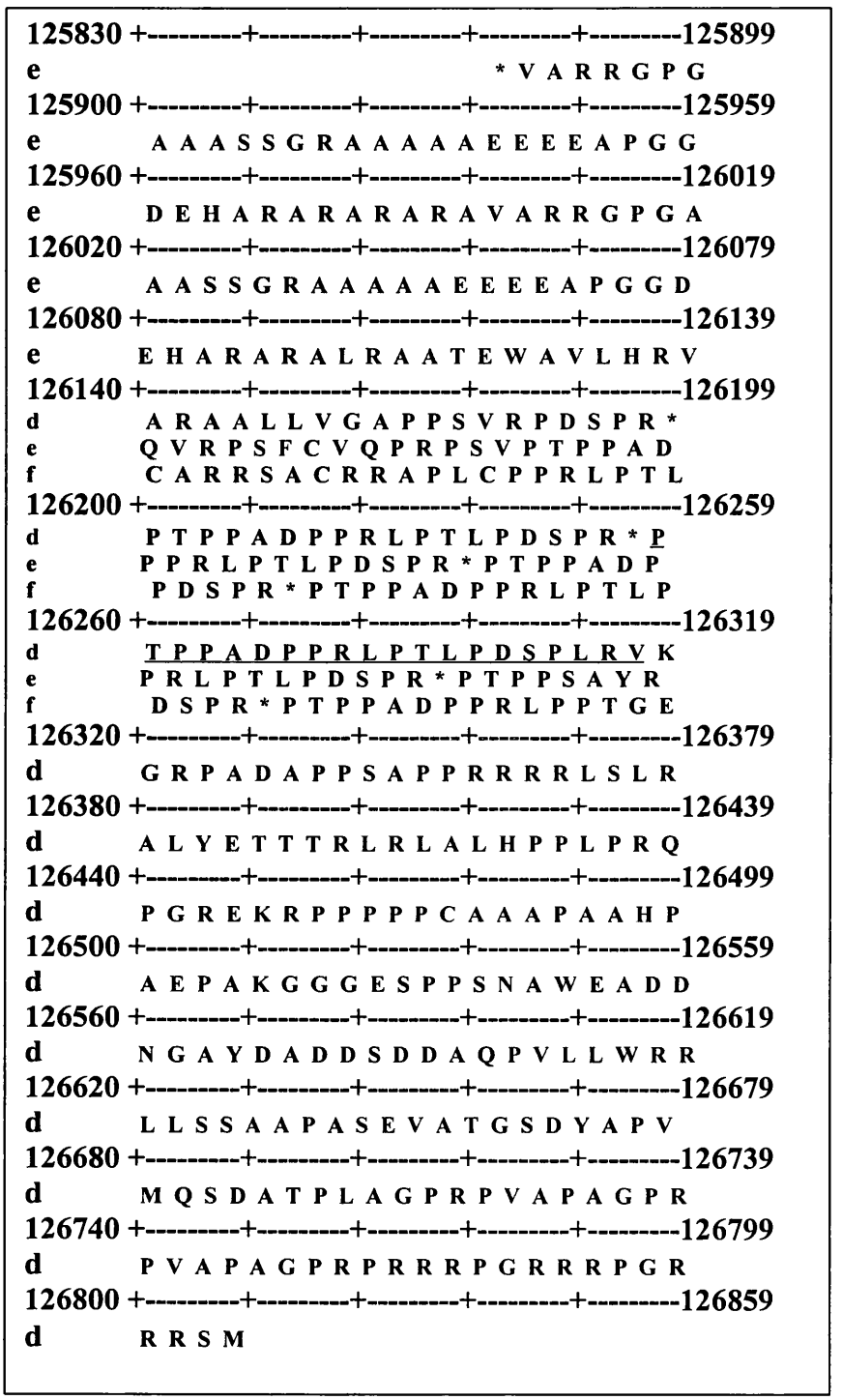


Figure 76. Amino acid sequence of ICP34.5 with the three forward reading frames in the intron. The IR_L ICP34.5 sequence from the initiating methionine is shown at the bottom of this figure. The amino acid sequence if the mRNA is not spliced and thus translation continues in the same frame (d) is underlined in the intron until reaching a stop codon at n.p. 126256.

There is no evidence of a polyA site in the intron (McGeoch *et al.*, 1991) to suggest expression of a truncated RL1 RNA.

An alternative possibility for the origin of ICP34.5A is expression of a protein, from a 5' truncated transcript, initiating in the intron and containing exon 2. Although this is less likely because there is no in-frame ATG in the intron to initiate translation, another HSV-2 gene, UL16, initiates with a Leu instead of the usual Met (Dolan *et al.*, 1998). Determination of the nature of ICP34.5A will require analysis of viruses with mutations either in exon 1, 2 or the intron. RNA mapping would also help determine the origin of ICP34.5A.

The generated antisera recognize ICP34.5 and ICP34.5A by Western blotting and immunoprecipitation indicating recognition of both denatured and native epitopes. Due to poor labelling with ^{35}[S]methionine, detection of immunoprecipitated ICP34.5 required Western blotting.

Similarly to the anti HSV-1 ICP34.5 sera, the anti-HSV-2 sera do not cross react with HSV-1 ICP34.5. This is probably because although the part of the protein with the highest homology is at the carboxy terminus most of the antibodies in both the HSV-1/2 antisera will be directed against the amino terminus (Brown *et al.*, 1997).

By constructing recombinant HSV-1 RL1 deleted viruses expressing the HSV-2 gene under a strong HSV-1 promoter (gD) of similar kinetic class to ICP34.5, we have also demonstrated that the HSV-2 gene can, at least *in vitro*, functionally complement the loss of the HSV-1 gene in a HSV-1 background. Future experiments will assess whether this complementation also applies *in vivo* regarding pathogenicity and latency.

The functional importance (if any) of ICP34.5A has yet to be determined. In all assays carried out both *in vitro* and *in vivo,* viruses with the RL1 intron deleted (1628 and 2624) and hence only expressing the 37 kDa ICP34.5 behave like wild type. However, in viruses containing the wild type gene (ie. including the intron) only low levels of ICP34.5 are detected by the antisera and the predominant protein detected is ICP34.5A. In contrast, viruses lacking the intron express ICP34.5 at much higher levels close to those of ICP34.5A in intron containing viruses (Fig. 76). It seems unlikely that a protein expressed at such high levels will not have some function in addition to that of ICP34.5 in the virus lifecycle.

The ability of the HSV-2 ICP34.5 to interact with PCNA as illustrated by GST pull-downs indicates at least one function in common with HSV-1 ICP34.5. This

interaction is likely to be mediated by the highly conserved 63 amino acid carboxy terminal region.

6. MUTANT VIRUSES RESULTS AND DISCUSSION

6.1. ORIENTATION OF INSERTED GENES IN HSV-1 UL43

6.1.1. Orientation Effect on Expression

One of the major aims of this investigation was to singly express a gene of interest, either RL1 or the antisense gene ORF P, in a novel site. This was achieved by insertion of a gene into the unique *Nsi*I site in the UL43 gene (Fig. 77). In 1996, an open reading frame antisense to UL43 was identified and named UL43.5. UL43.5 was shown to encode a protein with an apparent M_r of 38 kDa (Ward *et al.*, 1996). The UL43.5 gene is regulated as a late (γ_2) gene. Deletion of UL43 also deletes UL43.5 and does not alter replication *in vitro* or virulence *in vivo* and consequently they have been termed non-essential (MacLean, C. *et al.*, 1991). For this reason we thought this locus was a good candidate for insertion of expression cassettes.

In Section 4.2 and 4.3, 1622 and 1623 were described and characterised. To recapitulate, 1622 expresses the RL1 ORF and 1623 expresses the RL1 ORF and an additional 134 bp upstream sequence. These RL1 fragments were individually inserted in the UL43 *Nsi*I locus. Due to the considerable variation in ICP34.5 expression between 1622 and 1623 (64-fold), the orientation of the inserted RL1 sequence was examined. The restriction enzyme *Bam*HI was used to digest pHH1 and pHH2, the plasmids co-tranfected with 1716 to generate 1622 and 1623, respectively (Fig. 78a). There is one *Bam*HI site in the RL1 HSV-1 sequence located in the insert and a second *Bam*HI site at the end of the HSV-1 sequence in p35(PacI). The DNA digests were electrophoresed through a 1% (w/v) agarose gel (Fig. 78b). pHH1 gives two bands of 7.4 kbp and 5.8 kbp (lane 2), but pHH2 gives two bands of 8.5 kbp and 4.7 kbp (lane 3). These sizes were determined by comparison to a 1 kbp DNA ladder. This gel demonstrates that 1622 (pHH1) has the RL1 gene is in the same transcriptional orientation as UL43.5. However, the RL1 sequence in 1623 (pHH2) is in the opposite orientation (i.e. same transcriptional orientation as UL43).

Two additional recombinant viruses, 1627 and 1628, were constructed in the same manner as 1622 and 1623. The major difference is that the RL1 sequences in 1627 and 1628 are derived from HSV-2. However, the RL1 gene was inserted similarly in the UL43 locus. Orientation of these inserts was determined by *Nco*I digestion and Southern blotting of recombinant viral DNA (Fig. 79). 1627 and 1628 DNA could be directly examined since there is a single *Nco*I site within the insert (Fig. 78a,b). The Southern blot was probed with radiolabelled p35(PacI). The Southern blot revealed that the RL1 insert 1627

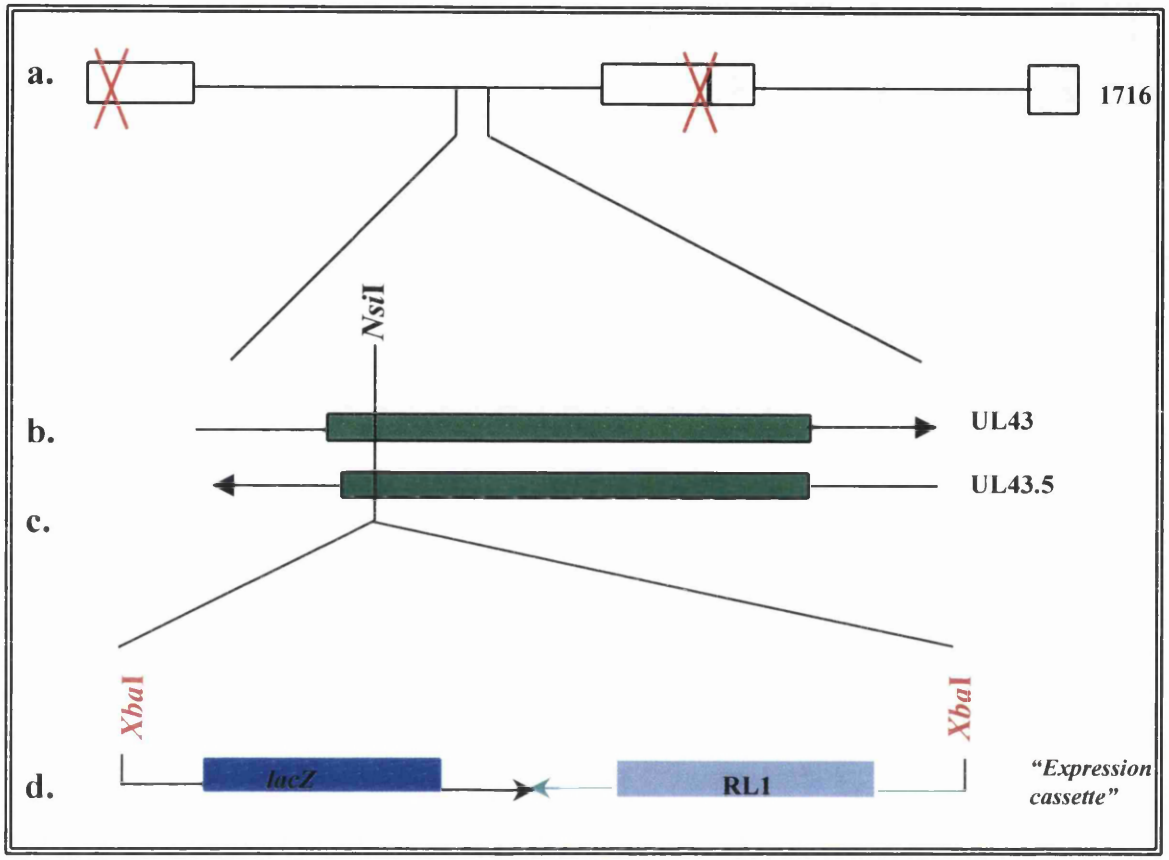


Figure 77. Schematic representation of genes inserted in the UL43 *Nsi*I site.

a. A diagram of the HSV-1 1716 genome. The X indicates that RL1/ORF P have been deleted in 1716. **b**. The position and transcriptional orientation of the UL43 ORF. **c**. The transcriptional orientation and ORF of UL43.5. **d**. The *Nsi*I location and example of the RL1 gene in the UL43.5 transcriptional orientation while the β-galactosidase gene, *lacZ*, is in the UL43 transcriptional orientation.

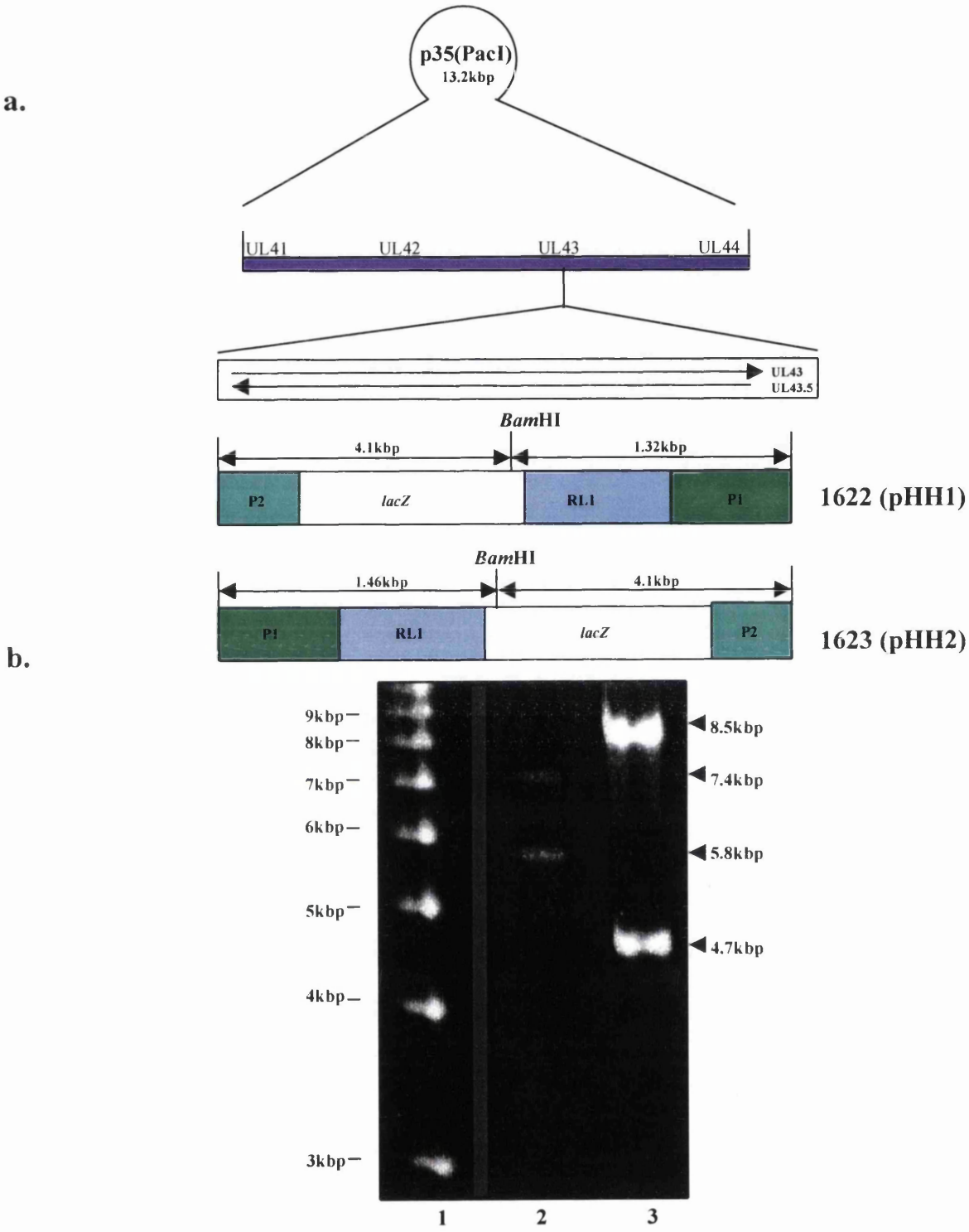


Figure 78. Schematic representation of *Bam*HI sites in HSV-1 and plasmid DNA.

p35(PacI) containing the UL41-UL44 HSV-1 sequences. **b**. The plasmids used to generate recombinant viruses 1622 (pHH1) and 1623 (pHH2) were digested with *Bam*HI and analysed by a 1% (w/v) agarose gel. Lane 1 contains a 1 kbp ladder. pHH1 (lane 2) gave a band of 7.4 kbp and 5.8 kbp. pHH2 (lane 3) gave two fragments of 8.5 kbp and 4.7 kbp.

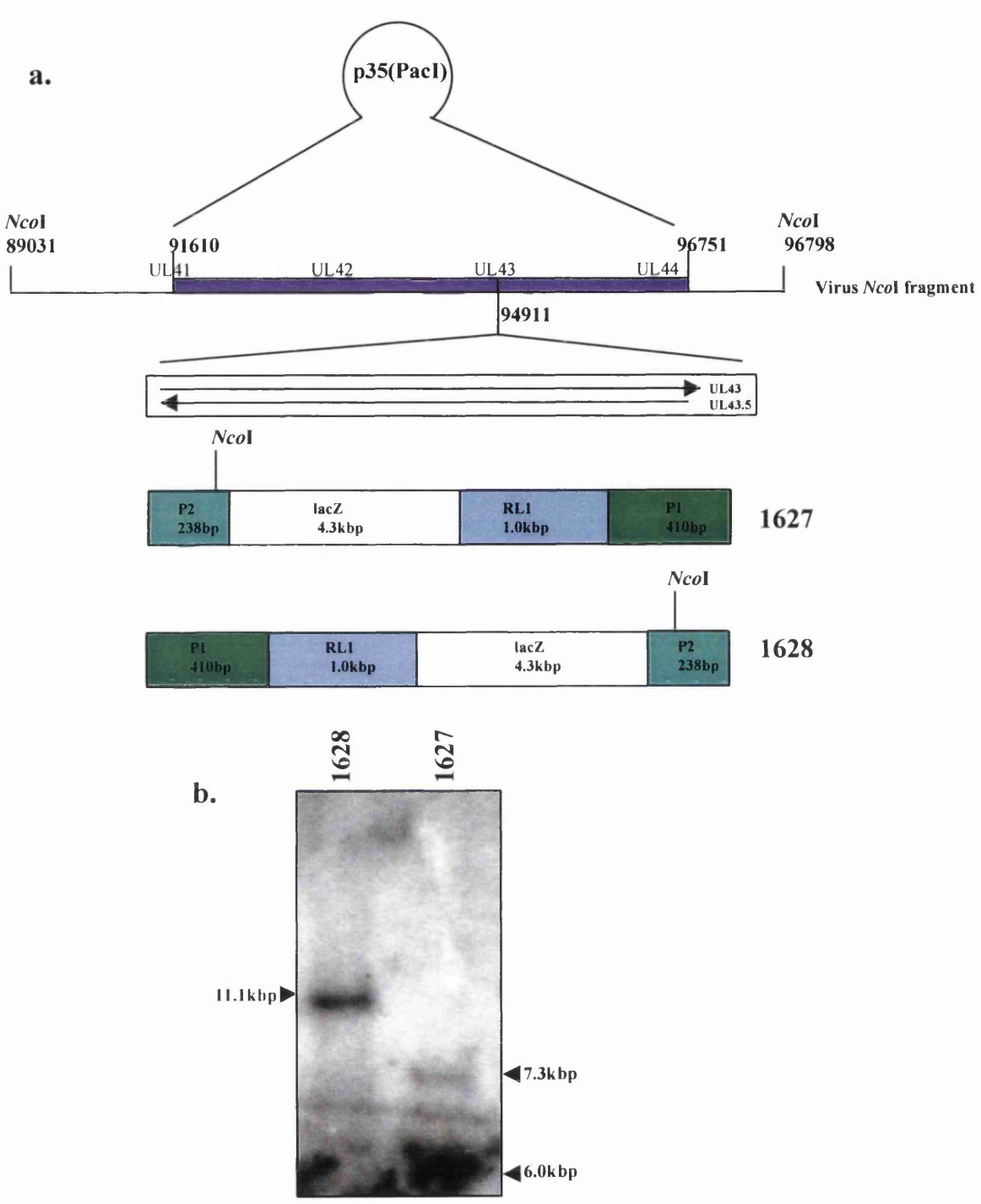


Figure 79. Southern blot of 1627 and 1628 DNA digested with *Nco*I.

a. Schematic representation of viral DNA within the p35(PacI) HSV-1 sequence UL41-UL44.
b. Viral DNA digested with *Nco*I was electrophoresed through a 1% (w/v) agarorse gel. The gel was Southern blotted and probed with radiolabelled p35(PacI). 1627 has one DNA fragment of 11.1 kbp recognised (the 2.1 kbp fragment is not shown). 1628 has two DNA fragments of 7.3 kbp and 6.0 kbp recognised.

was in the same transcriptional orientation as UL43.5 while the RL1 insert in 1628 was in the same orientation as UL43. 1627 has one visible band at 11.1 kbp one band 2.1 kbp, not seen. 1628 has two bands at 6.0 kbp and 7.3 kbp (Fig. 79b).

To clarify the orientation of RL1 and the opposing *lacZ* genes in each recombinant virus, a summary is given in Table 19.

There are currently no UL43 antisera available. However, Ward *et al* (1996) raised an antiserum against a MBP fusion protein from UL43.5 ORF. This antiserum was made available by Prof. B. Roizman for use in a Western blot (Fig. 80). Infected BHK cells were harvested at 6 and 16 h pi in SDS lysis buffer and samples were analysed by a 10% SDS-PAGE. As predicted, the UL43.5 antiserum detected UL43.5 in 17^+ only at 16 h pi, with an apparent M_r of 38 kDa (Fig 80). However, UL43.5 was not detected in any of the recombinant viruses. UL43.5 expression was also examined in 1716 but was not detected (data not shown). The UL43.5 antiserum is weak and does not readily detect UL43.5 expression from wild type by Western blotting.

All recombinant viruses described above contain an identical SV40 promoter driving the β-galactosodase gene. To determine if there was an orientation effect on expression levels of inserted genes, a Western blot was performed to examine the level of expression of β-galactosidase using an anti-β-galactosidase serum (Fig. 81). 17^+ and 1716 were included in this experiment as negative controls (Fig. 81). Three different cell lines were used (BHK, HeLa, and SK-N-SH) to eliminate the possibility that any observed orientation effect on expression was not cell type specific. All cells were infected at a m.o.i. of 20 p.f.u./cell. In BHK cells, 1623 and 1628, containing *lacZ* in the UL43.5 transcriptional orientation, show higher levels of β-galactosidase expression (Fig. 81a). In contrast, 1622 and 1627 have *lacZ* in the UL43 transcriptional orientation, express β-galactosidase at low levels, barely detectable by Western blot (Fig. 81). A similar expression pattern was also seen in HeLa cells (Fig 81b). Although lower cell density of SK-N-SH cells caused fewer cells to be loaded, the same *lacZ* expression profile among recombinant viruses as in BHK and HeLa cells was observed (Fig. 81c).

The above data suggests that orientation is one factor causing different expression levels from an inserted gene in the *Nsi*I UL43 locus. However, the difference in β-galactosidase expression may also have been a result of the overall length or sequence of the inserted gene. Although the inserts contained an identical β-galactosidase gene the

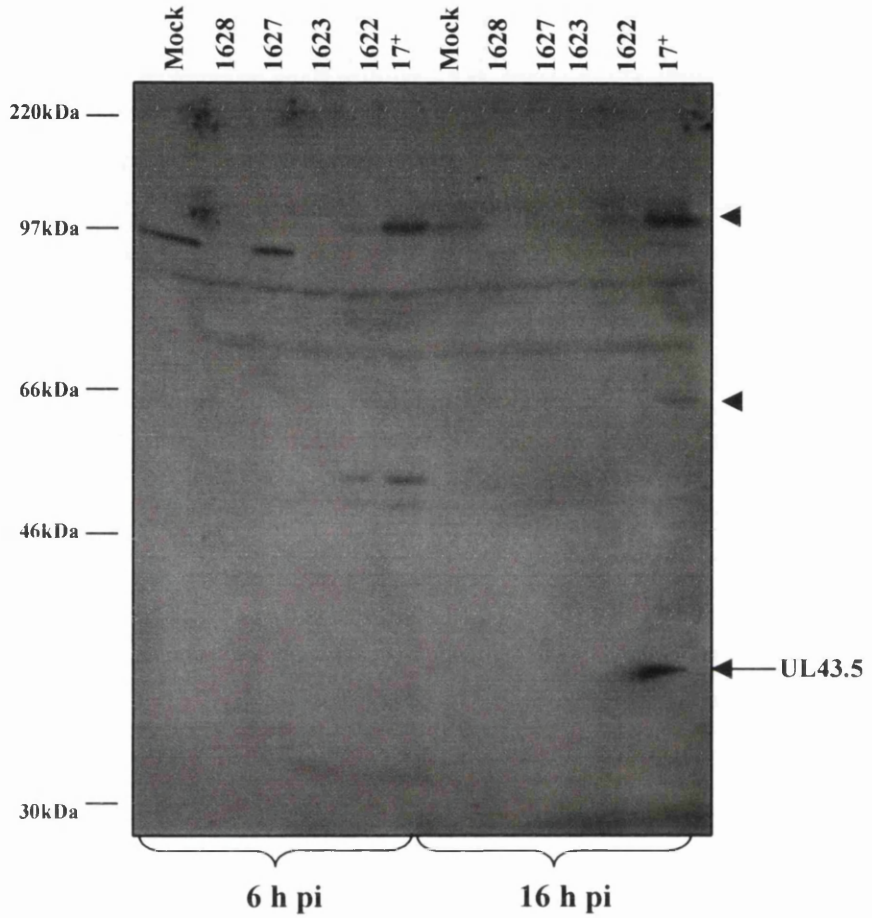


Figure 80. Western blot of UL43.5 using anti-UL43.5 sera.

