CHARACTERIZATION OF THE ROLE OF ICP34.5 AND ORF P IN THE HSV-1 LIFECYCLE

by

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Summary

The work carried out in this thesis comprises two main sections: i) characterisation of the role of ICP34.5 and ORF P in HSV-1 replication; and ii) identification of cellular and viral proteins which interact with ORF P.

A complication in the analysis of the role of ICP34.5 in the HSV-1 lifecycle is the presence of overlapping antisense genes, ORF O/P, which are also deleted in ICP34.5 negative mutants. In 1991, MacLean, A. *et al,* isolated 1716, a HSV-1 17^+ ICP34.5/ORF O/P deletion mutant. This mutant has been demonstrated to have specific characteristics both *in vitro* and *in vivo*. To attribute characteristics which were originally attributed solely to ICP34.5 to each of the genes, a number of HSV-1 recombinant viruses that express ICP34.5 and ORF O/P independently were constructed, purified and characterised. In all recombinant viruses an expression cassette was inserted into the 1716 UL43/UL43.5 locus containing the gene of interest under the control of the HSV-1 gD promoter and *β galactosidase* in the opposite orientation. Recombinants 1622 and 1625 express ICP34.5 and in both 1624 and 1624.5 ORF P is inserted in UL43 and UL43.5, respectively. Additionally, in 1625 ORF P is inserted in the non essential US5 gene.

Western blotting analysis of 1622 and 1625 infected BHK cells demonstrated at least an eight-fold increase in ICP34.5 levels compared to wild type 17^+. In the recombinants 1624, 1624.5 and 1625 no ORF P was detected, suggesting that these recombinants express low levels of ORF P, as the rabbit polyclonal sera used exhibited problems of detection with low amounts of ORF P from 17^+ but not from the overproducing ts mutant in ICP4, *ts*K. Also, it is possible that the recombinants do not express ORF P.

As we were not able to detect ORF P from our recombinant viruses, we proceeded to look for its RNA. A band with the size expected for ORF P RNA of about 700 bp was detected in 1624, 1624.5, and 1625. The 700 bp ICP34.5 transcript was also detected in 1622.

Multicycle replication kinetics of the recombinants was analysed in three cell lines: BHK, 3T6 and SK-N-SH cells. In BHK cells all recombinant viruses with the exception of 1624 exhibited a similar growth pattern to 17^{+}. In stationary state 3T6 cells, 17^{+} grew well whereas 1716, 1624 and 1624.5 failed to grow. 1622 and 1625 also grew, although platauing at a maximum titre of 10^{1} to 10^{2} fold lower than 17^{+} with 1625 slightly more impaired than 1622. In SK-N-SH cells, 17^{+} grew well. This cell line is semi permissive for 1716 and 1624.5, exhibiting limited growth with a maximum titre 10^{4} fold lower than 17^{+}. In agreement with its impaired growth in BHK cells, 1624 grew consistently 10 fold poorer than 1716 and1624.5. Both 1622 and 1625 grew similarly to wild type virus. Restoration of ICP34.5 function in both 1622 and 1625 was confirmed *in vitro* by the prevention of host cell protein synthesis shutoff in SK-N-SH cells. However, restoration of ORF P fails to prevent host cell protein synthesis shutoff. ICP34.5 seems to confer two functions of growth and maintenance of protein synthesis, with no apparent function in the *in vitro* viral lifecycle for ORF O/P.

An alternative way to analyse the function of ORF O/P is to identify those cellular and viral proteins with which they interact. This was carried out for ORF P using GST pulldowns. A major problem faced in carrying out pulldown experiments was the lack of expression of full length GST-ORF P fusion protein despite attempts to try and optimise this. A number of specific interacting proteins were detected. These proteins include at

least two viral and possibly one cellular protein with sizes of 27, 40 (viral) and 47 KDa (cellular). We then tried to identify these interacting cellular and viral gene proteins. Due to the previous association of ORF P with proteins involved in posttranscriptional control and its suggested role in HSV splicing, the pulldowns were screened with ten available antibodies against cellular proteins with a role in posttranscriptional regulation of an approximate molecular weight to those identified and antibodies against HSV ICP27 and thymidine kinase. Of these, only SC35 was demonstrated to interact specifically with ORF P. We demonstrated that both SC35 and ORF P are located in the nucleus and tried to determine if ORF P interacts with SC35 *in vivo* by carrying out immunoprecipitation of infected BHK cell extracts. Unfortunately, SC35 was non-specifically coimmunoprecipitated by the ORF P antisera and we were unable to immmunoprecipitate SC35 with the SC35 antibody. Thus we were unable to determine by coimmunoprecipitation if SC35 and ORF P interact *in vivo*.