BHK cells were infected at a m.o.i. of 20 pfu/cell and harvested in SDS lysis buffer at 6 h pi and 16 h pi. Samples were analysed by 10% SDS-PAGE and Western blotted with UL43.5 antiserum (Ward *et al.*, 1996). The positive control of 17^+ shows the UL43.5 expression at 16 h pi with a M_r of 38 kDa. Two unidentified bands are recognised from 17^+ infection 6 h and 16 h pi with apparent M_r 64 kDa and 98 kDa (lane 12;◀)

Table 19. Orientation of RL1 and *lacZ* genes in recombinant viruses.

	lacZ	RL1
1622	UL43	UL43.5
1623	UL43.5	UL43
1627	UL43	UL43.5
1628	UL43.5	UL43

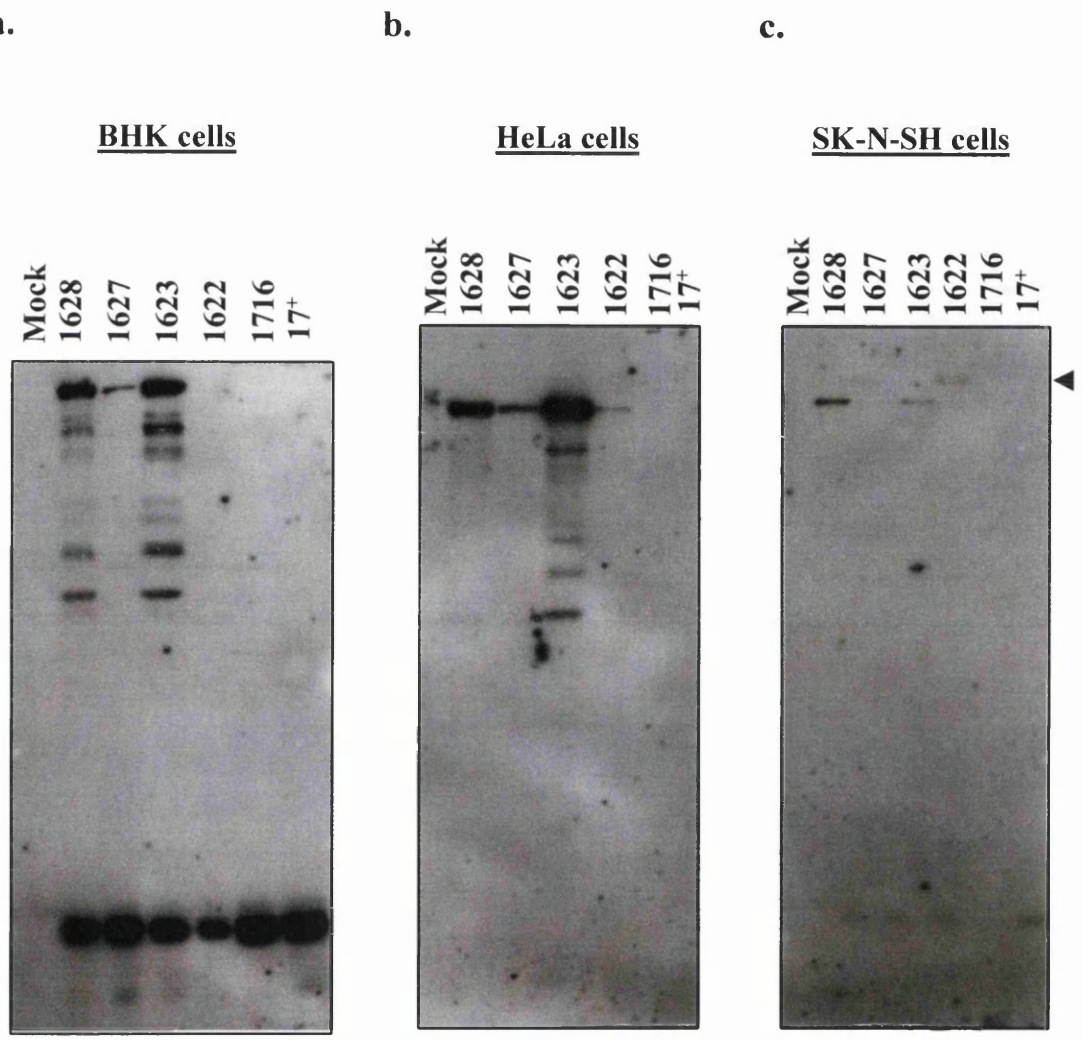


Figure 81. Western blot of β-galactosidase expression in different cell lines.

Cells were infected with different viruses at a m.o.i. of 20 p.f.u./cell. Infected cells were harvested into loading buffer 16 h pi. Samples were analysed using 10% SDS-PAGE and Western blotted with β-galactosidase antiserum.
a. In BHK cells, the β-galactosidase expression is predominant from 1628 and 1623 virus infection. **b**. In HeLa cells, the β-galactosidase expression predominant from 1628 and 1623 infections. **c**. In SK-N-SH cells, there is β-galactosidase expression from 1628 and 1623. 1622 has a non-specific band which migrates slower than β-galactosidase (◄).

RL1 gene in all four recombinant viruses varied in length by 100 to 150 bp. 1623 has a 134 bp larger RL1 insert than 1622 but 1627 has 154 bp more than 1628 due to the intron sequence.

6.1.2. Direct Comparison of Orientation and Expression of Genes Inserted into the *Nsi*I UL43 Locus

In order to confirm if the orientation of a gene inserted in the UL43 locus has an affect on expression of that gene, two new viruses were constructed using the original RL1 cassette from 1622 (Fig. 82a). pHH1 was digested with *Pac*I to separate the *Xba*I RL1/*lacZ* cassette. This cassette was re-cloned into the UL43 *Pac*I site in p35(PacI). Two new plasmids pHH4 and pHH5 (Fig 82b,c), were digested with *Bam*HI to determine the RL1 orientation. Digested DNA was separated by electrophoresis through a 1% (w/v) agarose gel (Fig. 83) and showed that pHH4 has one band of 7.4 kbp and another of 5.8 kbp determining that RL1 is in the same transcriptional orientation as UL43.5. pHH5 shows two bands of 8.5 kbp and 4.7 kbp determining that the RL1 gene is in the same transcriptional orientation as UL43.

pHH4 and pHH5 were separately co-transfected with 1716 DNA generating two new viruses 16(UL43.5)22 and 16(UL43)22, respectively. These viruses were purified once on BHK cells and their genomic structure analysed by Southern blotting (data not shown). BHK cells were infected at a m.o.i. of 10 pfu/cell and harvested 18 h pi. Extracts were analysed by 10% SDS-PAGE and Western blotted with 137 (Fig. 84). 16(UL43.5)22 and 17^+ express ICP34.5. The appearance that ICP34.5 from 16(UL43.5)22 is 1-2 kDa heavier than from 17^+, is probably due to uneven migration of the samples through the gel. The 70 kDa protein induced by 17^+ infection is not recognised from the recombinant viruses. Instead, in 16(UL43)22 and 16(UL43.5)22 a 98 kDa protein is detected. 16(UL43)22 shows no apparent ICP34.5 expression. This is expected since the RL1 gene is in the same transcriptional orientation as UL43. We conclude that there is a direct correlation between orientation and expression of an inserted gene in the UL43 *Nsi*I locus. However, caution must be taken when exerting the importance of orientation expression as this could not be quantitated due to time constraints and virus impurity.

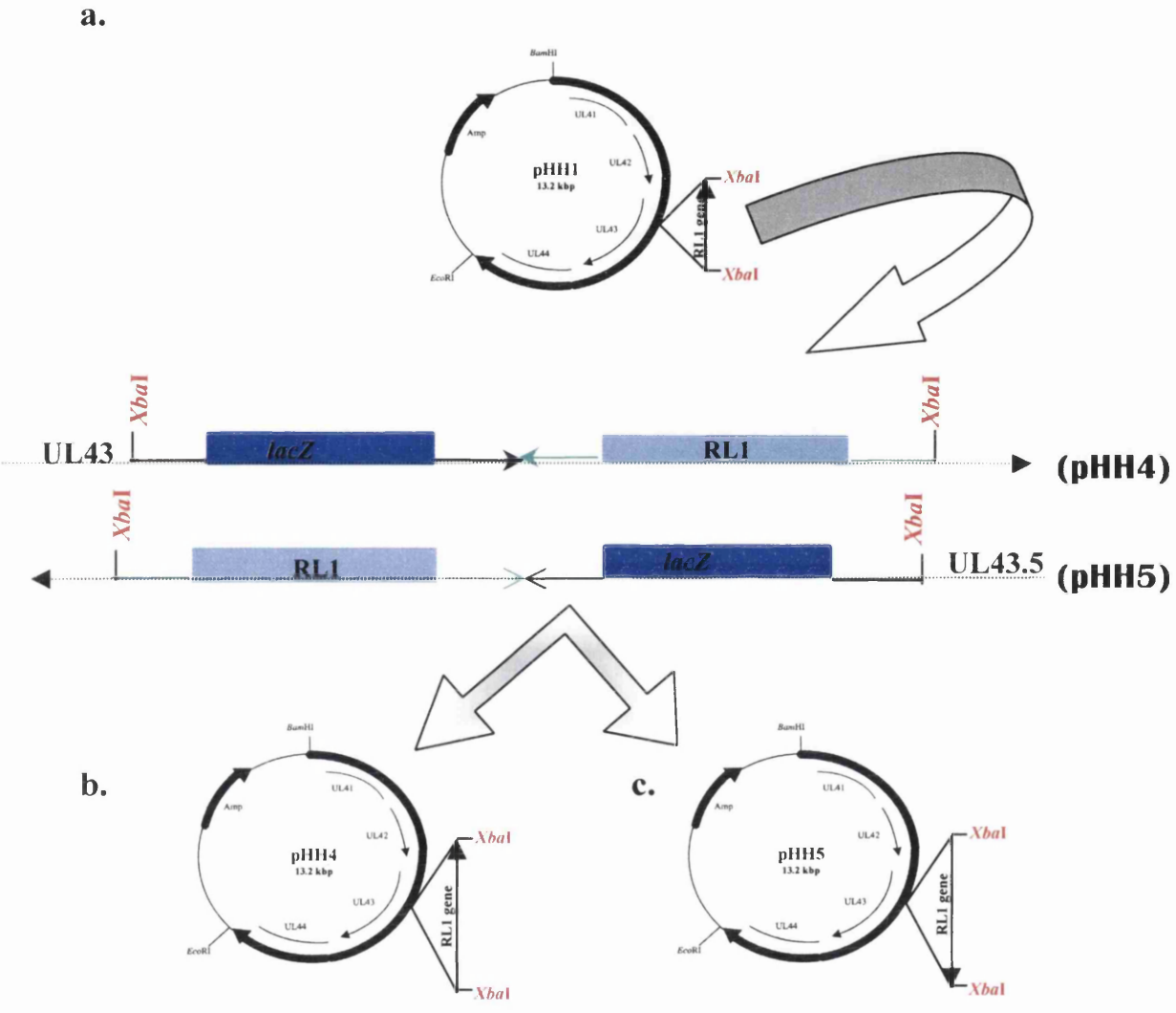


Figure 82. Schematic representation of cloning pHH4 and pHH5.

a. pHH1 was digested with *Pac*I to isolate the RL1/*lacZ* cassette from the plasmid backbone. **b.** and **c.** The RL1/ *lacZ* cassette was sub-cloned in opposite orientations into the UL43 *Pac*I site in UL43 in p35(PacI) generating pHH4 and pHH5.

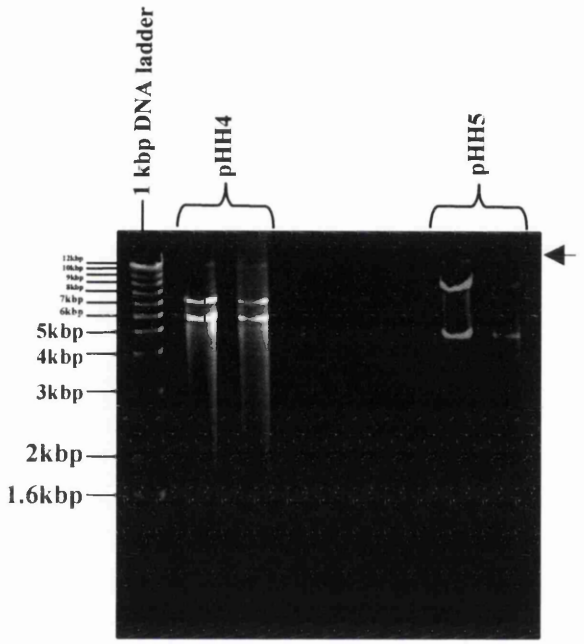


Figure 83. Agarose gel of 16(UL43)22 and 16(UL43.5)22.

A 1% (w/v) agarose gel of pHH4 and pHH5 which were used to generate recombinant viruses 16(UL43.5)22 and 16(UL43)22, respectively. *Bam*HI digests pHH4 into two bands, 7.4 kbp and 5.8 kbp. pHH5 digests into two bands 8.5 kbp and 4.7 kbp. Sizes were determined by comparison to a 1 kbp DNA ladder. Undigested plasmid DNA is also visible (◄).

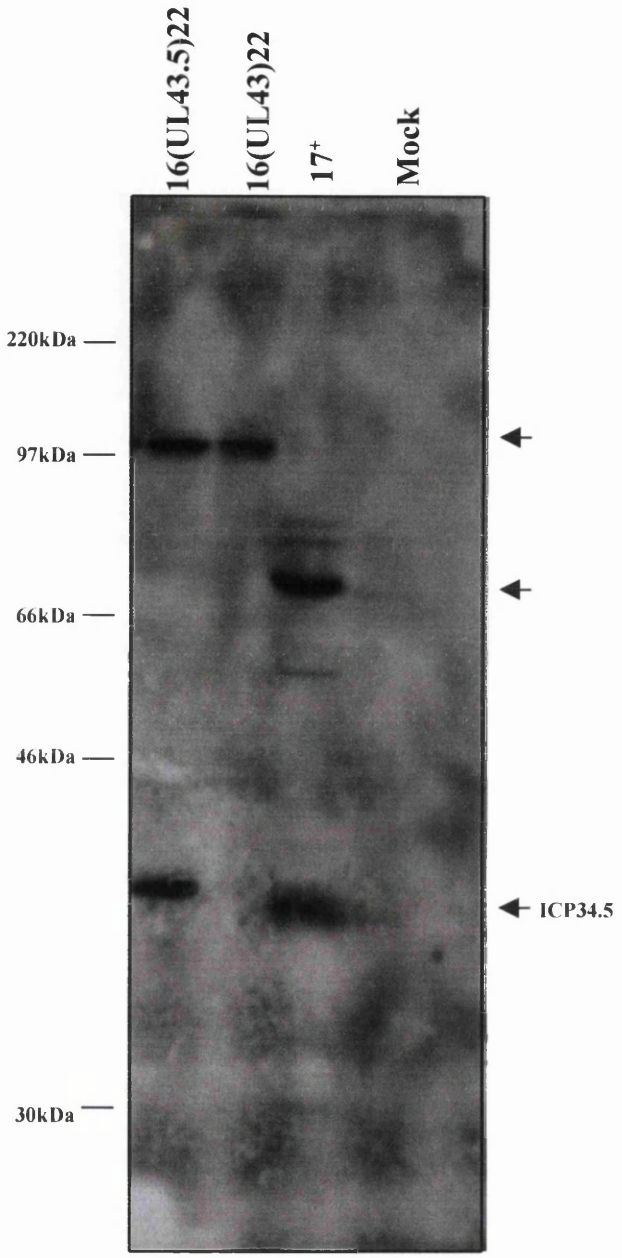


Figure 84. 16(UL43)22 and 16(UL43.5)22 recombinant viruses ICP34.5 expression by Western blotting.

A 10% SDS-polyacrylamide gel was Western blotted with 137 to analyse BHK cells infected with new recombinant viruses, 17^+, and mock, 18 h pi. 16(UL43.5)22 express ICP34.5 with an apparent 1-2 kDa more than 17^+ of 37 kDa. The 70 kDa virus induced protein is present from 17^+ infection. A 98 kDa protein is recognised from recombinant virus extracts.

6.1.3. Discussion

Before this study, it was not known that the orientation of an inserted gene in UL43/UL43.5 might have an effect on expression. Looking more closely at the *Nsi*I site in UL43/UL43.5 we see that by inserting a gene in the *Nsi*I locus the gene is placed at the immediate 5' terminus of UL43. Inserting a gene in this locus stops translation of UL43, however, it might allow translation of a truncated UL43.5. UL43.5 expression was analysed by a UL43.5 antiserum (Ward *et al.*, 1996) and although there was no apparent expression of UL43.5 by Western blot analysis we cannot conclude that a truncated protein not recognised by this antiserum may be expressed. A potential experiment for further investigation would be to look for possible recognition of a truncated UL43.5 transcript.

This finding has significant implications for HSV vectors. Several investigators are looking at nonessential genes such as UL43 within the HSV genome as sites to insert genes of interest to target different disorders for cancer and/or gene therapy. If the level of the desired gene expression is critical then this study suggests that the gene should be inserted in the same transcriptional orientation as UL43.5. Transcriptional studies of RNA could be carried out to assess this phenomenon. This orientation dependent effect might apply to other sites in the HSV genome.

6.2. RECOMBINANT VIRUSES AND PLAQUE MORPHOLOGY

6.2.1. Introduction

HSV induces cellular changes upon infection *in vitro* which include ballooning of the cells, the appearance of condensed nuclear chromatin and ultimately degeneration of the host cell nuclei. Throughout this study recombinant viruses were constructed using the RL1 deleted mutant 1716 as their parental strain. A striking feature of these recombinant viruses as well as 1716 itself is their plaque morphology in all cell types. Both permissive and non-permissive cells revealed mutant virus plaque sizes smaller than the wild type HSV-1 17^{+}. Although plaque size is somewhat arbitrary, dependent on time of infection and state of cell cycle, the plaque phenotype in these mutant viruses was so marked it is described and investigated below.

6.2.2. Plaque Phenotype in Different Cells

3T6 cells are non-permissive for ICP34.5 negative viruses whereas BHK cells are permissive. Cell monolayers (BHK and 3T6) were infected with serial 10-fold dilutions of 1622, 1716 and 17^{+}. After 1 h absorption the monolayers were washed twice with PBS and a methylcellulose/media overlay applied (Section 3.14). Infected cell monolayers were kept at 37°C 5%CO_2 for 72 h. After incubation the methylcellulose overlay was removed and the cells were fixed and stained with Giemsa. Plaques were visualised under a microscope at 4x magnification and photomicrographs were taken (Fig. 85). Small plaques from 1716 and 1622 were seen on 3T6 cells (Fig. 85) and on permissive BHK cells (Fig. 85). The small plaque morphology from 1716 on 3T6 cells has been documented previously (Brown *et al.*, 1994a,b). However, it was predicted that 1622 plaques would return to wild type size. The small plaque phenotype of 1716 and 1622 was observed in all cell lines examined in this study. The average plaque size for each virus on 3T6 cells was quantified

by measuring plaque size from 60mm plates and is shown in Table 20. Photomicrographs of plaques were precisely cut out and weighed. Average weights are given in grams and confirm that there is a distinct difference between 1716/1622 and 17^{+}.

Virus stocks were analysed to determine particle to p.f.u. ratios. It is important to note that stocks were not grown simultaneously (i.e. different passage number of BHK cells). The results in Table 21 show particle:p.f.u. ratios of all viruses were similar. This is in agreement with the observation that despite the apparently poor plaque formation,

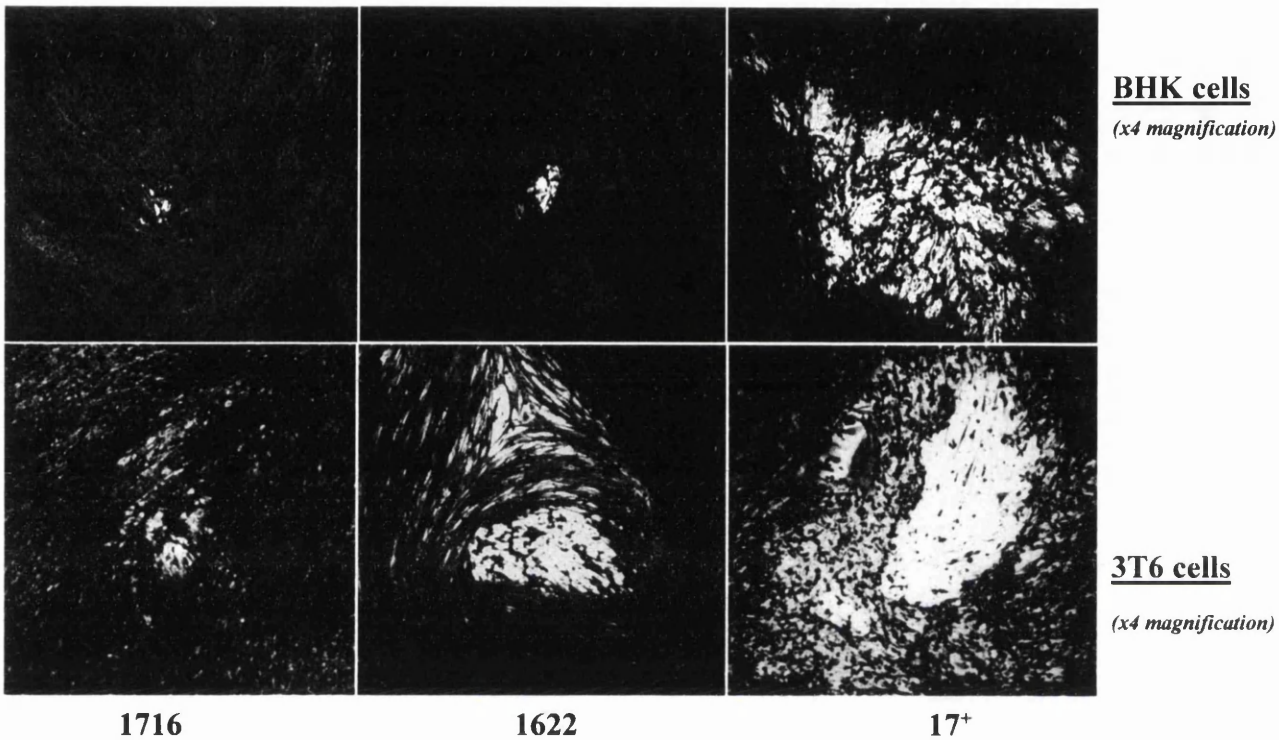


Figure 85. Plaque sizes between 17^+, 1716 and 1622 on BHK and 3T6.

Plaque size variation between 1716, 1622 and 17^+ is evident in BHK and 3T6 cells. Photomicrographs were taken at 4x magnification.

Table 20. Plaque size quantification in 3T6 cells.

Virus	Number of plaques tested	Ave. wt(g)	% of *wt* plaque weight
1622	26	0.3101	19%
1716	26	0.0926	6%
17^{+}	22	1.5975	100%

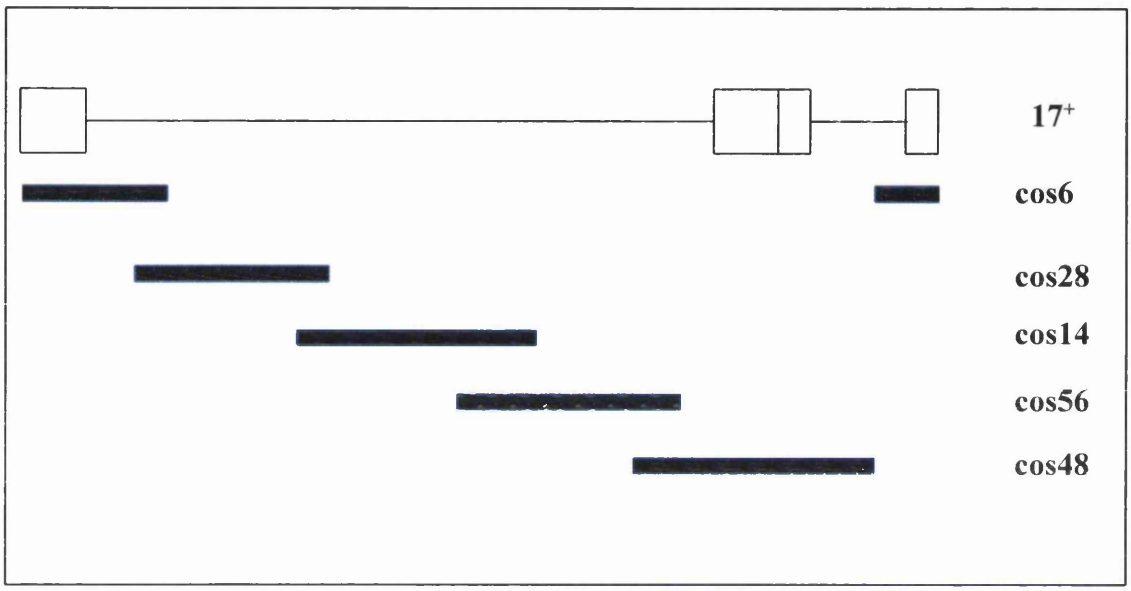


Figure 86. Diagrammatic representation of the "set c" cosmids.

The entire HSV-1 strain 17^+ genome fragments inserted into separate cosmids. (Cunningham and Davison, 1993). For exact map location and n.p. see Table 22.

mutant viruses were able to replicate normally in single-cycle growth experiments in BHK cells (Sections 4.2, 4.3 and 5.3).