ORF P insertion into the virus has not been conclusively proven and there is no evidence of expression at all. Therefore the conclusions made with regard to growth curves etc may be incorrect. 1625 may be (are) unstable due to multiple insertion of the gD promoter. The recombinants need to be properly characterised to conclude if the actually contain/express ORF P by using purified ORF P fragment as a probe.

CONTENTS

Chapter 5: Optimization of GST-P Pulldown experiments

Chapter 6: Identification of proteins which interact with ORF P

Chapter 7: Discussion

List of figures and tables **After Page**

Figures

Tables

Abbreviations

Ab	antibody
Amp	ampicillin
APS	ammonium persulphate
BHK	baby hamster kidney cells
bp	base pairs
BSA	bovine serum albumin
^0C	degrees Celsius
$CaPO_4$	calcium phosphate
$C_2H_4O_2$	acetic acid
CKII	casein kinase II
CO_2	carbon dioxide
cpe	cytopathic effect
CPSF	cleavage and polyadenylation specificity factor
CS	newborn calf serum
d	day
dH_2O	distilled water
DMSO	dimethyl sulphoxide
DMEM10	Dulbecco's modified Eagle's medium with 10% foetal calf serum
DNA	deoxyribonucleic acid
DRG	dorsal root ganglia
E	early
E.coli	*Escherichia coli*

EDTA	ethylenediaminetetra acetic acid
Emet/5C2	Eagle's medium with reduced methionine and 5% serum
ETC10	Eagle's media with 10% newborn calf serum
EtBr	ethidium bromide
EtOH	ethanol
FCS	foetal calf serum
h	hour (s)
HCl	hydrochloric acid
H_2O	water
hnRNP	heterogenous nuclear ribonucleoprotein
HRP	horseradish peroxide
HSV-1	herpes simplex virus 1
HSV-2	herpes simplex virus 2
ICP	infected cell protein
IE	immediate-early
IPTG	isopropyl-β-D-thiogalactoside
KAc	potassium acetate
Kb	kilobase pair (s)
KDa	kilodalton
$KHCO_3$	potasium bicarbonate
Lac Z	β-galactosidase
LAT	latency associated transcript
M	molar

mA	milliamp
Mab	monoclonal antibody
MeOH	methanol
mg	milligram
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
min	minute
ml	millilitre
mM	millimolar
mm	millimetre (s)
MOI	multiplicity of infection
mRNA	messenger RNA
NaAc	sodium acetate
NaOH	sodium hydroxide
NaP	sodium phosphate
ng	nanograms
nm	nanometre
NP40	Nonidet P-40
OD	optical density
PBS-A	phosphate buffered saline
PCR	polymerase chain reaction
pi	post infection
Pol	polymerase

Poly (A)	polyadenylated
Propan-2-ol	isopropanol
RNA	ribonucleic acid
RNaseA	Ribonuclease A
rpm	revolution per minute
RT	room temperature
s	second
SDS	sodium dodecyl sulphate
SDS-PAGE	denaturing polyacrylamide gel electrophoresis
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein
SV40	simian virus 40
TEMED	N,N,N,N ,-tetramethylethylene diamine
TG	trigeminal ganglia
Tris	tris (hydroxymethyl) aminomethane
U	unit (s)
UV	ultra violet light
µl	microlitre
µm	micromole
V	voltage
vhs	virion host shut off
wt	wild type
w/v	weight/volume (ratio)

w/w	weight/ weight (ratio)
v/v	volume/volume (ratio)
X-gal	5-chloro-4-bromo-3-indoyl-2-D-galatoside

1.1 The Herpesviridae family

This family are double stranded linear DNA enveloped viruses which are widely distributed in nature. So far, 112 herpesviruses have been identified which infect mainly vertebrates especially mammals, including cattle, pigs and man (Comps and Cochennec, 1993; Roizman and Sears, 1993).

1.2 Biological properties

The following biological properties are shared by all herpesviuses. (i) They encode a large array of enzymes involved in nucleic acid metabolism, DNA synthesis and possibly processing of proteins. (ii) They encode several transacting factors which regulate the temporal expression of viral genes controlling the phase of infection (Cann, 1993). (iii) Both the synthesis of viral DNAs and the assembly of capsids occur in the nucleus. (iv) Production of infectious progeny virus invariably leads to cell death. (v) Infection leads to establishment of a latent infection in their natural host (Roizman and Sears, 1996).

1.3 Classification:

The current classification of *herpesviridae* is on the basis of their biological properties and divides them into three subfamilies, the *alphaherpesvirinae*, the *betaherpesvirinae* and the *gamaherpesvirinae* (Roizman *et al.*, 1981).