6.2.3. Rescue of Plaque Size Phenotype using Cosmids

To ascertain whether the small plaque phenotype was the result of a second mutation elsewhere in the 1716 genome rescuants were constructed using cosmids spanning the entire HSV-1 17^+ genome (Cunningham and Davison, 1993). Cosmid set C was used in this study which contains the following cosmids: cos6, cos28, cos14, cos56 and cos48 (Fig. 86; Table 22). Individual cosmids were co-transfected with 1716 DNA and harvested transfectants were given a unique number: 1, 2, 3, 4, and 5 (Table 22). Positive and negative transfection controls were carried out using 1716 or 17^+ DNA alone, named 6 and 7, respectively.

Two cosmids co-transfected on BHK cells with 1716 DNA (1 and 5) and the positive control of 17^+ DNA alone (7) rescued large, wild type 17^+ plaques (data not shown). To test whether this was cell type specific the transfectants 1 and 5 along with control transfectants 6 and 7 were titrated on rabbit skin cells (data not shown). Similar to BHK cells only transfectants 1 and 5 along with 17^+ (7) produced interspersed wild type plaques. Cosmid tranfections were serially diluted and plated on 3T6 cell monolayers. The only tranfectants producing plaques were 1 and 5 and the positive control 17^+ (7) (Fig 87). Holes in the monolayers appeared from transfectants 2 and 3 at the periphery of the plate which were due to detached cells and not virus plaque formation (Fig. 87).

A semi-quantitative analysis was performed by taking photomicrographs of plaques at 4x magnification as described above (Section 6.2.2.). Individual transfectants were examined on BHK and 3T6 cells. The only tranfectants to produce plaques on 3T6 cells were 1 and 5 and the positive control 17^+, 7 (Table 23). In BHK cells, all tranfectants produced plaques but of varying sizes. The averages and percent comparison of weight between tranfectants are given in Table 22. The frequency with which wild type large plaques occurred from 1 and 5 was low due to the mixed population of the transfection plate containing 'wild type' and '1716' plaques.

6.2.4. Rescued Plaque Size Phenotype using pBAM'k'

The cosmid experiments determined that no other mutation outside TR_L and IR_L regions was causing a genuine small plaque phenotype. However, other mutant viruses,

Table 21. Plaque forming efficiencies of 17^+, 1716 and recombinant viruses on BHK cells.

Virus	Yield of Particles	Titre (p.f.u.)	Particle to p.f.u. ratios
17^+	2.295×10^{11}	1×10^{10}	23 (1)
1716	2.4×10^{10}	1×10^{9}	24 (1.0)
1622	3.3×10^{10}	1×10^{9}	33 (1.3)
1623	4.87×10^{10}	1×10^{8}	48.7 (2.4)
1627	6.0×10^{9}	1×10^{8}	60 (2.8)
1628	2.55×10^{10}	3×10^{9}	8.5 (0.37)

Table 22. Cosmids with n.p. and new transfectant names.

Cosmid	n.p.	1716/cosmid tranfectant name
cos6	141221-29733	1
cos28	24699-64405	2
cos14	54445-90477	3
cos56	79442-115152	4
cos48	107496-144681	5

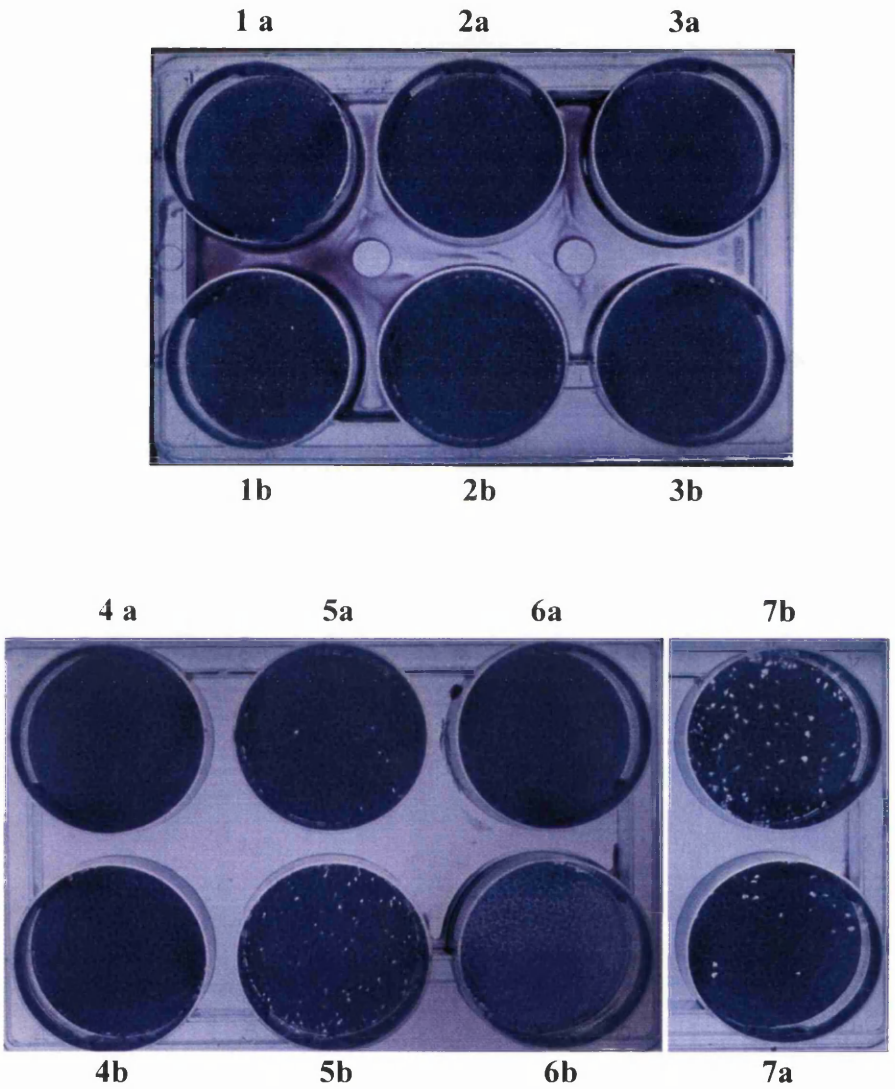


Figure 87. Transfectants titrated on 3T6 cells.

Each transfection was plated on 3T6 cells at (**a**) $1x10^1$ p.f.u./well or (**b**)1 pfu/well. Numbers refer to the cosmid 1716 combination (Table 22). 1, 5 and 7 (positive control) were the only transfections to forms plaques on 3T6 cell monolayers.

Table 23. Plaque sizes from cosmid and 1716 tranfectants on 3T6 and BHK cells.

3T6 cells

Transfectant number	Number of plaques tested	Ave. weight(g)	% of *wt* plaque weight
1	9	0.995	48%
5	8	1.20	58%
7	10	2.06	100%

BHK cells

Transfectant number	Number of plaques tested	Ave. weight(g)	% of *wt* plaque weight
1	3	0.400	22%
2	3	0.208	24%
3	3	0.516	28%
4	3	0.358	19%
5	3	0.535	29%
6	3	0.360	19%
7	4	1.86	100%

which contain stop codons in the RL1 gene do not cause small plaque phenotypes. 1771 and 1771R and 17termA and 17termAR were included in a single direct comparison experiment with 1716 and 17^+ to observe plaque size phenotype (data not shown). The 1716 rescuant virus 1716R, was originally generated using plasmid pBam'k' and produced a wild type 17^+ plaque phenotype.

6.2.5. HSV Glycoprotein Expression

Glycoproteins play an integral role in the viruses ability to spread from cell to cell causing syncitia and plaque formation. The glycoproteins gB, gD and gI have been heavily studied to demonstrate their role in HSV-1 infection. Western blots were performed to determine the expression of some of these glycoproteins during infection in BHK cells.

BHK cells were infected at 20 pfu/cell and harvested in SDS lysis buffer 16 h pi. Samples were visualised by SDS-PAGE. Glycoprotein D protein was expressed from all recombinant viral infections (Fig 88). A Western blot using gD antiserum showed expression of gD from all viruses (Fig. 88). Due to the poor quality of the lane, the 17^+ sample appears skewed. Different extracts were analysed on a second gel and the blot was exposed to autoradiograph film. A short exposure of the blot demonstrated that gD expression is better in 17^+ and 1628. However, a longer exposure showed that all recombinant viruses express gD (Fig. 89).

Infected BHK cells were harvested 16 h pi, analysed on a 10% SDS-PAGE, and Western blotted with gC antiserum (Fig. 90a) 17^+ and 1716 showed expression of gC but there was no detectable expression from 1622 (Fig. 90). The experiment was repeated using two time points of 6 h and 16 h pi (Fig 90b). 17^+ and 1716 show expression of gC at 6 h. By 16 h pi. 17^+ and 1716 express gC, however no recombinant viruses are expressing any detectable gC.

Viruses were assayed by Western blot to examine gI expression using a monoclonal antibody against gI (Fig 91). BHK cells were infected with 17^+, 1716 and 1622 at a m.o.i. of 20 pfu/cell and harvested at 16 h pi. All viruses show gI expression at similar levels (Fig. 91).

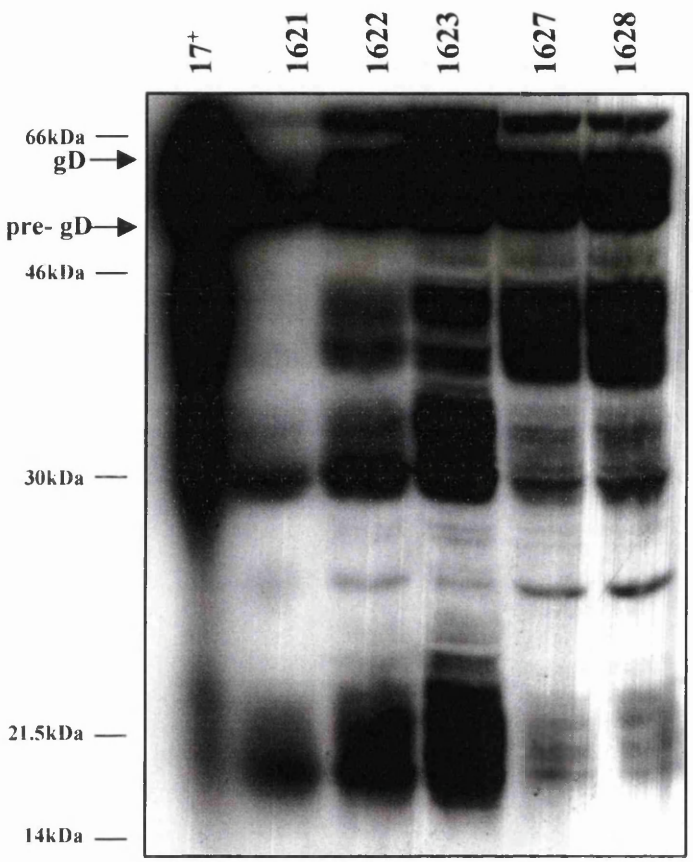


Figure 88. Western blotting with gD antiserum.

Infected BHK cells were analysed by SDS-PAGE and Western blotted with a gD antiserum.

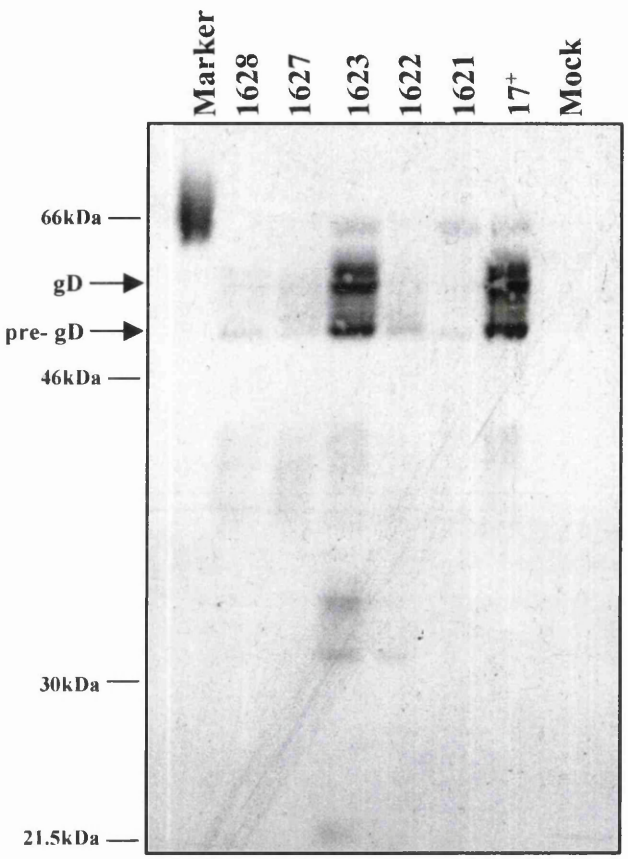


Figure 89. Western blot with gD antiserum.

Infected BHK cells visualised by SDS-PAGE which was Western blot with gD antiserum shows clearly gD expression in 17^+ and 1623.

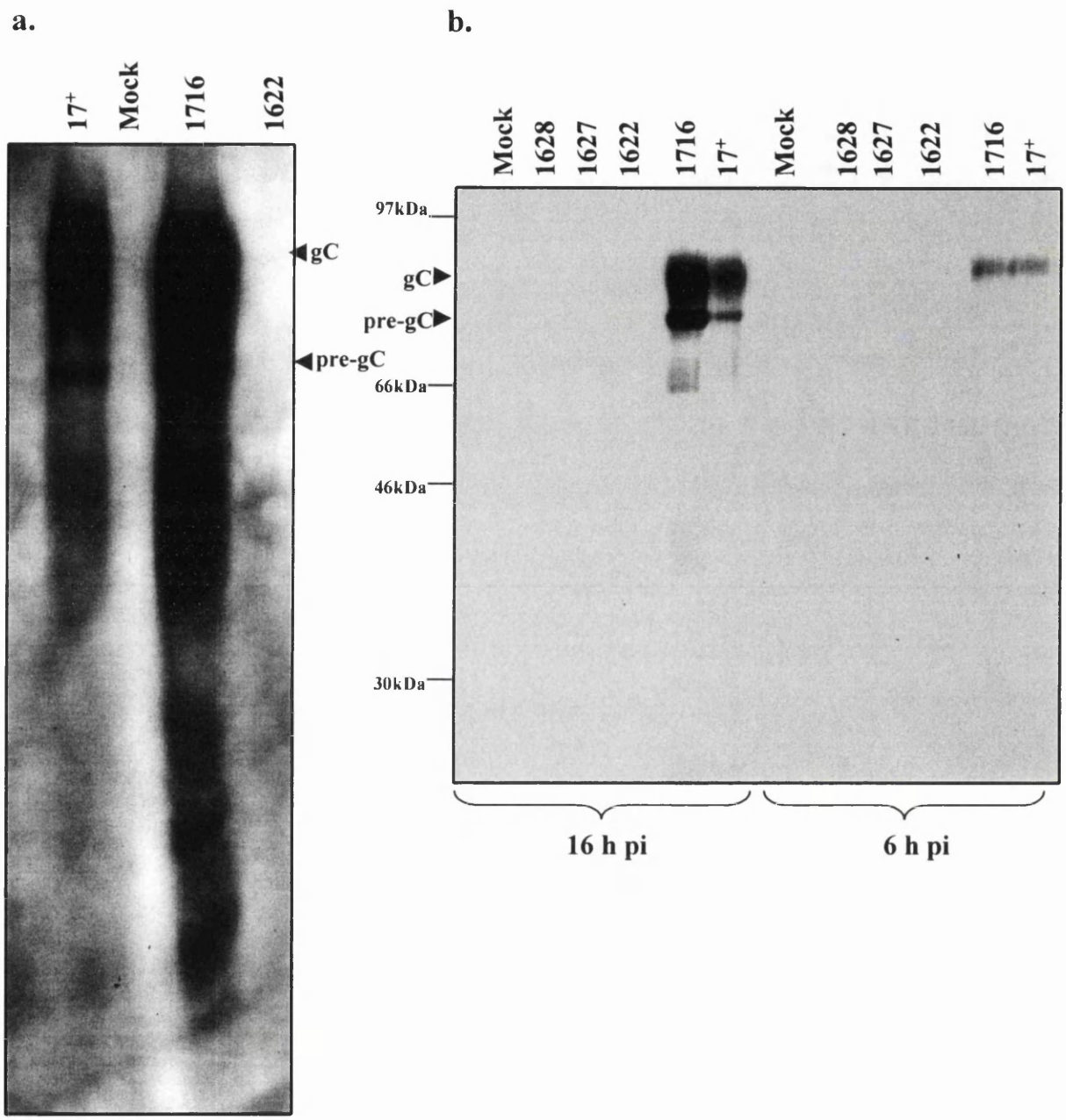


Figure 90. Western blotting with gC antiserum.

a. BHK cells were infected and harvested 16 h pi in SDS lysis buffer, analysed by 10% SDS-PAGE and Western blotted with gC antiserum. Glycoprotein C is detectable from 17^+ and 1716 infected extracts. **b**. BHK cells were infected at a m.o.i. of 20 p.f.u./cell and harvested at either 6 h pi or 16 h pi.

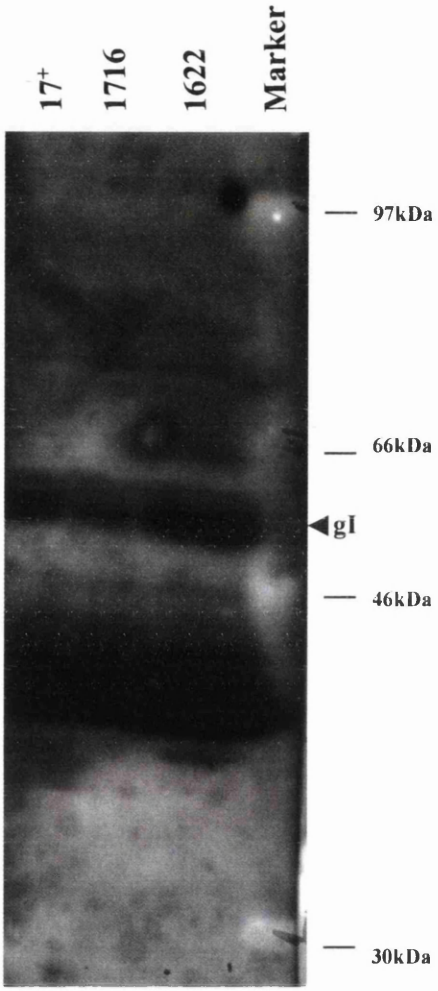


Figure 91. Western blotting with anti-gI serum.

BHK cells were infected at a m.o.i. of 20 pfu/cell and harvested 16 h pi. Samples were analysed by 10% SDS-PAGE, and Western blotted with gI mAb, 3104.

6.2.6. Discussion

HSV-1 mutations in several genes with a small plaque phenotype have been described. Brown *et al* (1994a) first reported a small plaque phenotype in BHK cells from the RL1 deleted virus 1716. Another mutant producing small plaques is the Vmw65 insertion mutant, *in*1814 (Ace *et al.*, 1989), which has a high particle to pfu ratio and is avirulent, but can establish latency and reactivate. A UL39 deleted mutant yields 4 to 5 fold less virus, has small plaque morphology and is avirulent (Goldstein and Weller, 1988).

Additionally, polykaryocyte formation caused by herpes simplex virus infection in a given cell line has been suggested to be due to alteration in at least 6 *syn* loci: S*yn*1 to 6 (Tognon *et al.*, 1988).

Romanelli *et al* (1989) characterized a mutant virus with different syncytial genes deleted. *Syn*1 maps to the *Bam*HI 'l' fragment affecting gK (Romanelli *et al.*, 1991). The *syn*3 mutation affects the 3' end of gB (DeLuca *et al.*, 1982; Kousoulas *et al.*, 1984). *Syn*5 is located within the *Bam*HI 'q' fragment affecting the UL24 gene. In 1991, Romanelli *et al* studied the *syn*6 and found that it lies in the junction fragment *Bam*HI *k*. Only *Syn*6 was able to induce syncitium formation in Hep-2, Vero, BHK and HEL cells. Romanelli *et al* (1991) mapped the *syn*6 mutation to a *Bam*HI/*Sac*I C (~1.6 kbp) fragment. This fragment includes the 5' end of RL2 and the putative polyadenylation signal of RL1. They concluded that the gene responsible for the *syn*6 mutation might be ICP0 or ICP34.5. They also suggest that the *syn*6 locus may be an unidentified gene which overlaps with the RL2 gene or lies on the 1 kbp region between the 5' end of RL2 and the *Sac*I site, the product of which is involved in fusion. Another attractive candidate is ORF P.

The lack of rescued plaque size in 1622 which overexpresses ICP34.5 indicates that small plaque formation is not due to level of ICP34.5 expression. It is also important to note that when ICP34.5 is not expressed in 1771 or 17termA, two RL1 stop codon mutants, plaque formation is identical to wild type 17^+ in all permissive cell types examined in this study. This implies that some sequence other than RL1 may be missing or mutated in 1716 and its 'rescuants' 1622, 1623, 1624, 1627, and 1628.

DNA sequence analysis experiments spanning the BamHI *k* region could be carried out to determine if there may, in fact, be an additional mutation in these viruses.

Certain glycoproteins associated with virus maturation and egress were examined in this study. Glycoprotein C was not expressed from all 1716 recombinant viruses, but expressed in 1716 and 17^+. This may be attributed to cloning in this region, as gC is

expressed from UL44. Recombinant viruses manipulated in UL43 may have resulted in a mutation stopping the expression of gC. However, other mutant viruses with mutation in UL43/UL43.5 do not demonstrate the described plaque morphology.

Another glycoprotein involved in egress is gI. Equal expression of gI from recombinant viruses, 1716 and 17^{+} was determined by Western blotting. Finally, gD expression from all viruses was shown by Western blotting.

7. FINAL DISCUSSION

7.1. ICP34.5 and ORF P Recombinant Viruses

A complicating feature of RL1 mutants has been the discovery of two overlapping antisense open reading frames (O and P) to RL1. Transcripts spanning the long short repeat region (L/STs) of HSV-1 were reported by two independent groups (Bohenzky *et al.*, 1993; 1995; Yeh and Schaffer, 1993). These RNAs are transcribed in the same orientation as LATs. A strong binding site for ICP4 is at the 5' terminus of the L/STs (Yeh and Schaffer, 1993; Samaniego *et al.*, 1997; Randall *et al.*, 1997) and mutagenesis of this site resulted in over production of ORF P and L/STs during normal lytic infection, demonstrating that ICP4 represses transcription of L/STs (Lagunoff and Roizman, 1995; Lee and Schaffer, 1998). Lagunoff and Roizman, (1995) hypothesised that expression of ORF O and P would only be expected in infected cells lacking functional ICP4, a condition satisfied by latency (Preston, 2000). The observation predicted that a positive feedback system could control HSV gene expression. During latency in the absence of ICP34.5, ORF O and P would be expressed and prevent the synthesis or function of IE proteins, thereby maintaining the remainder of the genome in a stable nontranscribed state. Reactivation could result from interference with ORF O or P function during latency (Preston, 2000), but is disputed by the observations that latency was established by a normal virus mutant which ORF P was specifically interrupted (Lee and Schaffer, 1998); mutants with derepressed synthesis of ORF P established latency inefficiently and were also highly attenuated (Bruni and Roizman 1996; Lee and Schaffer, 1998).

The RL1/ORF P deletion mutant, 1716, has been well characterised *in vitro* and *in vivo* and shown to be avirulent upon IC inoculation in BALB/c mice (MacLean *et al.*, 1991). 1716 infection resulted in "abortive" plaques on mouse embryo fibroblast 3T6 cell monolayers (Brown *et al.*, 1994 a,b; Brown *et al.*, 1997). 1716 infected cells were studied by electron microscopy which showed 1716 was not released from the lamellae of the cell. To gain a better understanding of the function of the deleted sequence in 1716 the two identified genes, RL1 and ORF P, shown to express proteins ICP34.5 and ORF P, respectively, were re-introduced independently into 1716.

The basic design of all recombinant viruses was the following: an expression cassette was inserted, into the 1716 UL43 locus, which contained the gene of interest under a HSV-1 gD promoter with a HSV-2 IE5 polyadenylation signal, and in the opposite orientation a *lacZ* gene under a SV40 promoter with a polyadenylation signal. Cloning of these recombinant viruses required several steps which have been described in Section 4.1.

The selection of the insertion locus, UL43, was based on previous studies (MacLean, C. *et al.*, 1991) which demonstrated deletion of this gene conferred no affect on virus replication *in vitro* or *in vivo*. The UL43 locus was found in this study to have additional points of interest which will be discussed in relation to Section 6.1. The HSV-1 gD promoter was used to express RL1 or ORF P as it was believed that this would express the gene of interest strongly with the same kinetics.

The first recombinant virus, 1622, expressed the 17^+ RL1 ORF singly. A second mutant, 1623, expressed RL1 with 134 bp additional upstream sequence.

The third recombinant virus, 1624, expressed the 17^+ ORF P ORF singly. 1624 was constructed to understand the individual role of ORF P. Unfortunately, there was an initial delay in cloning this gene (Section 4.4). Characterization of 1624 could not be done here. However, work is currently under way in the laboratory to purify and subsequently investigate its *in vitro* and *in vivo* properties.

Although 1624 was not characterised in this study, observations were made on ORF P expression from wild type strain 17^+ and other RL1 mutants. Infected cells extracts were analysed by SDS-PAGE and Western blotted using newly generated ORF P antisera (R. Reid). Expression of ORF P from 17^+ infected BHK cells was observed. Additionally, an abundant transcript of 2 kbp was detected following infection of BHK cells at 37°C by HSV-1 strain 17^+ and strain F, even though both produced functional ICP4 protein (McKie, personal communication). Previously published data suggested that only a mutated ICP4 binding site allowed synthesis of ORF P in strain F (Bruni and Roizman, 1996) and in strain KOS (Lee and Schaffer, 1998). Confirmation that ORF P expression from 17^+ was genuine was made when a tsK mutant also showed expression of a similar protein. A second confirmation of ORF P protein expression was made with a mutant virus, 1621. The ORF P protein migrated more slowly from 1621 infected BHK cells due to an epitope tag sequence at the 3' terminus of ICP34.5. ORF P was not detected from 1716 infected cells.

The fourth and fifth intertypic recombinant viruses, 1627 and 1628, contained the HSV-2 strain HG52 RL1 gene in the UL43 locus of 1716. These mutants were constructed to address the second main objective: the identification of HSV-2 ICP34.5 protein expression. 1627 and 1628 will be described in detail later in respect to HSV-2 characterization.

The first objective of this study was to individually express ICP34.5 in the 1716 background to ascertain its role in the 1716 phenotype. Western blotting analysis of 1622

infected BHK cells determined at least an 8-fold increase in ICP34.5 level compared with wild type, 17^+. 1623 infected BHK cells, unexpectedly demonstrated a reduction (8-fold) of ICP34.5 expression compared to 17^+ by Western blotting. One hypothesis for the reduction in ICP34.5 expression from 1623 infected cells is the additional 134 bp which includes three methionines. An experimental approach to examine this is to compare RNA levels in 1622 and 1623 infected cells. If the gD promoter is not as efficient as the wild type promoter and multiple methionines in the sequence could be competing, this may result in less ICP34.5 expression. Another strategy would be to construct a new mutant lacking the gD promoter but maintaining the RL1 ORF with the additional 134 bp. This mutant could demonstrate if the 134 bp leader sequence was sufficient to promote ICP34.5 expression. If this new mutant expressed ICP34.5 with normal kinetics, it could also be analysed in experiments *in vivo* to clarify two questions posed by 1622: (1) if the location of ICP34.5 in the UL43 locus is attributed to its small reduction in neurovirulence and/or (2) if gD promoting RL1 affected the time of ICP34.5 expression in 1622 resulted in decreased replication kinetics *in vivo*.

Expression of ICP34.5 from 1622 infected cells was believed to be responsible for the restored ability of 1716 to replicate to wild type levels in 3T6 cells. Additional cell lines were examined for replication kinetics of 1622, 1623, 1716 and 17^+. In HeLa cells demonstrated to be non-permissive for 1716 and 1623 infection at a low m.o.i., 1622 and 17^+ replicated with similar kinetics.

In permissive BHK cells, 1623 showed slightly impaired levels of replication. At high m.o.i., 3T6 cells failed to allow replication of 1623, with titres lower than 1716. In low m.o.i. infection of Vero cells, 1623 demonstrated replication with only a slight reduction (5-fold) compared to 1622 and 17^+ and replicated slightly better (2-fold) than 1716. In HeLa cells, at low m.o.i. 1716 and 1623 did not replicate. These results correlate with host and viral protein synthesis experiments. One hypothesis for the observed reduction in 1623 replication in 3T6 and Vero cells and lack of replication in HeLa cells is the lower level ICP34.5 expression. An alternative hypothesis for the poor replication of 1623 is the occurrence of a secondary mutation elsewhere in its genome. One approach to test this would be to carry out recombination experiments with cosmids spanning the entire HSV-1 genome and co-transfecting with 1623 DNA. This strategy was applied in this study to examine the 1716 genome (Section 6.2).

An interesting observation was made when examining the replication of several viruses in rabbit skin cells. Virus stocks which were grown on rabbit skin cells were able

to yield higher titres on these cells than virus stocks which were generated on BHK cells. There was no apparent replication defect for RL1 mutants on rabbit skin cells.

Restoration of ICP34.5 function in 1622 infection was confirmed *in vitro* with analysis of host cell and viral protein synthesis in neuroblastoma (SK-N-SH) and HeLa cells. R3616 had been previously shown to cause preclusion of host and viral protein synthesis in two human derived cells lines, SK-N-SH and HFFF. However, R3616 maintains protein synthesis similar to wild type in primate Vero cells. These studies had not previously been done using 1716. Here it is reported that 1716 also precludes protein synthesis in SK-N-SH cells and (to a lesser extent) in HeLa cells. It was not clear why total protein synthesis shutoff was not observed in HeLa cells. The replication kinetics experiments demonstrated an inability of 1716 to produce a productive infection in HeLa cells which suggests the lack of protein synthesis.

ICP34.5 is naturally a diploid gene located in the long repeats but in 1622 the single RL1 gene has been positioned in the middle of the unique long region. This may attribute to a difference in the transcription of ICP34.5 in 1622 and 1623 by different antisense and 5' leader sequences.

The ICP34.5 level expressed from 1623 is sufficient to maintain protein synthesis in neuronal SK-N-SH cells at late times (16 h) post infection but does not contribute to replication.

Due to the unexpected phenotype and questionable genotype of 1623 *in vitro*, it was not included in pathogenicity studies *in vivo*. Instead, 1622 which demonstrated a restored wild type *in vitro* phenotype was examined *in vivo*.

When this project was initiated there was no previous examination of the role of ICP34.5 independently from ORF P *in vivo*. During this study, two groups attempted to characterise the role(s) of ORF P in strain F (Lagunoff *et al.*, 1996) and in strain KOS (Lee and Schaffer, 1998). Attempts to define ORF P role(s) were carried out by site directed mutagenesis experiments and these mutants were characterised *in vivo* (see Introduction).

Initially, 1622 was used in intracranial LD_{50} experiments. LD_{50} values between HSV-1 strains and RL1 mutants vary considerably between groups. The LD_{50} reported for 17^+ is <3.5 pfu/mouse while in strain F it is 10^1 and KOS 10^3. The strain F, RL1 mutant, R4009, has an LD_{50} of >1 x 10^7 p.f.u./mouse (Chou *et al.*, 1990a), but the same RL1 mutant in strain 17^+, 17termA, has an LD_{50} of 1 x 10^4 (Bolovan *et al.*, 1994). Another RL1 stop codon mutant in strain 17^+, 1771, has an LD_{50} value of >1 x 10^6 pfu/mouse (McKie *et al.*, 1994), again comparable to the RL1 deletion mutant, 1716 (MacLean, A. *et al.*, 1991).

al., 1991). Although 1622 had a slightly increased LD_{50} value it was not significantly different from 17^+ in these studies. One aspect to consider when interpreting LD_{50} values is the difference in protocols between laboratories. In this laboratory, a LD_{50} is a measure of any neurological distress and/or death. In other laboratories neurological distress is not included in LD_{50} quantification and animals are left until death occurs, or in some rare instances, lytic infection is cleared.

The LD_{50} originally reported for 1716 was confirmed in this study. The LD_{50} for 1622 and 17^+ were similar. This determined that the function of ICP34.5 for allowing viral replication in the CNS was restored in 1622. Another study to examine the ability of 1622, 17^+ and 1716 to replicate at the periphery and to the ganglia was carried out in Dr. Sawtell's laboratory (Children's Hospital, Cincinnati). Samples were taken after initial infection (day 0) to determine an equivalent input dose of infection ($1x10^5$ pfu/ml) and virus replication was examined starting 2 d pi. All viruses replicate by day 2 in the snout, however, 17^+ was replicating dramatically better (10-fold) than 1622 and 1716. The replication of 17^+ continued and stayed constant throughout the study (4 d pi) but 1622 and 1716 failed to replicate. In the TG of these infected mice, replication of 17^+ and 1622 was observed but replication of 1622 was less and there was no observed replication of 1716.

1622, 17^+ and 1716 were studied in *in vitro* reactivation experiments. In a direct comparison using one dose, 1622 and 17+ reactivated equally well. One theory why the reactivation frequency from 1716 infected mice is reduced it that the amount of virus getting to the ganglia to establish latency is probably low. As seen by the immunohistochemistry experiments, the lack of HSV positive antigen in the ganglia of 1716 represents a lack of virus replication. An *in situ* experiment, looking at RNA levels of 1716 and 1622 in ganglia, could elucidate a more quantitative answer to the amount of 1716 and 1622 replicating to the ganglia.

7.2. HSV-2 ICP34.5 Expression

Another aim of this project was to verify expression of HSV-2 ICP34.5 and determine if HSV-2 ICP34.5 could complement HSV-1 ICP34.5.

This work provides the first demonstration that RL1 in HSV-2 strain HG52 expresses a homologue of HSV-1 ICP34.5. This is not surprising, as deletion of RL1 in both HSV-1 and 2 produces a similar avirulent phenotype (Taha *et al.*, 1989, MacLean, A., *et al.*, 1991). Antisera raised against the HSV-1 homologue did not cross-react with the HSV-2 ICP34.5 protein (Brown *et al.*, 1997). Therefore, we constructed a GST fusion

protein containing the whole of the HSV-2 RL1 ORF. This was used to raise rabbit polyclonal antisera 596 and 597. Recombinant viruses, 1627 and 1628, expressing the HSV-2 ICP34.5 with and without the 154 bp intron, respectively, were constructed to also aid in the detection of ICP34.5. 1627 and 1628 did not express detectable levels of ICP34.5 by Western blotting with HSV-1 ICP34.5 antisera. However, 596 and 597 specifically recognized two proteins of 27 and 37 kDa from wild type HSV-2 and 1627 infected cells. Infection with viruses expressing HSV-2 RL1 without the intron led to expression of only the 37 kDa protein. To indicate its close relationship to ICP34.5, we have named the 27 kDa protein ICP34.5A. The origin of the 27 kDa protein requires further investigation. One possible explanation is translation of a non spliced transcript, or one terminating before the intron acceptor site, to the first in frame stop codon in the intron. This would produce a 185 amino acid protein containing the first exon of ICP34.5 plus an additional 21 amino acids from the intron, approximately two-thirds of the 261 amino acid length and the M_r of ICP34.5. Alternatively, ICP34.5A is expressed from an unspliced or 5' truncated transcript, initiating in the intron and containing exon 2. This is less likely because there is no in-frame ATG in the intron to initiate translation. However, this type of transcript has been shown in another HSV-2 gene, UL16. In UL16, a protein is initiated with a Leu instead of the usual Met (Dolan *et al.*, 1998). This hypothesis requires experimental proof by RNA mapping. Determination of the nature of ICP34.5A will require analysis of viruses with mutations either in exon 1, 2 ,or the intron. The generated antisera recognize ICP34.5 and ICP34.5A by Western blotting and immunoprecipitation indicating recognition of both denatured and native epitopes. Similarly to the anti-HSV-1 ICP34.5 sera, the anti-HSV-2 sera do not cross react with HSV-1 ICP34.5. This is probably because the epitope with the highest homology is at the carboxy terminus and antisera are directed against the amino terminus (Brown *et al.*, 1997).

By constructing recombinant HSV-1 RL1 deleted viruses expressing the HSV-2 gene under a strong HSV-1 promoter (gD) of similar kinetic class to ICP34.5, we have also demonstrated that the HSV-2 gene can, at least *in vitro*, functionally complement the loss of the HSV-1 gene in a HSV-1 background. Future experiments will assess whether this complementation also applies *in vivo* regarding pathogenicity and latency.

The functional importance (if any) of ICP34.5A has yet to be determined. In all assays carried out, both *in vitro* and *in vivo,* a virus with the RL1 intron deleted and hence only expressing the 37 kDa ICP34.5 behaves like wild type. However, in viruses containing the wild type gene (i.e. including the intron) only low levels of the 37 kDa

ICP34.5 are detected by the antisera and the predominant protein is ICP34.5A. It seems unlikely that a protein expressed at such high levels does not have some function. The ability of HSV-2 ICP34.5 to interact with PCNA as illustrated by GST pull-downs indicates at least one common function with HSV-1 ICP34.5. This interaction is likely to be mediated by the highly conserved 63 amino acid carboxy terminal region.

7.3. 1716 and Recombinant Viruses

The locus used in this study for insertion of expression cassettes is the non-essential UL43 gene of HSV-1. If the level of the desired gene expression is critical, then this study suggests that the gene should be inserted in the UL43 *Nsi*I site in the same transcriptional orientation as UL43.5. Additional transcriptional studies of RNA could be carried out to assess this phenomenon., as well as sequence investigation to compare the UL43 and UL43.5 promoters. It was not known that the orientation in the UL43 *Nsi*I locus might cause an effect on inserted gene expression. Looking more closely at the UL43 and UL43.5 sequence we can see that by inserting a gene in the *Nsi*I locus the gene is placed at the immediate 5' terminus of UL43. Inserting a gene in this locus would stop translation of UL43, while allowing translation of UL43.5. UL43.5 expression was analysed by a UL43.5 antiserum (Ward *et al.*, 1996). Although there was no apparent expression of UL43.5 by Western blot analysis we cannot conclude that a truncated protein was not synthesised. An experiment for future would be to look at possible recognition of this truncated UL43.5 protein.

Initial studies of HSV mutants focussed on plaque morphology. Different genes have been studied which demonstrate a role in syncitia formation in certain cells (Section 1.12). Other genes expressing glycoproteins have been studied for their role in absorption and penetration into cells and cell to cell spread (Section 1.4.1). Antisera against gC, gD and gI were available at the Institute and used by Western blotting to examine expression from 1716 and recombinant virus infected BHK cells. The recombinant viruses did not express gC (UL44). Although gC is considered non-essential, it may, as yet, have unknown role(s) *in vivo*. The lack of gC expression could be contributing to the decrease in 1622 replication kinetics in the snout and TG. Expression of the other glycoproteins (gD and gI) was similar among viruses.

It was previously reported by Brown *et al* (1994 a,b) that RL1 is responsible for an unusual plaque morphology. 1716 gives small "abortive" plaques in 3T6 cells (Brown *et al.*, 1994a). The size of plaques was called abortive because 1716 did not replicate to wild

type levels on 3T6 cells. Here it is reported that 1716 and all recombinant viruses containing functional ICP34.5, produce small plaques on BHK and 3T6 cells, regardless of a productive infection or not. Analysis of the particle to p.f.u. ratio by electron microscopy did not elucidate any further evidence that 1716 recombinant viruses did not produce wild type particles. In an attempt to pin-point a second mutation in the 1716 genome, recombination experiments were carried out, using a series of cosmids with co-transfection of 1716 DNA. These experiments showed that wild type plaque size was rescued by recombination with sequences in the long repeats. However, additional recombination experiments using plasmids containing smaller sequences spanning the long repeat region could give a better definition of the mutated sequence. A final experiment to address if there may be a single amino acid mutation which results in this unusual phenotype could be carried out by sequencing the *Bam*HI *k* region.

7.4. Future work

Two novel findings from this study include (1) the construction and characterization of 1622 and (2) detecting expression of ICP34.5 and ICP34.5A from HSV-2 strain HG52 infected cells.

Before any definitive conclusions can be drawn from the 1622 expression of ICP34.5 and 1622 pathogenicity, characterization of 1624 must be carried out. This would allow a direct comparison between these mutants *in vitro* and *in vivo*. An experiment to examine the increased level of ICP34.5 expression from 1622 infection could include Northern blotting of ICP34.5 transcripts *in vitro* and *in situ* hybridisation *in vivo*. Western blotting with HSV-1 ICP34.5 antisera consistently recognised a virally induced 70 kDa protein from 17^+, 1716 and 1622 BHK infected cells. Brown *et al* (1997) identified this protein from virally infected BHK cells and only 17^+ infected 3T6 cells, but not mock or 1716 infected 3T6 cells. The 70 kDa protein is dependent upon productive infection and not dependent upon ICP34.5 expression (Brown *et al.*, 1997). The 70 kDa protein was not detected until 8 h pi and not induced in the presence of PAA, suggesting it is a late consequence of infection. Further investigation to identify this protein is currently underway by using cDNA libraries. The yeast-two-hybrid system could be used to analyse full protein interactions with ICP34.5.

Appendix A. List of the identified HSV-1 genes and proteins.
The staus of each gene is indicated as E=essential, NE=nonessential E?= proabably essential, E/NE=non-essential under certain conditions, ?=status unknown. (Adapted from McGeoch *et al.*, 1993; Fields, 1996.)

Gene Names	Protein Names	Status
RL1	ICP34.5, $\gamma_1$34.5	NE
ORF P	OPP	NE
ORF O	OPO	NE
RL2 (IE-1)	ICP0, Vmw110	NE
LAT		NE
UL1	gL	E
UL2		NE
UL3		NE
UL4		NE
UL5		E
UL6		E
UL7		?
UL8		E
UL8.5		?
UL9		E
UL9.5		?
UL10	gM	NE
UL11		NE
UL12		E
UL13		NE
UL14		?
UL15		E?
UL16		NE
UL17		E
UL18	VP23	E
UL19	VP5	E
UL20		E/NE
UL21		NE
UL22	gH	E
UL23	tk	NE
UL24		NE

UL25		E
UL26		E
UL26.5		E
UL27	gB	E
UL27.5		?
UL28		E
UL28.5		?
UL29	ICP8	E
UL30		E
UL31		?
UL32		E
UL33		E
UL34		?
UL35	VP26	E
UL36	Vmw273, VP1-2	E
UL37		?
UL38	VP19C	E
UL39	R1, Vmw136, ICP6	E/NE
UL40	R2, Vmw38	E/NE
UL41	vhs	NE
UL42		E
UL43		NE
UL43.5		NE
UL44	gC	NE
UL45		NE
UL46	VP11 and VP12	NE
UL47	VP13 and VP14	NE
UL48	Vmw65, VP16, αTIF	E
UL49	VP22	?
UL49A	VP22	NE
UL50		NE
UL51		E/NE
UL52		E
UL53	gK	E?
UL54 (IE-2)	Vmw63, ICP27	E
UL55		NE
UL56		NE
RS1 (IE-3)	Vmw175, ICP4	E
US1 (IE-4)	Vmw68, ICP22	E/NE

US1.5		NE
US2		NE
US3		NE
US4	gG	NE
US5	gJ	NE
US6	gD	E
US7	gI	NE
US8	gE	NE
US8.5		NE
US9		NE
US10		NE
US11		NE
US12 (IE-5)	Vmw12, ICP47	NE

Appendix B. Hyperthermia Induced Reactivation of Herpes Simplex Virus in a Mouse Model

This document was written for approval by the British Home Office of the "hyperthermia induced reactivation of HSV-1 in a mouse model". The contents are outlined below.

I. Introduction.
   A. The virulence of herpes simplex virus.
   B. Current *in vivo* methods to study latency and reactivation.
      i. Different species.
      ii. Mice.
II. Protocol.
   A. Description of existing procedure.
   B. Description of modified procedure for Glasgow.
III. Bibliography.

I. Introduction

Herpes simplex virus (HSV) is currently being studied in this laboratory to gain a better understanding of its molecular mechanisms in the establishment of latency and the reactivation from a latent infection. Reactivation is thought to be due to stresses such as heat and UV light. Several animal models have been developed to look at this. One method developed by Sawtell and Thompson (1992) closely mimics the host's natural response to reactivation of HSV.

In order to study the difference between genuine reactivation and a reduction in replication efficiencies we would like to use this method in addition to other *in vivo* and *in vitro* replication methods currently in studies using 17^+, 1716 and recombinant viruses singly expressing RL1 or ORF P.

A. The Virulence of Herpes Simplex Virus

The herpes simplex virus type 1 (HSV-1) genome consists of 2 unique sequences (U_L) and an unique short sequence (U_S) which are flanked by re-iterated repeat sequences. The repeat regions contain 3 categories of transcripts; 1) transcripts which are known to be translated (IE0, IE4 and RL1), 2) latency associated transcripts (LATs), and 3) transcripts that span the long/short junction of the repeat sequence (L/STs). It has been shown that the 2 immediate early genes (IE) which encode polypeptides, ICP0 and ICP4, are essential for the lytic replication of the virus and are thought to be significant in the establishment of latency.

The other translated gene located in the repeats, RL1, encodes the infected cell protein (ICP) 34.5. Deletion of RL1 results in mutant viruses that are incapable of replicating in the neurones in the central nervous system and do not cause encephalitis or death in mice. Both the HSV-1 mutant 1716 (759 bp deletion in RL1) and the mutant 1771 (stop codon insertion immediately downstream of the initiating methionine) grow as well as their parental virus in tissue culture, but do not replicate *in vivo* when injected into the cerebral hemisphere of mice (MacLean *et al.*, 1991; McKie *et al.*, 1993). Furthermore, ICP34.5 mutants have severe replication defects *in vivo* when injected in the periphery. We hypothesise that this replication defect may play a role in the viruses ability to cause a latent infection and reactivate.

B. Current *in vivo* Methods to Study Latency and Reactivation

i. Different Species

Some laboratories have experimented with animal model systems to study latency and reactivation of herpes simplex virus. Many initial *in vivo* studies were carried out in rabbits. There are several reasons why using rabbits is not a good practical method among them is that a significant level of spontaneous reactivation occurs in these animals and the expense and space incurred in the upkeep of rabbits. Publications which discuss this in more detail are as follows: Gerdes *et al.*, 1983; Hill *et al.*,1987; Gordon *et al.*, 1990; and Beyer *et al.*, 1990.

ii. Mice

Many laboratories have developed different methods for looking at reactivation of HSV using mice. Here they have shown a relatively low frequency of spontaneous reactivation and they are much easier to house and to handle economically and practically.

The method currently employed in Glasgow is called "co-cultivation" or "*in vitro* reactivation". In this method, mice are injected with HSV in the footpad and the virus travels by axonal transport to the dorsal root ganglia (DRG). Following incubation, mice are sacrificed, ganglia explanted and reactivating virus looked at in vitro.

In more detail, mice are injected in the right rear footpad and placed in cages for at least 42 d pi to allow the establishment of a true latent infection. Forty-two d pi, mice are sacrificed, dissected, and dorsal root ganglia (DRG) dissected using a microscope. Whole ganglia are individually placed in 96-well tissue culture plates containing media. The media is subsequently plated onto fresh baby hamster kidney (BHK21/C13) cell monolayers every two days to look for reactivating virus by cytopathic effect. Replacement of media on the DRG occurs every 2 d and is perpetuated for a subsequent 21 d (Robertson *et al.*, 1992). In this method, wild type virus, 17^+, shows reactivating ganglia starting at day 4 post explantation and continues to show c.p.e. up to day 21 post explantation. However, the ICP34.5 mutant virus, 1716, yields reactivating virus at lower level and is delayed from that of 17^+ by 2 weeks post explantation.

II. Protocol

A. Description of Procedure

This procedure was adapted by Sawtell and Thompson (Germain *et al.*, 1985 and German *et al.*, 1984). Thirty days after infecting outbred Swiss Webster mice via snout scarification and inoculation of HSV, mice are handled individually by the scruff and placed in a 50 ml Falcon tube with holes throughout (Fig. 1). In the original work, a temperature probe was placed 3 cm into the rectum. This step was done initially to ensure body core temperature but due to the critical timing in the bath this is not done routinely. A group of mice in their respective containers are carefully placed in a metal basket and slowly lowered into an equilibrated 43°C water bath (Fig. 2). The animals are carefully monitored in the water bath for exactly 10 min to watch for signs of stress caused by heat or high levels of water. If this occurs the animals are immediately removed from the water bath and from the experiment. Mice are generally complacent in this environment with some movement within the tube. After the water bath the mice are removed from the tubes and blotted dry with paper towels. They are put back in their appropriate cages placed in a warm incubator (34°C) for 30 min. to prevent hypothermia. Twenty-four h later mice are killed and the ganglia (trigeminal or dorsal root ganglia, depending on the site of infection) are removed asceptically and homogenised in 0.5 ml media. The homogenates are plated onto fresh rabbit skin cell monolayers and monitored for c.p.e.

Different times (14, 24 and 48 h) post hyperthermic stress have been studied by Sawtell *et al (*1992; personal communication) to see if there is a variation in the amount of reactivating virus. It was shown that 24 h is optimum for the highest levels of 17+ virus (60%). The length of time between hyperthermic treatment and reactivation is within 14 h post induction. This is expected because it is the time which is required for one round of viral replication in tissue culture in vitro. The results of time course studies also showed that the number of virus positive ganglia 48 h posttreatment was only half that at 24 h posttreatment. Using a light microscope to view individual neurones Sawtell and Thompson found that an immune response modulated destruction of the reactivating virus and/or neurones which is why there was a decrease in virus positive ganglia at 48 h posttreatment.

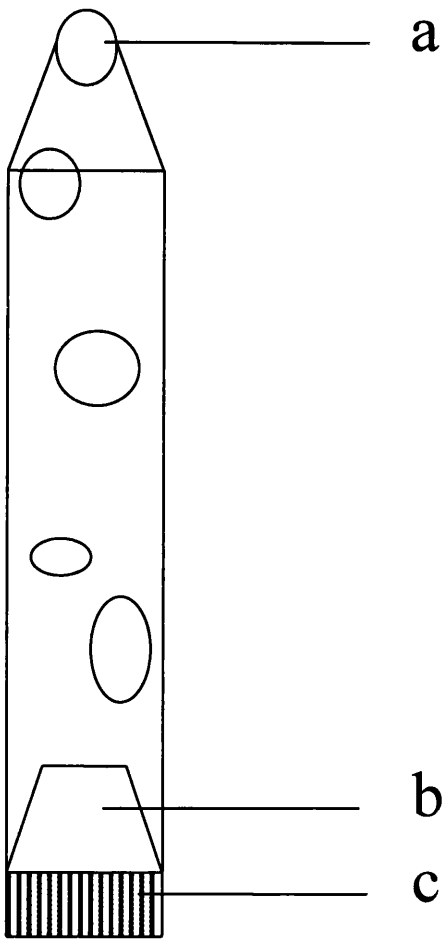


Figure 1. Scetch of a 50 ml Falcon tube used in the "HSV *in vivo* reactivation" method to contain individual mice when placed in the water bath. **a**. Holes cut out for water and air to enter the tube, **b**. Sponge to adjust the heigth of the mouse in the tube, and **c**. Lid with a slit cut out for the tail screwed on the the tube.

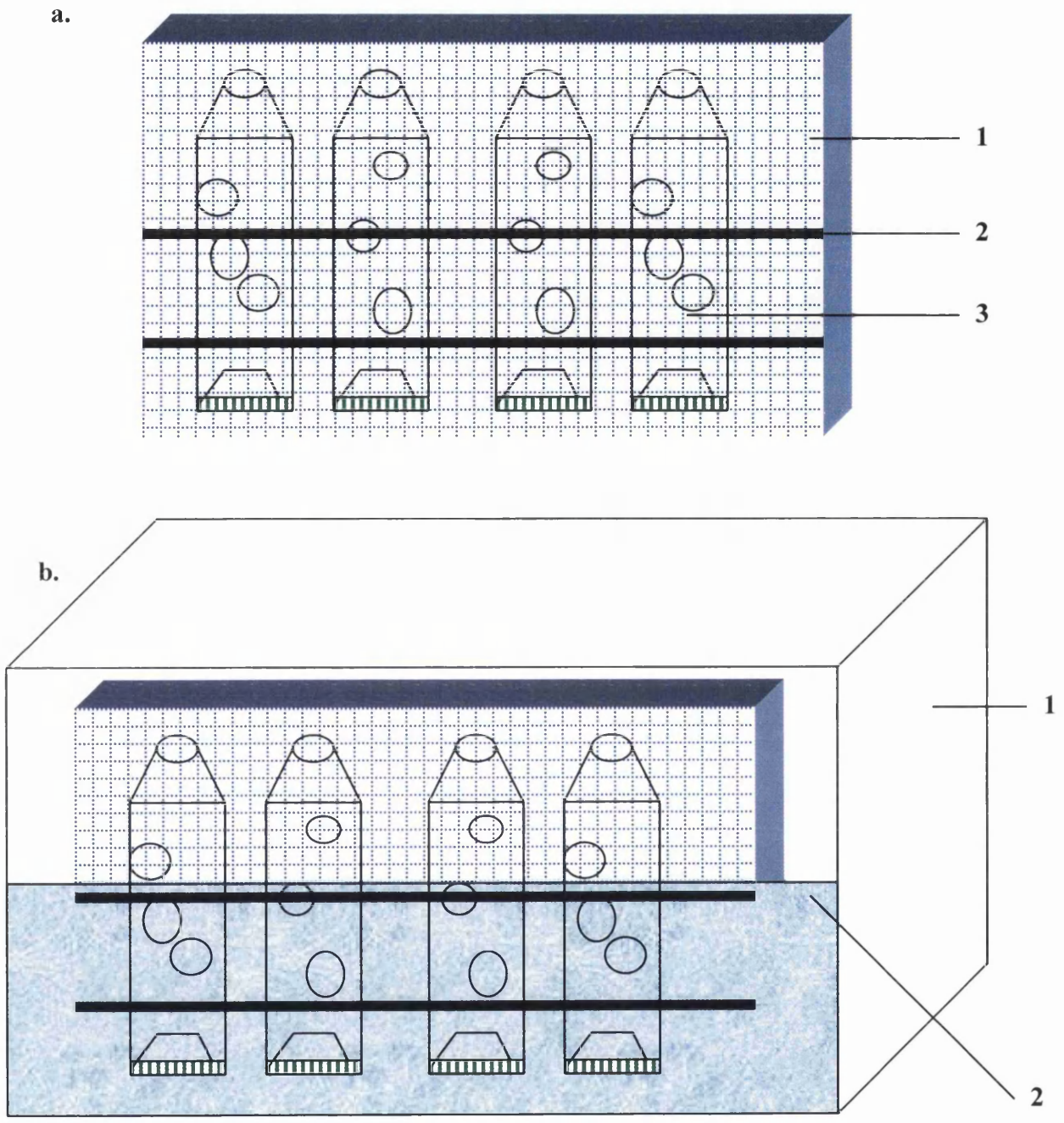


Figure 2. a. Metal basket representing components involved (1) metal basket, (2) rubber hose to contain the tubes with mice and (3) tubes containing mice. **b**. (1) water bath and (2) schematic representation of the level the water should be when metal basket is placed therein.

B. Description of Modified Procedure for Glasgow

Most of the procedure described above we are proposing to practice at the University of Glasgow's Central Research Facility (CRF) with a few minor changes which includes the following: three-week old BALB/c mice. This is the breed of animals we routinely use for all current work carried on HSV and allows a standardised conformity among results.

Due to the low frequency of reactivation each virus dose will include no fewer than 10 mice. The number of mice is particularly significant when studying mutant viruses as they tend to have lower amounts of reactivating virus. In the original publication by Sawtell and Thompson (1992), they reported, 1 to 3 HSV antigen positive neurones per ganglia, 24 h after heat stress.

As previously described in our project licence (No. 60/2215, "Herpes simplex latency and virulence"), HSV and mutant viruses derived from HSV-1, will be injected in the right rear footpad. This method is less traumatic for the animal. The mice will be housed for 30 d to allow a true latent establishment of HSV. The heat stress and thermal warming takes approximately 1 h per group and the animals are housed for a subsequent 24 h and killed.

The DRG are removed asceptically using a dissecting microscope and homogenised in 0.5 ml media. The homogenates are plated onto fresh BHK21/C13 cell monolayers and monitored for c.p.e.

Ace, C.I., T. McKee, J.M. Ryan, J.M. Cameron, and C.M. Preston. 1989. Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate early gene expression. *Journal of Virology* **63**:2260-2269.

Ackermann, M., J. Chou, M. Sarmiento, R.A. Lerner, and B. Roizman. 1986. Identification by antibody to a synthetic peptide of a protein specified by a diploid gene located in the terminal repeats of the L component of herpes simplex virus genome. *Journal of Virology* **58**:843-850.

Alvarez, R.D. and D.T. Curiel. 1997. A Phase I study of recombinant adenovirus vector mediated intraperitoneal delivery of herpes simplex virus thymidine kinase gene and intravenous gangcyclovir for previously treated ovarian cancer patients. *Human Gene Therapy* **8**:597-613.

Andreansky, S.S., B. He, G.Y. Gillespie, L. Soroceanu, J. Markert, J. Chou, B. Roizman, and R.J. Whitley. 1996. The application of genetically engineered herpes simplex viruses to the treatment of experimental brain tumors. *Proceedings of the National Academy of Sciences* **93**:11313-11318.

Arther, J.L., R. Everett, I. Brierley, and S. Efstathiou. 1998. Disruption of the 5' and 3' splice sites flanking the major latency-associated transcripts of herpes simplex virus type 1: evidence for alternate splicing in lytic and latent infections. *Journal of General Virology* **79:**107-116.

Aurelian, L. 1989. The pathogenesis of herpes simplex virus infection: latency. In *"Molecular Aspects Of Human Disease".* J.W. Gorrod, O.Albano, S.Papa et al. (eds.).Wilcons & Co New York, U.S.A.

Bancroft, A. 1982. In "Theory and Practice of Histological Techniques." Churchill Livingstone, Edinburgh.

Bauke, R.B., and P.G. Spear. 1979. Membrane proteins specified by herpes simplex virus V. identification of an Fc-binding domain. *Journal of Virology* **32**:779-787.

Behbakht, K., I. Benjamin, H. Chiu, S. Eik, O. Van Deerlin, S. Rubin, and J. Boyd. 1996. Adenovirus mediated gene therapy of ovarian cancer in a mouse model. *American Journal of Obtetrics and Gynecology* **175**:1260-1265.

Bell, S., Cranage, M., Borysiewicz, L., and Minson, T. 1990. Induction of immunoglobin-G Fc-receptors by recombinant vaccinia viruses expressing glycoprotein-E and glycoprotein-I of herpes simplex virus type 1. *Journal of Virology* **64**:2181-2186.

Bdour, S. 1995. Characterization of the functional domains of the herpes simplex virus (HSV-2) strain HG52 RL1 gene. PhD thesis, University of Glasgow.

Black, T.L., G.N. Barber, and M.G. Katze. 1998. Degradation of the interferon induced 68,000 M_r protein kinase by poliovirus requires RNA. *Journal of Virology* 67:791-800.

Bloom, D.C. 1998. HSV vectors for gene therapy. In "*Herpes simplex virus protocols*" Ed. S.M. Brown and A.R. MacLean. pp.369-386. Humana Press. Totonta, N.J.

Bloom, D.C., J.M. Hill, G. Devi-Rao, E.K. Wagner, L.T. Feldman, and J.G Stevens. 1996. A 348 base pair region in the latency associated transcript facilitates herpes simplex virus type 1 reactivation. *Journal of Virology* **70**:2449-2459.

Bloom, D.C., G.B. Devi-Rao, J.M. Hill, J.G. Stevens, and E.K. Wagner. 1994. Molecular analysis of herpes simplex virus type 1 during epinephrine - induced reactivation of latently infected rabbits in vivo. *Journal of Virology* **68**:1283-1292.

Bohenzky, R.A., A.G. Papavassiliou, I.H. Gelman, and S. Silverstein. 1993. Identification of a promoter mapping within the reiterated sequences that flank the herpes simplex virus type 1 U_L region. *Journal of Virology* **67**:632-642.

Bolon, P., N. Davispoynter, S. Bell, H. Atkinson, H. Brown, and T. Minson. 1994. An analysis of the in vitro and in vivo phenotypes of herpes simplex virus type 1 lacking glycoprotein G , gE, or gI or the putative gJ. *Journal of General Virology* **75**:1245-1258.

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

Bond, V.C. and S. Person. 1984. Fine structure physical map locations of alterations that affect cell fusion in herpes simplex virus type 1. *Virology* **132**:368-376.

Bonneau, R.H., and S.R. Jennings. 1989. Modulation of acute and latent herpes simplex virus type 1 infection in C57B1/6 mice by adoptive transfer of immune lymphocytes with cytolytic activity. *Journal of Virology* 63:1480-1484.

Bouler, D.M., S. Kenangat, W. Wire and B.T. Rouse. 1995. Characterization of herpes simplex virus type 1 infection and herpetic stomal keratitis development in IFN-γ knockout mice. *Journal of Immunology* **155**:3964-3971.

Bower, J.R., H. Mao, C. Durishin, E. Rozenbom, M. Detwiler, D. Rempinski, T.L. Karban, and K.S. Rosenthal. 1999. Intrastrain variants of herpes simplex virus type 1 isolated from neonate with fatal disseminated infection differ in the ICP34.5 gene, glycoprotein processing and neuroinvasiveness. *Journal of Virology* **73**:3843-3853.

Bradley, J.D., Y. Kataoka, S. Advani, S.M.Chung, R.B. Arani, G.Y. Gillespie, R.J. Whitley, J.M. Markert, B. Roizman, and R.R. Weichselbaum. 1999. Ionizing radiation improves survival in mice bearing intracranial high grade gliomas injected with genetically modified herpes simplex virus. *Clinical Cancer Research* **5**:1517-1522.

Brandt, C.R., P.D. Imesch, N.L. Robinson, N.A. Syed, S. Untawale, S.R. Darjatmoko, R.J. Chappell, P. Heinzelman, and D.M. Albert. 1997. Treatment of

spontaneously arising retinoblastoma tumors in transgenic mice with an attenuated herpes simplex virus mutant. *Virology* **229**:283-291.

Bravo, R., R. Frank, P.A. Blundell, and H. Macdonald-Bravo. 1987. Cyclin/PCNA is the auxiliary protein of DNA polymerase gamma. *Nature* **326**:515-517.

Brown, S.M., A.R. MacLean, E.A. McKie, and J. Harland. 1997. The herpes simplex virus virulence factor ICP34.5 and the cellular protein MyD116 complex with proliferating cell nuclear antigen through the 63-amino-acid domain conserved in ICP34.5, MyD116, and GADD34. *Journal of Virology* **71**:9442-9449.

Brown, S.M., J. Harland, A.R. MacLean, J. Podlech, and J.B. Clements. 1994a. Cell type and cell state determine differential in vitro growth of non-neurovirulent ICP34.5 negative herpes-simplex virus type-1 and type-2. *Journal of General Virology* **75**:2367-2377.

Brown, S.M., A.R. MacLean, J.D. Aitken, and J. Harland. 1994b. ICP34.5 influences herpes simplex virus type 1 maturation and egress from infected cells in vitro. *Journal of General Virology* **75**:3679-3686.

Brown, S.M., D.A. Ritchie, and J.H. Subak-Sharpe. 1975. Genetic studies with herpes simplex virus type 1: Isolation of temperature sensitive mutants, their arrangements into complementation groups and recombination analysis leading to a linkage map. *Journal of General Virology* **18**:329-346.

Brown, S.M. and J. Harland. 1987. Three mutants of herpes simplex virus type 2: one lacking the genes US10, US11 and US12 and two in which R_S has been extended by 6kb to 0.91 map units with loss of U_S sequence between 0.94 and the U_S/TR_S junction. *Journal of General Virology* **68**:1-18.

Brown S.M. and R.A. Ritchie. 1975. Genetic studies with herpes simplex virus type 1. Analysis of mixed plaque forming virus and its bearing on genetic recombination. *Virology* **64**:32-42.

Brunetti, C.R., K.S. Dingwell, C. Wade, F.L. Graham, and D.C. Johnson. 1998. Herpes simplex virus gD and virions accumulate in endosomes by mannose-6-phosphate dependent and independent mechanisms. *Journal of Virology* **72**:3330-3339.

Brunetti, C.R., RL. Burke, B. Hoflack, T. Ludwig, K.S. Dingwell, and D.C. Johnson. 1995. Role of mannose 6-phosphate receptors in herpes simplex virus entry into cells and cell to cell transmission. *Journal of Virology* **69**:3517-3528.

Bruni, R. and B. Roizman. 1996. Open reading frame P - a herpes simplex virus gene repressed during productive infection encodes a protein that binds a splicing factor and reduces synthesis of viral proteins made from spliced mRNA. *Proceedings of the National Academy of Sciences* **93**:10423-10427.

Buckmaster, E.A., U. Gompels, and A. Minson. 1984. Characterisation and physical mapping of an HSV-1 glycoprotein of approximately 115x10(3) molecular weight. *Virology* **139**:408-413.

Busby, D.W.G., W. House, and J.R. MacDonald. 1964. In "*Virological Techniques*". Churchill, London.

Bzik, D.J., B.A. Fox, N.A. Deluca, and S. Person. 1984. Nucleotide sequence of a region of the herpes simplex type 1 gB gene mutations affecting rate of virus entry and cell fusion. *Virology* **137**:185-190.

Cai, W. and P.A. Schaffer. 1989. Herpes simplex virus type 1 ICP0 plays a critical role in the de novo synthesis of infectious virus following transfection of viral DNA. *Journal of Virology* **63**:4579-4589.

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

Cal, W.H., B.H. Gu, and S. Person. 1988. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. *Journal of Virology* **62**:2596-2604.

Cann, A.J. 1993. In *"Principles of Molecular Virology"*. Academic Press, London.

Cantin, E.M., D.R. Hinton, J. Chen, and H. Openshaw. 1995. Gamma interferon expression during acute and latent infection by herpes simplex virus type 1. *Journal of Virology* **69**:4898-4905.

Carr, D.J.J., S. Noisakran, W.P. Halford, N. Lukacs, V. Asensio, and I.L. Campbell. 1998. Cytokine and chemokine production in HSV-1 latently infected trigeminal ganglion cell cultures: Effects of hyperthermic stress. Journal of Immunology 85:111-121.

Carroll, K., O. Elroy-Stein, B. Moss, and R. Jagus. 1993. Recombinant vaccinia virus K3L gene product prevents activation of double stranded RNA dependent initiation factor 2 specific protein kinase. *Journal of Biochemistry* **268**:12837-12842.

Carter, K.L., P.L. Ward, and B. Roizman. 1996. Characterization of the products of the UL43 gene of herpes simplex virus 1: potential implications for regulation of gene expression by antisense transcription. *Journal of Virology* **70**:7663-7668.

Cassady, K., M. Gross, and B. Roizman. 1998a. The herpes simplex virus US11 protein effectively compensates for the $\gamma_1$34.5 gene if present before activation of protein kinase R by precluding its phosphorylation and that of the α subunit of eukaryotic translation initiation factor 2. *Journal of Virology* **72**:8620-8626.

Cassady, K.A., M. Gross, and B. Roizman. 1998b. The second site mutation in the herpes simplex virus recombinants lacking the $\gamma_1$34.5 genes precludes shut off of

protein synthesis by blocking the phosphorylation of eIF-2α. *Journal of Virology* **72**:7005-7011.

Chen, I.,-T., M.L. Smith, P.M. O'Connor, and A. Fornace. 1995. Direct interaction of GADD45 with PCNA and evidence of competitive interaction of GADD45 and p21 $^{Waf1/Cip1}$ with PCNA. *Oncogene* **11**:1931-1937.

Chen, S., M.F. Kramer, P.A. Schaffer, and D.M. Coen. 1997. A viral function represses accumulation of transcripts from productive-cycle genes in mouse ganglia latently infected with herpes simplex virus. *Journal of Virology* **71**:5878-5884.

Chou, J. and B. Roizman. 1994. Herpes simplex virus 1 γ_1 34.5 gene function, which blocks the host response to infection, maps in the homologous domain of the genes expressed during growth arrest and DNA damage. *Proceedings of the National Academy of Sciences* **91**:5247-5251.

Chou, J. and B. Roizman. 1992. The $\gamma_1$34.5 gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells. *Proceedings of the National Academy of Sceiences* **89**:3266-3270.

Chou, J. and B. Roizman. 1990. The herpes simplex virus 1 gene for ICP34.5, which maps in inverted repeats, is conserved in several limited-passage isolates but not in strain 17syn+. *Journal of Virology* **64**:1014-1020.

Chou, J. and B. Roizman. 1986. The terminal *a* sequence of the herpes simplex virus genome contains the promoter of a gene located in the repeat sequences of the L component. *Journal of Virology* **57**:629-637.

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

Chou, J., A.P.W. Poon, J. Johnson, and B. Roizman. 1994. Differential response of human cells to deletions and stop codons in the $\gamma_1$34.5 gene of herpes simplex virus. *Journal of Virology* **68**:8304-8311.

Chou, J., E.R. Kern, R.J. Whitley, and B. Roizman. 1990. Mapping of herpes simplex virus-1 neurovirulence to $\gamma_1$34.5, a gene nonessential for growth in culture. *Science* **250**:1262-1266.

Claesson-Welsh, L., and P.G. Spear. 1986. Oligomerization of herpes simplex virus glycoprotein B. *Journal of Virology* **60**:803-806.

Clemens, M.J. and A. Elia. 1997. The double stranded RNA dependent protein kinase PKR: structure and function. *Journal of Interferon Cytokine Research.* **17**:503-524.

Clements, G.B. and J.H. Subak-Sharpe. 1988. Herpes simplex virus type 2 establishes latency in the mouse footpad. *Journal of General Virology* **69**:376-383.

Clements, G. and J.H. Subak-Sharpe. 1983. Recovery of herpes simplex virus 1 temperature sensitive mutants from the dorsal root ganglia of mice. In "*Immunology of Nervous System Infection*" P. Behan *et al.*, Elsevier.

Clements, J.B. and N.D. Stow. 1989. A herpes simplex virus type 1 mutant containing a deletion within immediate early gene 1 is latency competent in mice. *Journal of General Virology* **70**:2501-2506.

Coen, D.M., M. Kosz-Vnenchak, J.G. Jacobsen, D.A. Leib, C.L. Bogard, P.A. Schaffer, K.L. Tyler, and D.M. Knipe. 1989. Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia but do not reactivate. *Proceedings of the National Academy of Sciences* **86**:4736-4740.

Coffin, R.S., M.K. Howard, D.V.E. Cumming, C.M. Dollery, J. McEwan, D.M. Yellon, M.S Marber, A.R. MacLean, S.M. Brown, and D.S. Latchman. 1996a. Gene delivery to the heart in vivo and to cardiac myocytes and vascular smooth muscle cells in vitro using herpes virus vectors. *Gene Therapy* **3**:560-566.

Coffin, R.S., A.R. MacLean, D.S. Latchman, and S.M. Brown. 1996b. Gene delivery to the central and peripheral nervous systems of mice using HSV1 ICP34.5 deletion mutant vectors. *Gene Therapy* **3**:88-891.

Cool, V., B. Pirotte, C. Gerard, J.L. Dargent, N. Baudson, M. Levivier, S. Goldman, J. Hildebrand, J. Brotchi, and T. Velu. 1996. Curative potential of herpes simplex virus thymidine kinase gene transfer in rats with 9L gliosarcoma. *Human Gene Therapy* **7**:626-635.

Costa, J.V. 1990. African Swine Fever Virus. P.247-270. In "*Molecular biology of iridoviruses*" Ed. G. Darai, Kluwer Academic Publishers, Norwell, Mass.

Coukos, G., A. Makrigoannakis, E.H. Kang, D. Caparelli, I. Benjamin, L.R. Kaiser, S.C. Rubin, S.M. Albelda, and K.L. Molnar-Kimber. 1999. Use of carrier cells to deliver a replication-selective herpes simplex virus 1 mutant for the intraperitoneal therapy of epithelial ovarian cancer. *Clinical Cancer Research* **5**:1523-1537.

Cuddihy, A.R., S. Li, N.W.N. Tam, A.H.-T. Wong, Y. Taya, N. Abraham, J.C. Bell, and A.E. Koromilas. In press. The double stranded RNA protein kinase PKR physically associated with the tumor suppressor p53 and phosphorylates human p53 with serine^{392} in vitro *Oncogene*

Cuddihy, A.R., S. Li, N.W.N. Tam, A.H.-T. Wong, Y. Taya, N. Abraham, J.C. Bell, and A.E. Koromilas. 1999. Double-stranded-RNA-activated protein kinase PKR enhances transcriptional activation by tumor suppressor p53. *Molecular and Cellular Biology* **19**:2475-2484.

Culver, K.W., Z. Ram, S. Wallbridge, H. Ishi, E.H. Oldfield, and R.M. Blaese. 1992. In vivo gene transfer with retroviral vector producer cells for treatment of experimental brain tumors. *Science* **256**:1550-1552.

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

Danaher, R.J., R.J. Jacob, M.D. Chorak, C.S. Freeman, and C.S. Miller. 1999. Heat stress activates production of herpes simplex virus type 1 from quiesently infected neurally differentiated PC12 cells. *Journal of Neurovirology* **5:**374-383.

Dargan, D. 1986. The structure and assemble of herpes viruses. In "*Viral Structure*" Eds. J.R. Harris and R.W. Horne, New York p.359-437.

Davido, J.D. and D.A. Leib. 1996. Role of cis-acting sequences of the ICP0 promoter of herpes simplex virus type 1 in viral pathogenesis, latency and reactivation. Journal of General Virology **77**:1853-1863.

Davies, M.V., H.W. Chang, B.L. Jacobs, and R.J. Kaufman. 1993. The E3L and K3L vaccinia virus gene products stimulate translation through inhibition of the double stranded RNA dependent protein kinase by different mechanisms. *Journal of Virology* **67**:1688-1692.

Davison, A. 1981. Detailed structural aspects of the herpes simplex virus genome. In "PhD thesis", University of Glasgow

Davison, M.D., F.J. Rixon, and A.J. Davison. 1992. Identification of genes encoding two capsid proteins (VP24 and VP26) of herpes simplex virus type 1. *Journal of General Virology* 73:2709-2713.

Debroy, C., N. Pederson, and S. Person. 1985. Nucleotide sequence of a herpes simplex virus type 1 gene that causes cell fusion. *Virology* **145**:36-48.

Deiss, L., J. Chou, and N. Frenkel. 1986. Functional domains within the 'a' sequence involved in the cleavage and packaging of herpes simplex virus DNA. *Journal of Virology* **59**:605-618.

Deluca, N.D., D.J. Bzik, V.C. Bond, S. Person, and W. Snipes. 1982. Nucleotide sequences of herpes simplex virus type 1 affecting virus entry, cell fusion, and production of glycoprotein B (VP7). *Virology* **122**:411-423.

Desai, P., N.A. DeLucca, and S. Person. 1998. Herpes simplex virus type 1 VP26 is not essential for replication in cell culture but influences production of infectious virus in the nervous system of infected mice. *Virology* **247**:115-124.

Desai, P.J., P.A. Schaffer, and A.C. Minson. 1982. Excretion of non-infectious virus particles lacking gH by a temperature sensitive mutant of herpes simplex virus type 1: evidence that gH is essential for virion infectivity. *Journal of General Virology* **69**: .

Deshane, J. G.P. Sicgal, .D. Alvaez, M.H. Wang, M. Feng, G. Cabrera, T. Liu, M. Kay, and D. Curiel. 1995. Targetted tumor killing via an intracellular antibody against erbB-2. *Journal of Clinical Investigation* **96**:2980-2989.

Dick, J.W. and K.S. Rosenthal. 1995. A block of glycoprotein processing correlates with small plaque morphology and virion targetting to cell-cell junctions for an oral and an anal strain of herpes simplex virus type 1. *Archives of Virology* **140**:2163-2181.

Dingwell, K.S., L.C. Doering, and D.C. Johnson. 1995. Glycoprotein E and glycoprotein I facilitate neuron to neuron spread of herpes simplex virus. *Journal of Virology* **69**:7087-7098.

Dolan, A., F.E. Jamieson, C. Cunningham, B.C. Barnett, and D.J. McGeoch. 1998. The genome sequence of herpes simplex virus type 2. *Journal of Virology* **72**:2010-2021.

Dolan, A., E. McKie, A.R. MacLean, and D.J. McGeoch. 1992. Status of the ICP34.5 gene in herpes simplex virus type 1 strain 17. *Journal of General Virology* **73**:971-973.

Donzé, O., J. Dostie, and N. Sonenberg. 1999. Regulatable expression of the interferon-induced double-stranded RNA dependent protein kinase PKR induces apoptosis and Fas receptor expression. *Virology* **256**:322-329.

Drolet, B.S., G.C. Pering, J. Cohen, S.M. Slanna, A. Yukht, A.B. Lesburn, and S.L. Wechster. 1998. The region of the herpes simplex virus type 1 LAT gene involved in spontaneous reactivation does not encode a functional protein. *Virology* **242**:221-232.

Dyer, A.P., B.NW. Banfield, D. Martindale, D.M. Spannier, and F. Tufaro. 1997. Dextran sulfate can act as an artificial receptor to mediate a type specific herpes simplex virus infection via glycoprotein B. *Journal of Virology* **71**:191-198.

Efstathiou, S. H.J. Field, P.D. Griffiths, E.R. Kern, S.L. Sacks, N.M. Sawtell, and L.R. Stanberry. 1999. Herpes simplex virus latency and nucleoside analogues. *Antiviral Research* **41**:85-100.

Ejercito, P.M., E.K. Kieff, and B. Roizman. 1968. Characterisation of herpes simplex virus strain differing in their effect on social behavior of infected cells. *Journal of General Virology* **3**:357-364.

Elliot, G. and P. O'Hare. 1997. Intercellular trafficking and protein delivery by a herpesvirus structural protein. *Cell* **88**:223-233.

Elshami, A.A., A. Saavedra, H. Zhang, J.C. Kucharczuk, D.C. Spray, G.I. Fishman, K.M. Amin, L.R. Kaiser, and S.M. Albelda. 1996. Gap junctions play a role in the bystander effect of the herpes simplex virus thymidine kinase gancyclovir system in-vitro. *Gene Therapy* **3**:85-92.

Engel, J.P., T.C. Madigan, and G.M. Peterson. 1997. The transneuronal phenotype of herpes simplex virus type 1 infection of the mouse hind footpad. *Journal of Virology* **71**:2425:2435.

Esparza, J., D.J.M. Purifoy, P.A. Schaffer, and M. Benyesh-Melnick. 1974. Isolation, complementation and preliminary phenotypic characterization of temperature sensitive mutants of herpes simplex virus type 2. *Virology* **57**:554-565.

Everett, R. 1991. Construction and characterization of herpes simplex type 1 viruses without introns in immediate early gene 1. *Journal of General Virology* **72**:651-659.

Everett, R.D. 1989. Construction and characterisation of herpes simplex virus type 1 mutants with defined lesions in immediate early gene 1. *Journal of General Virology* **70**:1185-1202.

Everett, R.D. 1988. Promoter sequence and cell type can dramatically effect the efficiency of transcriptional activation induced by herpes simplex virus type 1 and its IE gene products VMW175 and VMW110. *Journal of Molecular Biology* **203**:739-751.

Everett, R. 1985. Activation of cellular promoters during herpes virus infection of biochemically transformed cells. *EMBO* **4**:1973-1980.

Everett, R.D. 1984. Transactivation of transcription by a herpes simplex virus product: requirement for two HSV-1 immediate early polypeptide for maximal activity. *EMBO Journal* **3**:3135-3141.

Everett, R.D. and G.G. Maul. 1994. HSV-1 IE protein Vmw110 causes redistribution of PML. *EMBO Journal* **13**:5062-5069.

Everett, R., A. Orr, and C. Preston. 1998. A viral activator of gene expression functions via the ubiquitin-proteasome pathway. *EMBO* **17**:7161-7169.

Everett, R., P. Freemont, H. Saitoh, M. Dasso, A. Orr, M. Katoria, and J. Parkinson. 1998. The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110 and proteosome dependent loss of several PML isoforms. *Journal of Virology* **72**:6581-6591.

Farrell, M., J.M. Hill, T.P. Margolis, J.G. Stevens, E.K. Wagner, and L.T. Feldman. 1993. The herpes simplex virus type 1 reactivation function lies outside the latency associated transcript open reading frame. *Journal of Virology* **67**:3653-3655.

Fawl, R.L. and B. Roizman. 1993. Induction of reactivation of herpes simplex virus in murine sensory ganglia in vivo by cadmium. *Journal of Virology* **67**:7025-7031.

Fawl, R.L., R.M. Gesser, T. Valyi-Nagi, and N.W. Fraser. 1996. Reactivation of herpes simplex virus from latently infected mice after administration of cadmium in mouse-strain-dependent. *Journal of General Virology* **77**:2781-2786.

Fink, D.J., N.A. DeLuca, W.F. Goins, and J.C. Glorioso. 1996. Gene transfer to neurons using herpes simplex virus-based vectors. *Annual Review of Neuroscience* **19**:263-287.

Fink, D.J. and J.C. Glorioso. 1997. Engineering herpes simplex virus vectors for gene therapy to neurons. *Nature Medicine* **3**:357-359.

Fornace Jr, A.J., D.W. Nebert, M.C. Hollander, J.D. Luethy, M. Papathanasiou, J. Fargnoli, and N.J. Holbrook. 1989. Mammalian genes coordinately regulated by growth arrest signals and DNA damaging agents. *Molecular and Cellular Biology* **9**:4196-4203.

Forrester, A., H. Farrell, G. Wilkinson, J. Kaye, N. Davispoynter, and T. Minson. 1992. Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. *Journal of Virology* **66**:341-348.

Fraefel, C., S. Song, F. Lim, P. Lang, L. Yu, Y. Wang, P. Wild, and A.I. Geller. 1996. Helper virus-free transfer of herpes simplex virus type 1 plasmid vectors into neural cells. *Journal of Virology* **70**:7190-7197.

Frame, M.C., H.S. Marsden, and D.J. McGeoch. 1986. Novel herpes simplex virus type 1 glycoproteins identified by antiserum against a synthetic oligopeptide from the predicted product of gene US4. *Journal of General Virology* **67**:745-751.

Fraser, N.W., T.M. Block, and J.G. Spivack. 1992. The latency associated transcripts of herpes simplex virus: RNA in search of function. *Virology* **191**:1-8.

French, S.W., M.C. Schmidt, and J.C. Glorioso. 1996. Involvement of a high mobility group protein in the transcriptional activity of herpes simplex virus latency-active promoter 2. *Molecular and Cellular Biology* **16**:5393-5399.

Friedmann, T. 1997. Overcoming the obstacle of gene therapy. *Scientific American Special report*:80-85.

Gallacher, W.R., D.B. Levitam, and H.H. Blough. 1973. Effect of 2-deoxy-D-glucose on cell fusion induced by Newcastle Disease and herpes simplex virus. *Virology* **55**:193-201.

Garber, D.A., P. A. Schaffer, and D. M. Knipe. 1997. A LAT-associated function reduces productive - cycle gene expression during acute infection of murine sensory neurons with herpes simplex virus type 1. *Journal of Virology* **71**:5885-5893.

Geller, A.I. and X.O. Breakefield. 1988. A defective HSV-1 vector expresses β-galactosidase in cultured peripheral neurons. *Science* **241**:1667-1669.

Gelman, I.H. and S. Silverstein. 1986. Co-ordinate regulation of herpes simplex virus gene expression is mediated by the functional interaction of two immediate early gene products. *Journal of Molecular Biology* **191**:395-409.

Gesser, R.M. and S.C. Koo. 1996. Oral inoculation with herpes simplex virus type 1 infects enteric neurons and mucosal nerve fibres within the gastrointestinal tract in mice. *Journal of Virology* **70**:4097-4102.

Gibson, W. and B. Roizman. 1972. Proteins specified by herpes simplex virus. VIII. Characterisation and composition of multiple capsid forms of subtypes 1 and 2. *Journal of Virology* **10**:1044-1052.

Glorioso, J.C., W.F. Goins, and D.J. Fink. 1992. Herpes simplex virus-based vectors. *Seminars in Virology* **3**:265-276.

Goldenburg, D., N. Mador, M.J. Ball, A. Panet, and I. Steiner. 1997. The abundant latency-associated transcripts of herpes simplex virus type 1 are bound to polyribosomes in cultured neuronal cells during latent infection in mouse trigeminal ganglia. *Journal of Virology* **71**:2897-2904.

Goldstein, D. and S.K. Weller. 1988. Herpes simplex virus type 1 induced ribonucleotide reductase is dispensable for virus growth and DNA synthesis: isolation and characterisation of an ICP6 lacZ insertion mutant. *Journal of Virology* **62**:196-205.

Griffiths, A., S. Renfrey, and T. Minson. 1998. Glycoprotein C-deficient mutants of two strains of herpes simplex virus type 1 exhibit unaltered adsorption characteristic on polarized or non-polarized cells. *Journal of General Virology* **79**:807-812.

Gudnadottir, M. 1964. Identification of a novel herpes simplex virus type 2 strain from a patient with multiple sclerosis. *Experimental Neurology* **9**:85.

Gulbis, J.M., Z. Kelman, J. Hurwitz, M. O'Donnell, J. Kuriyan. 1996. Structure of the C-terminal region of p21 (Waf1/Cip1) complexed with human Pcna. *Cell* **87**:297.

Hager, M. and T. Burgess. 1980. Elution of proteins from sodium dodecyl sulphate polyacrylamide gels and renaturation of enzymatic activity: results with sigma subunit of *E. coli* RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. *Annals of Biochemistry* **109**:76-86.

Halford, W.P., B.M. Gebhardt, and D.J.J. Carr. 1996. Mechanisms of herpes simplex virus type 1 reactivation. *Journal of Virology* **70**:5051-5060.

Hanash, S.M., L. Beretta, C.L. Barcroft, S. Sheldon, T.W. Glover, D. Ungar, and N. Sonenberg. 1993. Mapping of the gene for the interferon inducible double stranded RNA dependent protein kinase to chromosome region 2p21-22: a site of rearrangement in myoloproliferative disorders. *Genes Chromosomes and Cancer* **8**:34-37.

Harland, J.E., S. Bdour, S.M. Brown, and A.R. MacLean. 1996. The herpes simplex virus type 2 strain HG52 RL1 gene contains a 154 bp intron as predicted from sequence analysis. *Journal of General Virology* **77**:481-484.

Harland, J. and S.M. Brown. 1991. Abolition of the RL neurovirulence phenotype of herpes simplex virus type 2 strain HG52 does not require deletion of the DR1 element of the 'a' sequence. *Journal of General Virology* **72**:2777-2779.

Harris, R.A. and C.M. Preston. 1991. Establishment of latency in vitro by the herpes simplex virus type 1 mutant *in*1814. *Journal of General Virology* **72**:907-913.

Hay, K.A., W.A. Edris, A. Gaydos, and R.B. Tenser. 1998. Herpes simplex virus latency after direct ganglion virus inoculation. *Journal of Neurovirology* **4**:531-538.

He, B., J. Chou, D. A. Liebermann, B. Hoffman, and B. Roizman. 1996. The carboxyl terminus of the murine MyD116 gene substitutes for the corresponding domain of the gamma(1)34.5 gene herpes simplex virus to preclude the premature shutoff of total protein synthesis in infected human cells. *Journal of Virology* **70**:84-90.

He, B., J. Chou, R. Brandimarti, I. Mohr, Y. Gluzman, and B. Roizman. 1997. Suppression of the phenotype of $\gamma_1$34.5- herpes simplex virus 1: failure of activated RNA-dependent protein kinase to shut off protein synthesis is associated with a deletion in the domain of the α47 gene. *Journal of Virology* **71**:6049-6054.

He, B., M. Gross, and B. Roizman. 1997. The $\gamma_1$34.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1a to dephosphoylate the a subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase. *Proceedings of the National Academy of Sciences* **94**:843-848

He, B., M. Gross, and B. Roizman. 1998. The $\gamma_1$34.5 protein of herpes simplex virus 1 has the structural and functional attributes of a protein phosphatase 1 regulatory subunit and is present in a high molecular weight complex with the enzyme in infected cells. *Journal of Biological Chemistry* **273**:20737-20743.

Hendricks, R.L., P.C. Weber, J.L. Taylor, A. Doumbis, T.M. Tumpey, and J.C. Glorioso. 1991. Endogenously produced interferon α protects mice from herpes simplex virus type 1 corneal disease. *Journal of General Virology* **72**:1602-1620.

Hill, J.M., J.B. Dudley, Y. Shimomura, and H.E. Kaufman. 1986. Quantitation and kinetics of adrenergic induced herpes simplex virus type 1 ocular shedding. *Current Eye Research* **5**:241-246.

Hill, J.M., B.M. Gabhardt, R. Wen, A.M. Bouterie, H.W. Thompson, R.J. O'Callaghan, W.P. Halford, and H.E. Kaufman. 1996. Quantification of herpes simplex virus type 1 DNA and latency associated transcripts in rabbit trigeminal ganglia demonstrates a stable reservoir of viral nucleic acids during latency. *Journal of Virology* **70**:3137-3141.

Hill, T.J., H.J. Field, and W.A. Blyth. 1975. Acute and recurrent infection with herpes simplex virus in the mouse: a model for studying latency and recurrent disease. *Journal of General Virology* **28**:341-353.

Howard, M.K., R.S. Coffin, A.R. MacLean, S.M. Brown, D. Bailey, P.N. Anderson, G. Burnstock, and D.S. Latchman. 1997. Gene delivery to rat enteric neurons using herpes simplex virus based vectors. *Journal of Molecular Neuroscience* **9**:65-74.

Hsu, S.-M., L. Raine, and H. Fanger. 1981. Use of Avidin-Biotin-Peroxidase complex (ABC) in immunoperoxidase techniques. *Journal of Histochemistry and Cytochemistry* **29**:577-580.

Hutchinson, L., K. Goldsmith, D. Snoddy, H. Ghosh, F.L. Graham, and D.C. Johnson. 1992. Identification and characterisation of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion. *Journal of Virology* **66**:5603-5609.

Hutchinson, L., C. Roopbeauchamp, and D.C. Johnson. 1995. Herpes simplex virus glycoprotein-K is known to influence fusion of infected cells, yet is not on the cell surface. *Journal of Virology* **69**:4556-4563.

Izquierdo, M., M. Cortes, P. DeFelipe, V. Martin, J. Diezquerra, A.A. Talavera, and A. Perez-Higueras. 1995. Long term rat survival after malignant brain tumor regression by retroviral gene therapy. *Gene Therapy* **2**:66-69.

Jackman, J., I. Alamo, and A.J. Fornace. 1994. Genotoxic stress confers preferential and coordinate messenger RNA stability on the 5 GADD genes. *Cancer Research* **54**:5656-5662.

Jamieson, D.R., L.H. Robinson, J.I. Daksis, M.J. Nicholl, and C.M. Preston. 1995. Quiescent viral genomes in human fibroblasts after infection with herpes simplex virus type 1 Vmw65 mutants. *Journal of General Virology* **76**:1417-31.

Jayachandra, S., A. Baghian, and K.G. Kousoulas. 1997. Herpes simplex virus type 1 glycoprotein K is not essential for infectious virus production in actively replicating cells but is required for efficient envelopment and translocation of infectious virions from the cytoplasm to the extracellular space. *Journal of Virology* **71**:5012-5024.

Jeffery, K.J.M., S.J. Read, T.E.A. Peto, R.T. Mayon-White, and C.R.M. Bangham. 1997. Diagnosis of viral infections of the central nervous system: clinical interpretation of PCR results. *Lancet* **349**:313-317.

Johnson, P.A., C. MacLean, H.S. Marsden, R.G. Dalziel, and R.D. Everett. 1986. The product of gene US11 of herpes simplex virus type 1 is expressed as a true ate gene. *Journal of General Virology* **67**:871-883.

Johnson, P.A. and R.D. Everett. 1986. The control of herpes simplex virus type 1 late gene transcription: a 'TATA-box'/cap site region is sufficient for fully efficient regulated activity. *Nucleic Acid Research* **14**:8247-8264.

Joki, T., M. Nakamura, and T. Ohno. 1995. Activation of the radiosensitive EGR-1 promoter induces expression of the herpes simplex virus thymidine kinase gene and sensitivity of human glioma cells to ganciclovir. *Human Gene Therapy* **6**:1507-1513.

Kanangat, S., J. Thomas, S. Gangappa, J.S. Babu, and B.T. Rouse. 1996. Herpes simplex virus type 1 mediated up regulation of IL-12 (p40) mRNA expression. *Journal of Immunology* **156**:1110-1116.

Kaplitt, M.G., P. Leone, R.J. Samulski, X. Xiao, and D.W. Pfaff. 1994. Long term gene expression and phenotype correction using adenoassociated virus vectors in the mammalian brain. *Nature Genetics* **8**:148-154.

Katze, M. 1995. Regulation of interferon induced PKR: can viruses cope? *Trends in Microbiology* **3**:75-78.

Kawagachi, Y., C. VanSant, and B. Roizman. 1997. Herpes simplex virus 1 α regulatory protein ICP0 interacts with and stabilizes the cell cycle regulator cyclin D3. *Journal of Virology* **71**:7328-7336.

Keller, J.M. 1976a. The expression of the syn gene of herpes simplex virus type 1. I. Morphology of infected cells. *Virology* **69**:490-499.

Keller, J.M. 1976b. The expression of the syn gene of herpes simplex virus type 1. II. Requirements of macromolecular synthesis. *Virology* **69**:402-499.

Kennedy, P.G. 1997. Potential use of herpes simplex virus (HSV) vectors for gene therapy of neurological disorders. *Brain* **120**:1245-1259.

Kenny, J.J., S. Millhouse, M. Wotring, and B. Wigdahl. 1997. Upstream stimulatory factor family binds to the herpes simplex virus type 1 and latency-associated transcript promoter. *Virology* **230**:381-391.

Kesari, S., T.M. Lasner, K.R. Balsara, B.P. Randezzo, V.M.-Y. Lee, J.Q. Trojanowski, and N.W. Fraser. 1998. A neuroattenuated ICP34.5-deficient herpes simplex virus type 1 replicates in ependymal cells of the murine central nervous system. *Journal of General Virology* **79**:525-536.

Kesari, S., B.P. Randazzo, T. Valyi-Nagy, Q.S. Huang, S.M. Brown, A.R. MacLean, V.M.Y. Lee, J.Q. Trojanowski, and N.W. Fraser. 1995. Therapy of experimental human brain tumors using a neuroattenuated herpes simplex virus mutant. *Laboratory Investigation* **73**:636-648.

Kesari, S. V.M. Y. Lee, S.M. Brown, J.Q. Trojanowski, and N.W. Fraser. 1996. Selective vulnerability of mouse CNS neurons to latent infection with a neuroattenuated herpes simplex virus 1. *The Journal of Neuroscience* **16**:5644-5653.

Kesari, S., T.M. Lasner, K.R. Balsara, B.P. Randazzo, V.M.-Y. Lee, J.Q. Trojanowski, and N.W. Fraser. 1998. A neuroattenuated ICP34.5 deficient herpes simplex virus type 1 replicates in ependymal cells of murine central nervous system. *Journal of General Virology* **79**:525-536.

Khodarev, N.N., S.J. Advani, N. Gupta, B. Roizman and R.R. Weichselbaum. 1999. Accumulation of specific RNAs encoding transcriptional factors and stress response proteins against a background of severe depletion of cellular RNAs in cells infected with herpes simplex virus 1. *Proceedings of the National Academy of Sciences* **96**:12062-12067.

Kill, I.R., J.M. Bridger, K.H. Cambell, G. Maldona-Codina, and C.J. Huchison. 1991. The timing of the formation and usage of replicase clusters in S phase nuclei of human diploid fibroblasts. *Journal of Cell Science* **100**:869-876.

Klatzmann, D., S. Herson, P. Cherin, O. Chosidow, F. Baillet, G. Bensimon, O. Boyer, and J.L. Salzmann. 1996. Gene therapy for metastic malignant melanoma

evaluation of tolerance to intratumoral injection of cells producing recombinant retroviruses carrying the herpes simplex virus type 1 thymidine kinase gene, to be followed by ganciclovir administration. *Human Gene Therapy* **7**:255-267.

Knotts, F.B., M.L. Cook, and J.G. Stevens. 1974. Pathogenesis of herpetic encephalitis in mice following ophthalmic inoculation. *Journal of Infectious Diseases* **130**:16-27.

Knowles, R.W. and S. Person. 1976. Effects of 2-deoxyglucose glucosamide and mannose on cell fusion and glycoproteins of herpes simplex virus. *Journal of Virology* **18**:644-651.

Konno, S. W.D. Taylor, and A.H. Dardiri. 1971. Acute African Swine Fever proliferative phase in lymphoreticular tissue and the reticullendothelial system. *Cornell Veterinarian* **61**:71-84.

Koromilas, A.E., C. Cantin, A.W. R. Jagus, J. Hiscott, and N. Sonenberg. 1995. The interferon inducible protein kinase PKR modulates the transcriptional activation of immunoglobin k gene. *Journal of Biological Chemistry* **270**:25426-25434.

Kosz-Vnenchak, M., J. Jacobson, D.M. Coen, and D.M. Knipe. 1993. Evidence for a novel regulatory pathway for herpes simplex virus gene expression in trigeminal ganglion neurons. *Journal of Virology* **67**:5383-5393.

Kousoulas K.G., P.E. Pellett, L. Pereira, and B. Roizman. 1984. Mutations affecting conformation or sequence of neutralizing epitopes identified by reactivity of viable plaques segregate from syn and ts domains of HSV-1(F) gB gene. *Virology* **135**:379-94.

Kramer, M.F. and D.M. Coen. 1995. Quantification of transcripts from the ICP4 and thymidine kinase genes in mouse ganglia latently infected with herpes simplex viruses. *Journal of Virology* **69**:1389-1399.

Kramm, C.M., N.G. Rainov, M. Senaesteves, M. Chase, P.A. Pechan, E.A. Chiocca, and X.O. Breakefield. 1996. Herpes vector mediated delivery of marker genes to disseminated central nervous system tumors. *Human Gene Therapy* **7**:291-300.

Kritas, S.K., M.B. Pensaert, and T.C. Mettenleiter. 1994a. Invasion and spread of single glycoprotein deleted mutants of Aujesky's disease virus in the trigeminal nervous pathway of pigs after intranasal inoculation. *Veterinary Microbiology* **40**:323-334.

Kritas, S.K., M.B. Pensaert, and T.C. Mettenleiter. 1994b. Role of envelope glycoproteins gI, gp63 and gC in the invasion and spread of Aujesky's disease virus in the olfactory nervous pathway of the pig. *Journal of General Virology* **75**:2319-2327.

Kucharczuk, J.C., B. Randazzo, A.A. Alshami, D.H. Sterman, K.A. Amin, K.L. Molnar-Kimber, S.M. Brown, L.A. Litzky, N.W. Fraser, S.M. Arbedda, and K.L. Kaiser. 1997. Use of a replication restricted recombinant herpes virus to treat localized human malignancy. *Cancer Research* **57**:466-471.

Kuklin, N. M. Daheshia, K. Karem, E. Manickan, and B.T. Rouse. 1997. Induction of mucosal immunity against herpes simplex virus by plasmid DNA immunization. *Journal of Virology* **71**:3138-3145.

Kwong, A.D. and N. Frenkel. 1984. Herpes simplex virus amplicon: effect of size on replication of constructed defective genomes containing eucaryotic DNA sequences. *Journal of Virology* **51**:595-603.

Lachmann, R.H. and S. Efstathiou. 1999. Use of herpes simplex virus type 1 for transgene expression within the nervous system. *Clinical Science* **96**:533-541.

Lachmann, R.H. and S. Efstathiou. 1997. Utilization of the herpes simplex virus type 1 latency associated regulatory region to drive stable reporter gene expression in the nervous system. *Journal of Virology* **71**:3197-3207.

Lachmann, R.H., C. Brown, and S. Efstathiou. 1996. Murine RNA polymerase 1 promoter inserted into the herpes simplex virus type 1 genome is functional during lytic, but not latent, infection. *Journal of General Virology* **77**:2575-2582.

Lagunoff, M., G. Randall, and B. Roizman. 1996. Phenotypic properties of herpes simplex virus 1 containing a derepressed open reading frame P gene. *Journal of Virology* **70**:1810-1817.

Lagunoff, M. and B. Roizman. 1995. The regulation of synthesis and properties of the protein product of open reading frame P of the herpes simplex virus 1 genome. *Journal of Virology* **69**:3615-3623.

Lagunoff, M. and B. Roizman. 1994. Expression of a herpes simplex virus 1 open reading frame antisense to the $\gamma_1$34.5 gene and transcribed by an RNA 3' coterminal with the unspliced latency associated transcript. *Journal of Virology* **68**:6021-6028.

Laing, X.P., L.A. Babiuk, S.V. Littlevandenhurk, D.R. Fitzpatrick, and T.J. Zamb. 1991. Bovine herpesvirus 1 attachment to permissive cells is mediated by its major glycoprotein gI, gC, and gE. *Journal of Virology* **65**:1124-1132.

Laing, X.P., L.A. Babiuk, and T.J. Zamb. 1991. Pseudorabies virus gC and bovineherpes gC chare complementary functions. *Journal of Virology* **65**:5553-3337.

Laycock, K.A., S.F. Lee, R.H. Brady, and J.S. Pepose. 1991. Characterization of a murine model of recurrent herpes simplex viral keratitis induced by ultraviolet B radiation. *Investigative Ophthalomogy and Visual Science* **32**:2741-2746.

Lee, L.Y. and P. Schaffer. 1998. A virus with a mutation in the ICP4 binding site in the L/ST promoter of herpes simplex virus type 1 but not a virus with a mutation in open reading frame P, exhibits cell-type specific expression of $_1$34.5 transcripts and latency associated transcripts. *Journal of Virology* **72**:4250-4264.

Lee, T.G., N. Tang, S. Thompson, J. Miller, and M.G. Katze. 1994. The 38,000 dalton cellular inhibitor of the interferon inducible double stranded RNA activated

protein kinase (PKR) is a member of the tetratripeptide repeat. *Molecular and Cellular Biology* **14**:2331-2342.

Lehman , I.R. and P.E. Boehmer. 1999. Replication of herpes simplex virus DNA. *Journal of Biological Chemistry* **274**:28059-28062.

Leib, D.A., D.M. Coen, C.L. Bogard, K.A. Hicks, D.R. Yager, D.M. Knipe, K.L. Tyler, and P.A. Schaffer. 1989. Immediate-early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency. *Journal of Virology* **63**:759-768.

Li, Z., R. Hromchak, A. Mudipalli, and A. Bloch. 1998. Tumor suppressor proteins as regulators of cell differentiation. *Cancer Research* **14**:2361-2371.

Lin, X.H., M.A. Ali, H. Openshaw, and E.M. Cantin. 1996. Deletion of the carboxy-terminus of herpes simplex virus type 1 (HSV-1) gB does no effect oligomerisation heparin-binding activity, or its ability to protect against HSV challenge. *Archieves of Virology* **141**:1153-1165.

Link, C.J., D. Moerman, T. Seregina, J. Levy, and K.A. Schabold. 1996. Phase I trial of in vivo gene therapy with the herpes simplex thymidine kinase/gancyclovir system for the treatment of refractory or recurrent ovarian cancer. *Human Gene Therapy* **7**:1161-1179.

Liu, T., Q. Tang, and R.L. Hendricks. 1996. Inflammatory infiltration of the trigeminal ganglion after herpes simplex virus type 1 corneal infection. *Journal of Virology* **70**:264-271.

Locker, H. and H. Frenkel. 1979. BamHI, KpnI and SalI restriction enzyme maps of the DNAs of herpes simplex virus strains Justin and F: occurrence of heterogeneities in defined regions of the viral DNA. *Journal of Virology* **32**:424-441.

Longnecker, R., B. Roizman, and B. Meizner. 1988. Herpes simplex viruses as vectors: Properties of the prototype vaccine strains suitable for use as vectors. In *"Viral Vectors"* Y. Glutman et. al. (ed). Current Communications in Molecular Biology, Cold Springs Harbor Laboratory, U.S.A.

Longnecker, R., S. Chatterjee, R.J. Whitley, and B. Roizman. 1987. Identification of a herpes simplex virus 1 glycoprotein gene within a gene cluster dispensible for growth in cell culture. *Proceedings of the National Academy of Science* **84**:4304-4307.

Longnecker, R. and B. Roizman. 1986. Generation of an inverting herpes simplex virus type 1 mutant lacking the L-S junction *a* sequence, an origin of DNA synthesis, and several genes including those specifying glycoproteins E and α47. *Journal of Virology* **58**:583-591.

Lord, K.A., B. Hoffman-Liebermann, and D.A. Liebermann. 1990. Sequence of MyD116 cDNA-a novel myeloid differentiation primary response gene induced by IL6. *Nucleic Acids Research* **18**:2823.

Lornsdale, D.M., S.M. Brown, J.H. Subak-Sharpe, K.G. Warren, and H. Koprowski. 1979. The polypeptide and the DNA restriction enzyme profiles of spontaneous isolates of Herpes simplex virus type 1 from explants of human trigeminal ganglia, superior cervical and vagus ganglia. *Journal of General Virology* **43**:151-171.

Loutsch, J.M., G.-C. Perng, J.M. Hill, X. Zheng, M.E. Marquart, T.M. Block, H. Ghiasi, A.B. Nesburn, and S.L. Wechsler. 1999. Identical 371-base pair deletion mutations in the LAT genes of HSV1 McKrae and 17syn+ result in different in vivo reactivation phenotypes. *Journal of Virology* **73**:767-771.

Lu, Y. M. Wanbash, M.G. Katze, and R.M. Krug. 1995. Binding of the influenza virus NS1 protein to the protein kinase that phosphorylates the eIF-2 translation initiation factor. *Virology* **214:222-228.**

MacDermid, A. 1998. Dying Scots aid brain tumour battle. *The Herald*; City ed. **116**:1-2.

McGeoch, D. and B. Barnett. 1991. Neurovirulence factor. *Nature* (London) **353**:609.

McGeoch, D.J., C. Cunningham, G. McIntyre, and A. Dolan. 1991. Comparative sequence analysis of the long repeat regions and adjoining parts of the long unique regions in the genomes of herpes simplex viruses types 1 and 2. *Journal of General Virology* **72**:3057-3075.

McGeoch, D.J., M.A. Dalrymple, A.J. Davison, A. Dolan, M.C. Frame, D. McNab, L.J. Perry, J.E. Scott, and P. Taylor. 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *Journal of General Virology* **69**:1531-1574.

McKay, E.M., B. McVey, H.S. Marsden, S.M. Brown, and A.R. MacLean. 1993. The herpes simplex virus type 1 strain 17 open reading frame RL1 encodes a polypeptide of apparent M(r) 37K equivalent to ICP34.5 of herpes simplex virus type 1 strain F. *Journal of General Virology* **74**:2493-2497.

McKie, E.A., R. Reid, A.R. MacLean, and S.M. Brown. 2000. Herpes simplex virus ORF P transcription and translation can be independent of ICP4 repression and are cell type dependent (*In preperation*)

McKie, E.A., D.I. Graham, and S.M. Brown. 1998. Selective astrocytic transgene expression in vitro and in vivo from the GFAP promoter in a HSV RL1 null mutant vector - potential glioblastoma targeting. *Gene Therapy* **5**:440-450.

McKie, E.A., A.R. MacLean, A.D. Lewis, G. Cruickshank, R. Rampling, S.C. Barnett, P.G.E. Kennedy, and S.M. Brown. 1996. Selective in-vitro replication of herpes-simplex virus type-1 (HSV-1) ICP34.5 null mutants in primary human CNS tumors evaluation of a potentially effective clinical therapy. *British Journal of Cancer* **74**:745-752.

McKie, E.A., R.G. Hope, S.M. Brown, and A.R. MacLean. 1994. Characterisation of the herpes simplex virus type 1 strain 17(+) neuro-virulence gene RL1 and its expression in a bacterial system. *Journal of General Virology* **75**:733-741.

McKie, E.A. 1993. Characterisation of the HSV-1 strain 17(+) neurovirulence gene RL1 and its expression in a bacterial system. In "Ph.D. thesis", University of Glasgow.

McLauchlan, J., K. Liefkens, and N. Stow. 1994. The herpes simplex virus type 1 UL37 gene product is a component of virus particles. *Journal of General Virology* **75**:2047-2052.

MacLean, A., X.-Q. Wei, F.-P. Huang, U.A.H. Al-Alem, W.L. Chan, and F.Y. Liew. 1998. Mice lacking inducible nitric oxide synthase are more susceptible to herpes simplex virus infection despite enhanced Th1 cell responses. *Journal of General Virology* **79**:825-830.

MacLean, A., L. Robertson, E. MacKay, and S.M. Brown. 1991. The RL1 neurovirulence locus in herpes simplex virus type 2 strain HG52 plays no role in latency. *Journal of General Virology* **72**:631-639.

MacLean, A.R., M.Ul-Fareed, L. Robertson, J. Harland, and S.M. Brown. 1991. Herpes simplex virus type 1 deletion variants 1714 and 1716 pinpoint neurovirulence-related sequences in Glasgow strain 17+ between immediate early gene 1 and the 'a' sequence. *Journal of General Virology* **72**:631-639.

MacLean, A.R. 1988. XbaI site loss mutants and deletion/duplication variants of herpes simplex virus type 1: isolation, characterisation and recombination studies. In "Ph.D. thesis", University of Glasgow.

MacLean, C.A., A. MacLean, S. Efstathiou, M.L. Elliott, F.E. Jamieson, and D.J. McGeoch. 1991. Investigation of herpes simplex virus type 1 genes encoding multiply inserted membrane proteins. *Journal of General Virology* **72**:897-906.

McMenamin, M.M., A.P. Byrnes, H.M. Charlton, R.S. Coffin, D.S. Latchman, and M.J.A. Wood. 1998. A $\gamma_1$34.5 mutant of herpes simplex 1 causes severe inflammation in the brain. *Neuroscience* **83**:1225-1237.

McMenamin, M.M., A.P. Byrnes, F.G. Pike, H.M. Charlton, R.S. Coffin, D.S Latchman, and M.J.A. Wood. 1998. Potential and limitations of a $\gamma_1$34.5 mutant of herpes simplex 1 as a gene therapy vector in the CNS. *Gene Therapy* **5**:594-604.

McNabb, D.S. and R.J. Courtney. 1992a. Identification and characterization of the herpes simplex virus type 1 virion protein encoded by the UL35 open reading frame. *Journal of Virology* **66**:2653-2663.

McNabb, D.S. and R.J. Courtney. 1992b. Posttranslational modification and subcellular localization of the p12 capsid protein of herpes simplex virus type 1. *Journal of Virology* **66**:2653-2663.

Maggioncalda, J., A. Mehta, Y.H. Su, N.W. Fraser, and T.M. Block. 1996. Correlation between herpes simplex virus type 1 rate of reactivation from latent infection and the number of infected neurons in trigeminal ganglia. *Virology* **225**:72-81.

Makarova, O., G. Gorneva, F. Wu, V. Farutin, B. Villeponteau, L. Poliani, D. Fink, and M. Levine. 1996. Incorporation of nuclear matrix attachment regions into the herpes simplex virus type 1 genome does not induce long term expression of a foreign gene during latency. *Gene Therapy* **3**:829-833.

Manservigi, R., P. Spear, and A. Buchan. 1977. Cell fusion induced herpes simplex virus is promoted and suppressed by different viral glycoproteins. *Proceedings of the National Academy of Sciences* **74**:3913-3917.

Marconi, P., D. Krisky, T. Oligino, P.L. Poliani, R. Ramakrishnan, W.F. Goins, D.J. Fink, and J.C. Glorioso. 1996. Replication-defective herpes simplex virus vectors for gene transfer in vivo. *Proceedings of the National Academy of Sciences* **93**:11319-11320.

Markovitz, N.S., D. Baunoch, and B. Roizman. 1997. The range and distribution of murine central nervous system cells infected with the γ_1 mutant of herpes simplex virus 1. *Journal of Virology* **71**:5560-5569.

Marsden, H.S., N.D. Stow, V.G. Preston, M.C. Timbury, and N. Wilkey. 1978. Physical mapping of herpes simplex virus induced polypeptides. *Journal of Virology* **28**:624-642.

Marshall KR, Lachmann RH, Efstathiou S, Rinaldi A, and C.M. Preston. 2000. Long-term transgene expression in mice infected with a herpes simplex virus type 1 mutant severely impaired for immediate-early gene expression. *Journal of Virology* **74**:956-964.

Martin, D.W. and P.C. Weber. 1998. Role of the DR2 repeat array in the regulation of the ICP34.5 gene promoter of herpes simplex virus type 1 during productive infection. *Journal of General Virology* **79**:517-523.

Martin, D. and P.C. Weber. 1996. The *a* sequence is dispensable for isomerization of the herpes simplex virus type 1 genome. *Journal of Virology* **70**:8801-8812.

Martuza, R.L., A. Malick, J.M. Markert, K.L. Ruffner, and D.M. Coen. 1991. Experimental therapy of human glioma by means of a genetically engineered virus mutant. *Science* **252**:854-856.

Mathews, M.B., and T. Schenk. 1991. Adenovirus associated RNA and translation control. *Journal of Virology* **65**:5657-5662.

Maul, G.G. and R.D. Everett. 1994. The nuclear location of PML, a cellular member of the C3HC4 zinc binding domain protein family, is rearranged during herpes simplex virus infection by the viral protein ICP0. *Journal of General Virology* **75**:1223-1233.

Maul, G.G., H.H. Guldner, and J.G. Spivak. 1992. Modification of discrete nuclear domains induced by herpes simplex virus type 1 immediate early gene 1 product ICP0. *Journal of General Virology* **74:**2679-2690.

Mebus, C.A. 1988. African Swine Fever. *Advances in Virus Research* **35**:251-269.

Mebus, C.A. 1987. Pathobiology and pathogenesis. p.21-29. *In* Y. Beaker (ed.), African swine fever. Martinus Nijhoff Publishing, Boston.

Merideth, M. A. Orr, and R.D. Everett. 1994. Herpes simplex virus type 1 immediate early protein Vmw110 binds strongly and specifically to a 135 kDa cellular protein. *Virology* **200**:457-469.

Miller, D.G. and A.D. Miller. 1992. Tunicamycin treatment of CHO cells abrogates multiple blocks to retrovirus infection, one of which is due to a secreted inhibitor. *Journal of Virology* **66**:78-84.

Minagawa, H., S. Tanaka, Y. Toh, and R. Mori. 1994. Detection of herpes simplex virus type 1-encoded RNA by polymerase chain reaction: different pattern of viral RNA detection in latently infected murine reactivation. *Journal of General Virology* **75**:647-650.

Mineta, T., S.D. Rabkin, T. Yazaki, W.D. Hunter, and R.L. Martuza. 1995. Attenuated multi-mutated herpes simplex virus 1 for the treatment of malignant gliomas. *Nature Medicine* **1**:938-943.

Mineta, T., S.D. Rabkin, and R.L. Martuza. 1994a. Treatment of malignant gliomas using gancyclovir hypersensitive ribonucleotide reductase deficient herpes simplex viral mutant. *Cancer Research* **54**:3963-3966.

Mineta, T., J.M. Markert, Y. Takamiya, D.M. Coen, S.D. Rabkin, and R. Martuza. 1994b. CNS tumor therapy by attenuated herpes simplex viruses. *Gene Therapy* **1**:578.

Minshell, J., J. Pines, R. Golsteyn, N. Standart, S, MacKie, A. Colman, J. Brown, J.V. Ruderman, M. Wu, and T. Hunt. 1989. The role of cyclin synthesis, modification and destruction in the control of cell division. *Journal of Cell Science Supplement* **12**:77-97.

Mitchell, P.S., M.J. Espy, T.F. Smith, D.R. Toal, P.N. Rys, E.F. Berbari, D.R. Osmon, and D.H. Persing. 1997. Laboratory diagnosis of central nervous system infections with herpes simplex virus by PCR performed with cerebrospinal fluid specimens. *Journal of Clinical Microbiology* **35:**2873-2877.

Mitchell, W., I. Steiner, A.M. Brown, A.R. MacLean, J.H. Subak-Sharpe, and N.W. Fraser. 1990a. A herpes simplex virus type 1 variant, deleted in the promoter region of the latency-associated transcripts does not produce any detectable minor RNA species during latency in the mouse trigeminal ganglion. *Journal of General Virology* **71**:953-957.

Mitchell, W.J., S.L. Deshmane, A. Dolan, D.J. McGeoch, and N.W. Fraser. 1990b. Characterization of herpes simplex virus type 2 transcription during latent infection of mouse trigeminal ganglia. *Journal of Virology* **64**:5342-5348.

Mitchell, W., R.P. Lirette, and N.W. Fraser. 1990c. Mapping of low abundance latency-associated RNA in the trigeminal ganglia of mice latently infected with herpes simplex virus type 1. *Journal of General Virology* **71**:125-132.

Mittenleiter, T.C. 1989. Glycoprotein C deletion mutants of pseudorabies virus are impaired in virus entry. *Virology* **171**:623-625.

Mocarski, E.S. and B. Roizman. 1982. Structure and role of the herpes simplex virus DNA termini in inversion, circularizationand generation of virion DNA. *Cell* **31**:89-97.

Mohr, I. and Y. Gluzman. 1996. A herpesvirus genetic element which affects translation in the absence of the viral GADD34 function. *EMBO* **15**:4759-4766.

Montgomery, R.I., M.S. Warner, B.J. Lum, and P.G. Spear. 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* **87**:427-436.

Moolten, F.L. 1986. Tumor chemosensitivity conferred by inserted herpes thymidine kinase gene: Paradigm for a prospective cancer chemotherapy. *Cancer Research* **46**:5276-5281.

Moolten, F.L. 1994. Drug sensitivity ("suicide") genes for selective cancer chemotherapy. *Cancer Gene Therapy* **1**:279-287.

Moriya, A., A. Yoskiki, . Kita, S. Fushiki, and J. Imanishu. 1994. Heat shock induced reactivation of herpes simplex virus type 1 in latently infected mouse trigeminal ganglia cells in dissociated culture. *Archives of Virology* **135**:419-425.

Moulton, J. and L. Coggins. 1968. Comparison of lesions in acute and chronic African swine fever. *Cornell Veterinarian* **58**:364-388.

Mulvey, M., J. Popper, A. Ladd, and I. Mohr. 1999. A herpesvirus ribosome-associated, RNA-binding protein confers a growth advantage upon mutants deficient in a GADD34-related function. *Journal of Virology* **73**:3375-3385.

Nash, A.A., A. Tayasurya, J. Relan, S.P. Cobbold, H. Waldmann, and T. Prospero. 1987. Different roles of L3T4+ and Lyt2+ T-cell subsets in the control of an acute herpes simplex virus infection of the skin and nervous system. *Journal of General Virology* **68**:825-833.

Navarro, D., P. Paz, and L. Pereira. 1992. Domains of herpes simplex virus I glycoprotein B that function in virus penetration, cell-to-cell spread, and fusion. *Virology* **186**:99-112.

Neidhardt, H., C.H. Schroder, and H.C. Kaerner. 1987. Herpes simplex virus type 1 glycoprotein E is not indispensable for viral infectivity. *Journal of Virology* **61**:600-603.

Neipel, F., J.-C. Albrecht, and B. Fleckenstein. 1997. Cell-homologous genes in the kaposi's sarcoma-associated rhadinovirus human herpesvirus 8: determinants of its pathogenicity? *Journal of Virology* **71**:4187-4192.

Nichol, P.F., J.Y. Chang, E.M. Johnson Jr., and P.D. Olivo. 1996. Herpes simplex virus gene expression in neurons: viral DNA synthesis is a critical regulatory event in the branch point between the lytic and latent pathways. *Journal of Virology* **70**:5476-5486.

Nilheden, E., S. Jeansson, and A. Vahlne. 1985. Amplification of herpes simplex virus resistance in mouse neuroblastoma (C1300) cells. *Archives of Virology* **83**:269-283.

Nishiyama, Y., R. Kurachi, T. Daikoku, and K. Umene. 1993. The US9, 10, 11, 12 genes of herpes simplec virus type 1 are of no importance for its neurovirulence and latency in mice. *Virology* **194**:419-423.

Noisakran, S., W.P. Halford, L. Veress, and D.J.J. Carr. 1998. Role of the hypothalamic pituitary adrenal axis and IL-6 in stress-induced reactivation of latent herpes simplex virus type 1. *Journal of Immunology* **160**:5441-5447.

Novotny, M.J., M.L. Parish, and P.G. Spear. 1996. Variability of herpes simplex virus 1 gI and anti-gI antibodies that inhibit cell fusion but not infectivity. *Virology* **221**:1-13.

Oligino, T., P.L. Poliani, Y. Wang, S.Y. Tsai, B.W. O'Malley, D.J. Fink, and J.C. Glorioso. 1998. Drug inducible transgene expression in brain using a herpes simplex virus vector. *Gene Therapy* **5**:491-496.

O'Neill, F.J. 1997. Prolongation of herpes simplex virus latency in cultured human cells by temperature sensitive elevation. *Journal of General Virology* **24**:41-46.

O'Neill, F.J., P.J. Goldberg, and F. Rapp. 1972. Herpes simplex virus latency in cultured human cells following treatment with cytosine arabinoside. *Journal of General Virology* **14**:189-197.

O'Rouke, G. Elliott, M. Papworth, R. Everett, and P. O'Hare. 1998. Examination of determinants for intranuclear localization and transactivation within the RING finger of herpes simplex virus type 1 IE110 protein. *Journal of General Virology* **79**:537-548.

Padilla, J., M. Yamada, Y. Takahashi, T. Tsukazaki, J. Nakamura, M. Yoshida, F. Uno, Y. Arao, and S. Nii. 1997. In vitro selection of variants of herpes simplex virus type 1 which differ in cytopathic changes. *Microbiology and Immunology* **41**:203-207.

Palella, T.D., L. Silverman, C.T. Schroll, F.L. Homa, M. Levine, and W.N. Kelley. 1988 *Molecular and Cellular Biology* **8**:457-460.

Peeters, B., J. Pol, A. Gielkens, and R. Moormann. 1993. Envelope glycoproteins-gp50 pseudorabies virus is essential for virus entry but is not required for viral spread in mice. *Journal of Virology* **67**:170-177.

Pennisi, E. 1996. Will a twist of cancer fate lead to a new cancer treatment? *Science* **274**:342-343.

Perng, G.C., H. Ghiasi, S.M. Slanina, A.B. Nesburn, and S.L. Wechsler. 1996a. High dose ocular infection with a herpes-simplex virus type-1 ICP34.5 deletion mutant produces no corneal disease or neurovirulence yet results in wild type levels of spontaneous reactivation. *Journal of Virology* **70**:2883-2893.

Perng, G.C., K. Chokephaibulkit, R.L. Thompson, N.M. Sawtell, S.M. Slanina, H. Ghiasi, A.B. Nesburn, and S.L. Wechsler. 1996b. The region of the herpes simplex virus type 1 LAT gene that is colinear with the ICP34.5 gene is not involved in spontaneous reactivation. *Journal of Virology* **70**:282-291.

Perng, G.C., R.L. Thompson, N.M. Sawtell, W.E. Taylor, S.M. Slanina, H. Ghiasi, and R. Kaiwar. 1995. An avirulent ICP34.5 deletion mutant of herpes-simplex virus type-1 is capable of in-vivo spontaneous reactivation. *Journal of Virology* **69**:3033-3041.

Poffenberger, K.I., E. Tabares, and B. Roizman. 1983. Characterisation of a viable, noninverting herpes simplex virus 1 genome derived by insertion and deletion of sequences at the junction of components L and S. *PNAS* **80**:2690-2694.

Poon, A.P. and B. Roizman. 1997. Differentiation of the shutoff of protein synthesis by virion host shutoff and mutant $\gamma_1$34.5 genes of herpes simplex virus 1. *Virology* **229**:98-105.

Poguegeile, K.L. and Spear, P.G. 1987. The single base pair substitution responsible for *syn* phenotype of herpes simplex virus type-1 strain Mp. *Virology* **157**:67-74.

Prelich, G. C.K. Tan, M. Kostura, M.B. Mathews, A.G. So, K.M. Downey, and B. Stillman. 1987. Functional identity of proliferating of proliferating cell nuclear antigen and a DNA polymerase-delta auxiliary protein. *Nature* **326**:517-520.

Preston, C.M. 2000. Repression of viral transcription during herpes simplex virus latency. *Journal of General Virology* **81**:1-19.

Preston, C.M. and M.J. Nicholl. 1997. Repression of gene expression upon infection of cells with herpes simplex virus type 1 mutants impaired for immediate early protein synthesis. *Journal of Virology* **71**:7807-7813.

Preston, C.M., A. Rinalds, and M.J. Nicholl. 1998. Herpes simplex virus type 1 immediate early gene expression is stimulated by inhibition of protein synthesis. *Journal of Virology* **79**:117-124.

Preston, C.M., R. Mabbs, and M.J. Nicholl. 1997. Construction and characterization of herpes simplex virus type 1 mutants with conditional defects in immediate early gene expression. *Virology* **229**:228-239.

Preston, C.M., J. Russell, R.A. Harris, and D.R. Jamieson. 1994. Herpes simplex virus latency in tissue culture cells. *Gene Therapy* **Suppl1**:S49-50.

Pyles, R.B., N.M. Sawtell, and R.L. Thompson. 1992. Herpes simplex virus type 1 dUTPase mutants are attenuated for neurovirulence, neuroinvasiveness, and reactivation from latency. *Journal of Virology* **66**:6706-6713.

Quinlan, M.P. and D.M. Knipe. 1985. Stimulation of expression of a herpes simplex virus DNA binding protein by two viral functions. *Molecular and Cellular Biology* **5**:957-963.

Quinn, J.P. and D.J. McGeoch. 1985. DNA sequences of the region in the genome of herpes simplex virus type 1 containing the genes for DNA polymerase and the major DNA binding protein. *Nucleic Acid Research* **13**:8143-8163.

Rainov, N.G., C.M. Kramm, K. Aboody-Guterman, M. Chase, K. Ueki, D.N. Louis, G.R. Harsh, A. Chiocca, and X.O. Breakfield. 1996. Retrovirus mediated gene therapy of experimental brain neoplasms using the herpes simplex virus thymidine kinase ganciclovir paradigm. *Cancer Gene Therapy* **3**:99-106.

Ralph, Jr, W.M., M.S. Cabatingan, and P.A. Schaffer. 1994. Induction of herpes simplex virus type 1 immediate early gene expression by a cellular activity expressed in vero and NB41A3 cells after growth arrest release. Journal of Virology **68**:6871-6882.

Rampling, R. G. Cruickchanks, A. MacLean, and S. Brown. 1998. Therapeutic replication competent herpes simplex virus. *Letter to the Editor:Nature Medicine* **4**:122.

Randall, G., M. Lagunoff, and B. Roizman. 1997. The product of ORF O located within the domain of herpes simplex virus 1 genome transcribed during latent infection binds to and inhibits in vitro binding of infected cell protein 4 to its cognate DNA site. *The Procedings of the National Academy of Science* **94**:10379-10384.

Randall, G. and B. Roizman. 1997. Transcription of the depressed open reading frame P of herpes simplex virus 1 precludes the expression of the antisense gamma(1)34.5 gene and may account for the attenuation of the mutant virus. *Journal of Virology* **71**:7750-7757.

Randazzo, B.P., J.C. Kucharczuk, L.A. Litzky, L.R. Kaiser, S.M. Brown, A. MacLean, A.M. Albelda, and N.W. Fraser. 1996. Herpes simplex 1716 - an ICP34.5 mutant is severely replication restricted in human skin xenografts in vivo. *Virology* **223**:392-395.

Randazzo, B.P., S. Kesari, R.M. Gesser, D. Aslop, J.C. Ford, S.M. Brown, A. MacLean, and N.W. Fraser. 1995. Treatment of experimental intracranial murine

melanoma with a neuroattenuated herpes simplex virus 1 mutant. *Virology* **211**:94-101.

Randazzo, B., S. Kesari, N. Fraser, and S.M. Brown. 1997. Treatment of experimental subcutaneous human melanoma with a replication restricted herpes simplex virus mutant. *Journal of Dermatology* **108**:933-937.

Ravi, V., P.G. Kennedy, and A.R. MacLean. 1998. Functional analysis of the herpes simplex virus type 2 strain HG52 RL1 gene: the intron plays no role in virulence. *Journal of General Virology* **79**:1613-1617.

Rebordosa, X., J. Pinol, A. Perezpons, J. Lloberas, J. Naval, X. Serrahartmann, E. Esana, and E. Querol. 1996. Glycoprotein E of bovine herpesvirus type 1 is involved in virus transmission by direct cell to cell spread. *Virus Research* **45**:59-68.

Reed, L.J. and H. Meunch. 1938. A simple method of estimating fifty percent endpoints. *American Journal of Hygeine* **27**:493-497.

Richman, D.D., P.H. Cleveland, and M.N. Oxman, 1982. A rapid enzyme immunofiltration technique using monoclonal antibodies to serotype herpes simplex virus. *Journal of Medical Virology* **9**:299-305.

Ritchie, D.A., S.M. Brown, J.H. Subak-Sharpe, and A.T. Jamieson. 1977. Heterozygous and genetic recombination in herpes simplex type 1 virus. *Virology* **82**:323-333.

Rixon, F.J. and J. McLauchlan. 1990. Insertion of DNA sequences at a unique restriction enzyme site engineered for vector purposes into the genome of herpes simplex virus type 1. *Journal of General Virology* **71**:2931-2939.

Robertson, L.M., A.R. MacLean, and S.M. Brown. 1992. Peripheral replication and latency reactivation kinetics of the non-neurovirulent herpes simplex virus type 1 variant 1716. *Journal of General Virology* **73**:967-970.

Roizman, B. 1999. Herpes simplex virus gene functions: What have we learned that could be generally applicable to its near and distant cousins? *Acta Virologica* **43**:75-80.

Roizman, B. 1996. The function of herpes simplex virus genes: A primer for genetic engineering of novel vectors. *Proceedings of the National Academy of Sciences* **93**:11307-11312.

Roizman, B. 1992. The family Herpesviridae: An update. *Archives of Virology* **123**:425-449.

Roizman, B. 1979. The structure and isomerization of herpes simplex virus genomes. *Cell* **16**:481-494.

Roizman, B. and W. Batterson. 1996. *Herpes virus and their replication.* In Field's "Virology".

Roizman, B. and A.E. Sears. 1996. Herpes simplex virus and their replication. In "*Fields: Virology*", B.N. Fields, D.M. Knipe, P. Howley, R.M. Charnock, M.S. Hirsch, J.L. Melnick, T.P. Monath, and B. Roizman.

Roizman, B. and A. Sears. 1987. An inquiry into the mechanisms of herpes simplex virus latency. *Annual Reviews of Microbiology* **41**:543-571.

Rødahl, E. and L. Haarr. 1997. Analysis of the 2-kilobase latency-associated transcript expressed in PC12 cells productively infected with herpes simplex virus type 1: evidence for a stable, nonlinear structure. *Journal of Virology* **71**:1703-1707.

Roller, R.J., L.L.Monk, D. Stuart, and B. Roizman. 1996. Structure and function in the herpes simplex virus 1 RNA binding protein US11: Mapping of the domain required for ribosomal and nucleolar association and RNA binding in vitro. *Journal of Virology* **70**:2842-2851.

Romanelli M.G., E.M. Cattozzo, L. Faggioli, and M. Tognon. 1991. Fine mapping and characterization of the Syn 6 locus in the herpes simplex virus type 1 genome. *Journal of General Virology* **72**:1991-5.

Ross, B.D., B. Kim, and B.L. Davidson. 1995. Assessment of gancyclovir toxicity to experimental intracranial gliomas following recombinant adenoviral mediated transfer of the herpes simplex virus thymidine kinase gene by magnetic resonance imaging and protein magnetic resonance spectroscopy. *Clinical Cancer Research* **1**:651-657.

Russell, J. and C.M. Preston. 1986. An *in vitro* latency system for herpes simplex virus type 2. *Journal of General Virology* **67**:397-403.

Ruyechan, W.T., L.S. Morse, D.M. Knipe, and B. Roizman. 1979. Molecular genetics of herpes simplex virus II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behaviour of infected cells. *Journal of Virology* **29**:677.

Samaniego, L.A., N. Wu, and N.A. DeLuca. 1997. The herpes simplex virus immediate-early protein ICP0 affects transcription from the viral genome and infected cell survival in the absence of ICP4 and ICP27. *Journal of Virology* **71**:4614-4625.

Samaniego, L.A., L. Neiderhiser, and N.A. DeLuca. 1998. Persistence and expression of the herpes simplex virus genome in the absence of immediate early proteins. *Journal of Virology* **72**:3307-3320.

Sawtell, N.M. and R. L. Thompson. 1992a. Herpes simplex virus type 1 latency-associated transcription unit promotes anatomical site-dependent establishment and reactivation from latency. *Journal of Virology* **66**:2157-2169.

Sawtell, N.M. and R.L. Thompson. 1992b. Rapid in vivo reactivation of herpes simplex virus in latently infected murine ganglionic neurons after transient hyperthermia. *Journal of Virology* **66**:2150-2156.

Sawtell, N.M. 1997. Comprehensive quantification of herpes simplex virus latency at the single-cell level. *Journal of Virology* **71**:5423-5431.

Sawtell, N.M., D.K. Poon, C.S. Tanksy, and R.L. Thompson. 1998. The latent herpes simplex virus type 1 genome copy number in individual neurons is virus strain specific and correlates with reactivation. *Journal of Virology* **72**:5343-5350.

Schreurs, C., T.C. Mettenleiter, F. Zuckermann, N. Sugg, and T. Benporat. 1988. Glycoprotein C of pseudorabies virus is multifunctional. *Journal of Virology* **62**:2251-2257.

Schroder, C., G. Linde, F. Fehler, and G.M. Keil. 1997. From essential to beneficial-glycoprotein D loses importance for replication of bovine herpesvirus 1 in cell culture. *Journal of Virology* **71**:25-33.

Schwartz, R.M. and M.O. Dayhoff. 1979. Dayhoff table. p.353-358. *In* M. Dayhoff (ed.) Atlas of protein sequence and structures National Biomedical Research Foundation, Washington, D.C.

Seth, P., W.E. Rauls, R. Duff, F. Rapp, E. Adam, and L.J. Milnick. 1974. Antigenic differences between isolates of herpesvirus type 2. *Intervirology* **3**:1-14.

Shimeld, C., J.L. Whiteland, N.A. Williams, D.L. Easty, and T.J. Hill. 1996. Reactivation of herpes simplex virus type 1 in the mouse trigeminal ganglion: an *in vivo* study of virus antigen and immune cell infiltration. *Journal of General Virology* **77**:2583-2590.

Simmons, A., R. Bowden, and B. Slobedman. 1997. Retention of herpes simplex virus DNA sequences in the nuclei of mouse footpad keratinocytes after recovery from primary infection. *Journal of Virology* **78**:867-871

Soares, K., D.-Y., Hwang, R. Ramakrishana, M.C. Schmidt, D.J. Fink, and J.C. Glorioso. 1996. Cis-acting elements involved in transcriptional regulation of the herpes simplex virus type 1 latency associated promoter 1 (LAP1) in vitro and in vivo. *Journal of Virology* **70**:5384-5394.

Soubrane, C., R. Mouawad, O. Rixe, V. Calvez, A. Ghoumari, O. Verola, M. Weil, and D. Khayat. 1996. Direct gene transfer of a plasmid carrying the herpes simplex virus thymidine kinase gene (HSV-TK) in transplanted murine melanoma: *in vivo* study. *European Journal of Cancer* **32A**:691-695.

Spatz, S.J., E.C. Nordby, and P.C. Weber. 1997. Construction and characterization of a recombinant herpes simplex virus type 1 which overexpresses the transrepressor protein ICP0R. *Virology* **228**:218-228.

Spear, P.G. 1993. Entry of alphaherpesviruses into cells. *Seminars in Virology* **4**:167-180.

Speck, P.G., S. Efstathiou, and A.C. Minson. 1996. In vivo complementation studies of a glycoprotein H-deleted herpes simplex virus-based vector. *Journal of General Virology* **77**:2563-2568.

Spivack, J.G., G.M. Woods, and N.M. Fraser. 1991. Identification of a novel latency-specific splice donor signal within the herpes simplex virus type 1 2.0 kilobase latency associated transcript (LAT): translation inhibition of LAT open reading frames by the intron within the 2.0 kilobase LAT. *Journal of Virology* **65**:6800-6810.

Spivack, J.G., M.U. Fareed, T. Valyi-Nagy, T.C. Nash, J.S. O'Keefe, R.M. Gesser, E.A. McKie, A.R. MacLean, N.W. Fraser, and S.M. Brown. 1995. Replication, establishment of latent infection, expression of the latency-associated transcripts and explant reactivation of herpes simplex virus type 1 $\gamma_1$34.5 mutants in a mouse eye model. *Journal of General Virology* **76**:321-332.

Stannard, L.M., S. Himmelhoch, and S. Wynchank. 1996. Intra-nuclear localization of two envelope proteins, gB and gD, of herpes simplex virus. *Archives of Virology* **141**:505-524.

Starr, P.A., F. Lim, F.D. Grant, L. Trask, P. Lang, L. Yu, and A.I. Geller. 1996. Long term persistence of defective HSV-1 vectors in the rat brain is demonstrated by reactivation of vector gene expression. *Gene Therapy* **3**:615-623

Steiner, I., J.G. Spivak, S.L. Deshmare, C.I. Ace, C.M. Preston, and N.W. Fraser. 1990. A herpes simplex virus type 1 mutant containing a nontransducing Vmw65 protein establishes latent infection in vivo in the absence of viral replication and reactivates efficiently from explanted trigeminal ganglia. *Journal of Virology* **64**:1630-1638.

Steller, H. 1995. Mechanisms and genes of cellular suicide. *Science* **267**:1445-1449.

Stevens, J.G. and Cook, M.L. 1974. Maintenance of latent herpetic infection. An apparent role for antiviral IgG. *Journal of Immunology* **113**:1685-1693.

Stevens, J.G., Nesburn, A.B., and Cook, M.L. 1972. Latent herpes simplex virus from trigeminal ganglia of rabbits. *Immunology* 216-217.

Steven, A.C. and P.G. Spear. 1996. Herpesvirus capsid assembly and envelopment *In* "Structural Biology of Viruses" (R. Burnett, W. Chiu, and R. Gracea, Eds), pp.312-351. Oxford Univ. Press, New York.

Stevens, J.G. 1993. HSV-1 neuroinvasiveness. *Intervirology* **35**:152-163.

Stow, N.D. 1982. Localization of an origin of DNA replication within the TRS/IRS repeated region of the herpes simplex virus type 1 genome. *EMBO* **7**:863-867.

Stow, N.D. 1978. Physical mapping of herpes simplex virus type 1 mutations. In "PhD Thesis", University of Glasgow.

Stow, N.D. and E.C. Stow. 1986. Isolation and characterisation of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110. *Journal of General Virology* **67**:2571-2585.

Stow, N. and McMonagle. 1983. Characterization of the TRs IRs origin of DNA replication of herpes simplex virus type 1. *Virology* **130**:427-438.

Stow, N., E. McMonagle, and A. Davison. 1983. Fragments from both termini of the herpes simplex virus type 1 genome contain signals required for encapsidation of viral DNA. *Nucleic Acid Research* **11**:8205-8219.

Stow, N.D., J.H. Subak-Sharpe, and N.M. Wilkie. 1978. Physical mapping of herpes simplex virus type 1 mutations by marker rescue. *Journal of Virology* **28**:182-192.

Stow, N.D. and N.M. Wilkie. 1976. An improved technique for obtaining enhanced infectivity with herpes simplex virus type 1 DNA. *Journal of General Viroloy* **33**:447-458.

Strelow, L., T. Smith, and D. Leib. 1997. The virion host shut off function of herpes simplex virus type 1 plays a role in corneal invasion and functions independently of the cell cycle. *Virology* **231**:28-34.

Strelow, L.I. and D. Leib. 1995. Role of the virion host shut off (vhs) of herpes simplex virus type 1 in latency and pathogenesis. *Journal of Virology* **69**:6779-6786.

Stuats, H.F. and R.N. Lausch. 1993. Cytokine expression in vivo during murine herpetic stromal kerititis. *Journal of Immunology* **151**:277-283.

Studier, F.W. and B.A. Moffat. 1986. Use of T7 RNA polymerase to direct selective high-level expression of cloned genes. *Journal of Molecular Biology* **189**:113-130.

Subak-Sharp, J.H., and D.J. Dargan. 1998. HSV molecular biology: general aspects of herpes simplex virus molecular virology. *Virus Genes* **16**:239-251.

Sussman, M.D., Z. Lu, G. Kutish, C.L. Afonso, P. Roberts, and D.L. Rock. 1992. Identification of an African swine fever virus gene with similarity to a myeloid differentiation primary response gene and a neuro-virulence associated gene of herpes-simplex virus. *Journal of Virology* **66**:5586-5589.

Taha, M.Y., G.B. Clements, and S.M. Brown. 1989. The herpes simplex virus type 2 (HG52) variant JH2604 has a 1488 bp deletion which eliminates neurovirulence in mice. *Journal of General Virology* **70**:3073-3078.

Tal-Singer, R., W. Podrzuki, T.M. Lasner, A. Skokotas, J. Leary, N.W. Fraser, and S.L. Berger. 1998. Use of differential display reverse transcription-PCR to reveal cellular change during stimuli that result in herpes simplex virus type 1 reactivation from latency: regulation of immediate-early cellular response genes TIS7, interferon, and interferon regulatory factor-1. *Journal of Virology* **72:**1252-1261.

Tanaka, S., H. Minagawa, Y. Toh, Y. Liu, and R. Mori. 1994. Analysis by RNA-PCR of latency and reactivation of herpes simplex virus in multiple neuronal tissues. *Journal of General Virology* **75**:2691-2698.

Tapscott, S.J., A.D. Miller, J.M. Olson, M.S. Berger, M. Groudine, and A.M. Spence. 1994. Gene therapy of rat 9L gliosarcoma tumors by transduction with selectable genes does not require drug selection. *Proceedings of the National Academy of Sciences* **91**:8185-8189.

Tew, E.M., P.N. Anderson, and G. Burnstock. 1992. Implantation of the myenteric plexus into the corpus striatum of adult rats: survival of the neurons and glia and interaction with host brain. *Restoratic Neurology Neuoscience* **4**:311-321.

Tew, E.M., P.N. Anderson, M.J. Saffrey, and G. Burnstock. 1994. Transplantation of the postnatal rat myenteric plexus into adult rat corpus striatum: an electron microscopic study. *Experiments in Neurology* **129**:120-129.

Thomas, S., R.S. Coffin, P. Watts, G. Gough, and D.S. Latchman. 1998. The TAATGARAT motif in the herpes simplex virus immediate early gene promoters can confer both positive and negative responses to cellular octamer binding proteins when it is located within the viral genome. *Journal of Virology* **72**:3495-3500.

Thompson, R.L. and N.M. Sawtell. 1997. The herpes simplex virus type1 latency-associated transcript gene regulates the establishment of latency. *Journal of Virology* **71**:5432-5440.

Thompson, R.L., S.K. Rogers, and M.A. Zehrhusen. 1989. Herpes simplex virus neurovirulence and productive infection of neural cells is associated with a function which maps between 0.82 and 0.832 map units on the HSV genome. *Virology* **172**:435-450.

Thompson, R.L. and J.G. Stevens. 1983. Biological characterization of a herpes simplex virus intertypic recombinant which is completely and specifically non-neurovirlent. *Journal of General Virology* **131**:171-179.

Thompson, R.L., E.K. Wagner, and J.G. Stevens. 1983. Physical location of a herpes simplex virus type-1 gene function(s) specifically associated with a 10 million-fold increase in HSV neurovirulence. *Virology* **131**:180-192.

Timbury, M.C. 1971. Temperature sensitive mutants of herpes simplex virus type 2. *Journal of General Virology* **13**:373-376.

Timbury, M.C., A. Theriault, and R.A. Elton. 1974. A stable syncytial mutant mutant of herpes simplex type 2 virus. *Journal of General Virology* **23**:219-224.

Tognon, M., R. Manservigi, M.G. Romanelli, A. Rotola, R. Gatti, L. Foa-Tomasi, and G. Campadelli-Fiume. 1988. A herpes simplex virus type 1 mutant resistant to benzhydrazone, an inhibitor of glycoprotein synthesis in herpesvirus-infected cells. Preliminary mapping of benzhydrazone-resistance and a novel syncytial mutation. *Archives of Virology* **98**:199-212.

Trousdale, M.D., I. Steiner, J.G. Spivack, S.L. Deshmane, S.M. Brown, A.R. MacLean, J.H. Subak-Sharpe, and N.W. Fraser. 1991. In vivo and in vitro reactivation of herpes simplex virus type 1 latency associated transcript variant in a rabbit eye model. *Journal of Virology* **65**:6989-6993.

Tumpey, T.M., S.-H. Chen, J.E. Oakes, and R.N. Lausch. 1996. Neutrophil mediated suppression of virus replication after herpes simplex virus type 1 infection of the murine cornea. *Journal of Virology* **70**:898-904.

Ugolini, G., H.G.J.M. Kuypers, and P.L. Strick. 1989. Transneuronal transfer of herpes virus from peripheral nerves to cortex and brainstem. *Science* **24**:89-90.

Umene, K. 1986. Conversion of a fraction of the unique sequence to part of the inverted repeats in the S component of the herpes simplex virus type 1 genome. *Journal of General Virology* **67**:1035-1048.

Valyi-Nagy, T., S.L. Deshmane, J.G. Spivak, I. Steiner, C.I. Ace, C.M. Preston, and N. Fraser. 1991. Investigation of herpes simplex virus type 1 (HSV-1) gene expression and DNA synthesis during the establishment of latent infection by an HSV-1 mutant, in1814, that does not replicate in mouse trigeminal ganglia. *Journal of General Virology* **72**:641-649.

Valyi-Nagy, T., M.U. Fareed, J.S. O'Keefe, R.M. Gesser, A.R. MacLean, S.M. Brown, J.G. Spivack, and N.W. Fraser. 1994. The herpes simplex virus strain 17+ γ34.5 deletion mutant 1716 is avirulent in SCID mice. *Journal of General Virology* **75**:2059-2063.

Valyi-Nagy, T., S.L. Deshame, J.G. Spivak, I. Steiner, C.I. Ace, C.M. Preston, and N.W. Fraser. 1991. Investigation of herpes simplex virus type 1 (HSV-1) gene expression and DNA synthesis during the establishment of latent infection by an HSV-1 mutant, in1814, that does not replicate in mouse trigeminal ganglia. *Journal of General Virology* **72**:641-649.

Vanhee-Brossollet, C., and C. Vaquero. 1998. Do natural antisense transcripts make sense in eukaryotes? *Gene* **211**:1-9.

Visalli, R.J., R.J. Courtney, and C. Meyers. 1997. Infection and replication of herpes simplex virus type 1 in an organotypic epithelial culture system. *Virology* **230**:236-243.

Vlazny, D.A. and N. Frenkel. 1981. Replication of herpes simplex virus DNA: localization of replication recognition signals within defective virus genomes. *Proceedings of the National Academy of Sciences* **78**:742-746.

Wagner, E.K., G. Devi-Rao, A.T. Dobson, Y.-F. Zhang, W. Flanagon, and J.G. Stevens. 1988. Physical characterisation of the herpes simplex transcript in neurons. *Journal of Virology* **62**:1194-1202.

Wagner, M.M. and W.C. Summers. 1978. Structure of the joint region and the termini of the DNA of herpes simplex virus type 1. *Journal of Virology* **27**:374-387.

Walz, M.A., R.W. Price, and A.L. Notkins. 1974. Latent infection with herpes simplex virus type 1 and type 2 viral reactivation in vivo after neuroectomy. *Science* **184**:1185-1187.

Wang, K., P.R. Krause, and S.E. Strauss. 1995. Analysis of the promoter and cis-acting elements regulating expression of herpes simplex virus type 2 latency associated transcripts. *Journal of Virology* **69**:2873-2880.

Warbrick, E., D.P. Lane, D.M. Glover, and L.S. Cox. 1995. A small peptide inhibitor of DNA replication defines the site of interaction between the cyclin dependent kinase inhibitor $p21^{Waf1}$ and proliferating cell nuclear antigen. Current Trends in Biology **5**:275-282.

Ward, P.L., D.E. Barker, and B. Roizman. 1996. A novel herpes simplex virus 1 gene, UL43.5, maps antisense to UL43 gene and encodes a protein which colocalizes in nuclear structures with capsid proteins. *Journal of Virology* **70**:2684-2690.

Wardlaw, A.C. 1987. Practical statistics for experimental biologists. Wiley-Interscience Publications.

Weber, P.C., S.J. Spatz, and E.C. Nordby. 1999. Stable ubiquitination of the ICP0R protein of herpes simplex virus type 1 during productive infection. *Virology* **253**:288-298.

Weber, P.C., M. Levine, and J.C. Glorioso. 1987. Rapid identification of nonessential genes of herpes simplex virus type 1 by mutagenesis. *Science* **236**:576-579.

Wigdahl, B.L., R.J. Ziegler, M. Snere, and F. Rapp. 1983. Herpes simplex virus latency and reactivation in isolated rat sensory neurons. *Virology* **127**:159-167.

Wigdahl, B.L., H.C. Isom, E. DeClerq, and F. Rapp. 1982. Activation of herpes simplex virus type 1 genome by temperature sensitive mutants of herpes simplex virus type 2. *Virology* **116**:468-479.

Wigdahl, B.L., H.C. Isom, and F. Rapp. 1981. Repression and activation of the genome of herpes simplex viruses in human cells. *Proceedings of the National Academy of Sciences* **78**:6522-6526.

Wilcox, C.L. and E.M. Johnson, Jr. 1988. Characterization of nerve growth factor-dependent herpes simplex virus latency in neurons in vitro. *Journal of Virology* **62**:393-399.

Wildy, P., W.C. Russell, and R.W. Horne. 1960. The morphology of the herpes virus. *Virology* **12**:204-222.

Willey, D.E., M.D. Trousdale, and A.B. Nesburn. 1984. Reactivation of murine latent HSV infection by epinephrine iontophoresis. *Investigative Ophthalmology and Visual Science* **25**:945-950.

Williams, K. 1999. The role of glycoprotein C in adsorption of herpes simplex virus type 1 (HSV-1). Master's thesis University of Glasgow.

Williams, R.K. and S.E. Straus. 1997. Specificity and affinity of binding of herpes simplex virus type 2 glycoprotein B to glycosaminoglycans. *Journal of Virology* **71**:1375-1380.

Willis, S.H., A.H. Rux, C. Peng, J.C. Whitbeck, A.V. Nicola, H. Lou, W.F. Hou, L. Salvador, R.J. Eisenberg, and G.H. Cohen. 1998. Examination of the kinetics of herpes simplex virus gD binding to the herpesvirus entry mediator, using surface plasmon resonance. *Journal of Virology* **72**:5937-5947.

Wu, N., S.C. Watkins, P.A. Schaffer, and N.A. DeLuca. 1998. Prolonged gene expression and cell survival after infection by a herpes simplex virus mutant defective in the immediate early genes encoding ICP4, ICP27, and ICP22. *Journal of Virology* **70**:6358-6369.

Wu, S., K.U. Kumar, and R.J. Kaufman. 1998. Identification and requirement of three ribosome binding domains in ds-RNA-dependent protein kinase (PKR). *Biochemistry* **37**:13816-13826.

Wu, T-T., Y.-H. Su, T.M. Block, and J.M. Taylor. 1998. Atypical splicing of the latency associated transcripts of herpes simplex type 1. *Virology* **243**:140-149.

Yang, Y., F.A. Nunes, K. Berenois, E. Goczol, J.F. Engelhardt and J. Wilson. 1994. Inactivation of E2a in recombinant adenovirus improves the prospect for gene therapy in cystic fibrosis. *Nature Genetics* 7:362-369.

Yeh, L. and P.A. Schaffer. 1993. A novel class of transcripts expressed with late kinetics in the absence of ICP4 spans the junction between the long and short segments of the herpes simplex virus type 1 genome. *Journal of Virology* **67**:7373-7382.

Yin, J., M.G. Kaplitt, A.D. Kwong, and D.W. Pfaff. 1998. In situ PCR for in vivo detection of foreign genes transfected into rat brain. *Brain Research* **783**:347-354.

Yoshikawa, T., L.R. Stanberry, N. Bourne, and P.R. Krauese. 1996. Downstream regulatory elements increase acute and latent herpes simplex virus type 2 latency-associated transcript expression but do not influence recurrence phenotype or establishment of latency. *Journal of Virology* **70**:1535-1541.

Youdim, M.B.H. and P. Reiderer. 1997. Understanding Parkinson's Disease. *Scientific American* 38-45.

Youssouffian, H., S.M. Hammer, M.S. Hirsch, and C. Mulder. 1982. Methylation of the viral genome in an in vitro model of herpes simplex virus latency. *Proceedings of the National Academy of Sciences* **79**:2207-2210.

Yuhasz, S.A. and J.G. Stevens. 1993. Glycoprotein B is a specific determinant of herpes simplex virus type 1 neuroinvasiveness. *Journal of Virology* **67**:5948-5954.

Zabolotny, J.M., C. Krummenacher, and N.W. Fraser. 1997. The herpes simplex virus type 1 2.0-kilobase latency-associated transcript is a stable intron which branches at a guanosine. *Journal of Virology* 71:4109-4208.

Zelus, B.D., R.S. Stewart, and J. Ross. 1996. The virion host shutoff of herpes simplex virus type 1: messenger ribonucleolytic activity in vitro. *Journal of Virology* **70**:2411-2419.

Zhan, Q.M., K.A. Lord, I. Alamo, M.C. Hollander, F. Carrier, D. Ron, K.W. Kohn, B. Hoffman, D.A. Liebermann, and A.J. Fornace. 1994. The GADD and MYD genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth. *Molecular and Cellular Biology* **14**:2361-2371.

Zhang, X., H. O'Shea, C. Entwisle, M. Bousnell, S. Efstathiou, and S. Inglis. 1998. An efficient selection for packaging herpes simplex virus amplicons. *Journal of General Virology* **79**:125-131.

Zhong, L. and G.S. Hayward. 1997. Assembly of complete, functionally active herpes simplex virus DNA replication compartments and recruitment of associated viral and cellular proteins in transient cotransfection assays. *Journal of Virology* **71**:3146-3160.

Zhu, J. L. Zhang, U.K. Hanisch, P.L. Felgner, and R. Reszka. 1996. A continuous intracerebral gene delivery system for in vivo liposome-mediated gene therapy. *Gene Therapy* **3**:472-476.

Zhu, Z., W. Cai, and P.A. Schaffer. 1994. Cooperativity among herpes simplex virus type 1 immediate early regulatory proteins: ICP4 and ICP27 affect the intracellular localization of ICP0. *Journal of Virology* **68**:3027-3040.

Zsak, L., Z. Lu, G.F. Kutish, J.G. Neilan, and D.L. Rock. 1996. An African swine fever virus virulence-associated gene *NL-S* with similarity to the herpes simplex virus ICP34.5 gene. *Journal of Virology* **70**:8865-8871.

Zsak, L., F. Zuckermann, N. Sugg, and T. Bunporat. 1992. Glycoprotein I of pseudorabies virus promotes cells fusion and virus spread via direct cell to cell transmission. *Journal of Virology* **66**:2316-2325.