1.3.1 *Alphaherpesvirinae*

Members of this subfamily have a variable host range, relatively short replication cycle, rapid spread in culture, efficient destruction of infected cells and capacity to establish latent

infections primarily, but not exclusively, in sensory ganglia of the peripheral nervous system (PNS). Members of this subfamily include herpes simplex virus types 1 and 2 (HSV-1/2) and varicella zoster virus (VZV).

1.3.2 *Betaherpesvirinae*

These viruses are slow growing and may be cytomegalic (massive enlargment of infected cells), have a variable host range, a long reproductive cycle and infect cells slowly in culture and become latent in secretory glands and kidneys. Examples include human cytomegalovirus (HCMV).

1.3.3 *Gammaherpesvirinae*

These viruses infect and become latent in lymphoid cells. Viruses have tropism for either B or T lymphocytes where lytic or latent infection is frequently found without production of infectious progeny. Epstein-Barr virus (HHV5) infects B cells and is classified in the lyptocryptovirus genus. Human herpes virus 8, Kaposi's sarcoma-associated herpesvirus, infects T cells and is classified in the rhadinovirus genus (Greensill *et al.*, 2000).

1.4 Morphology

The virion has a similar morphology in all herpesviruses which led to their classification into one family. The size of the virion is variable ranging from 120 nm to 300 nm in diameter. (Fig.1.1). The virion consists of a core containing a double stranded linear DNA genome encased in an icosohedral capsid (Wildy *et al.*, 1960). An amorphous, sometimes asymmetric, tegument surrounds the icosohedral capsid (Roizman and Furlong, 1974).

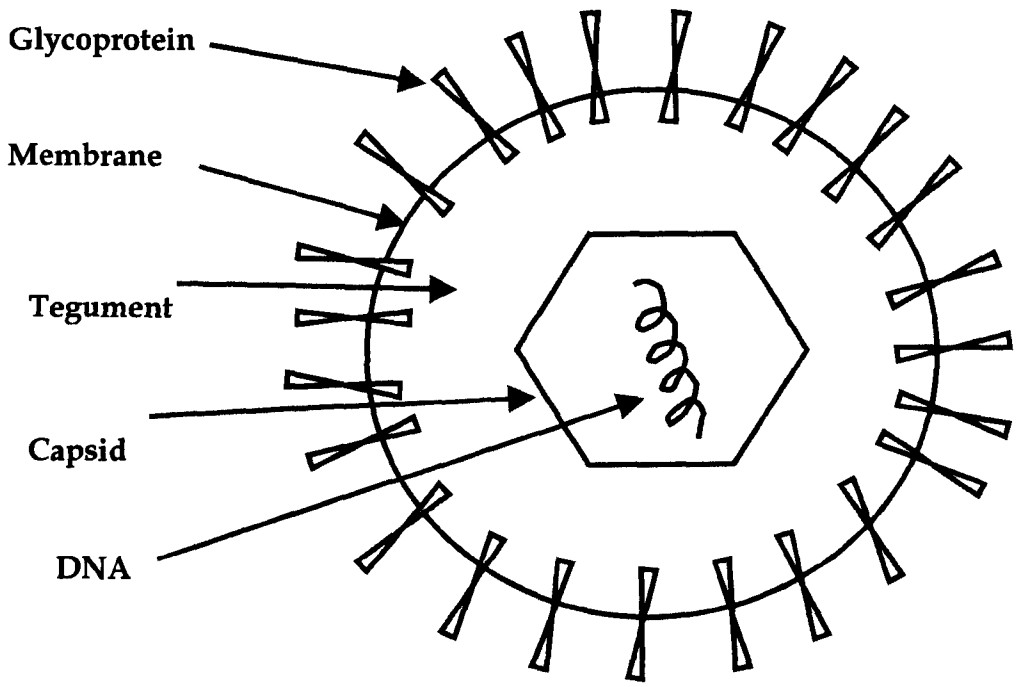


Figure 1.1 Structure of HSV-1 virion

Representation of virion morphology of herpesviridae, showing the outer envelope layer containing glycoprotein molecules, the tegument and icosahedral capsid surrounding the central core of double stranded DNA (Adapted from Rixon, 1993).

Surrounding the tegument is an envelope containing viral glycoprotein spikes on its surface (Spear and Roizman, 1972). The virion structure is reviewed in Rixon (1993).

1.4.1 Core

Herpesvirus DNAs are double stranded linear molecules, which can circularise immediately upon release into the host cell nuclei. DNA size and base composition are variable between different herpesviruses. DNA size ranges between 120 kbp and 230 kbp and base composition varies from 31% to 75% G+C. Genomic size varies within all herpesviruses to a minimal but significant extent. Both variation in the copy number of terminal and internal repeated sequences and spontaneous deletions contribute to this variation (Roizman, 1996).

Based on the presence and location of reiterated sequences, herpesviruses have been split into the six groups A-F (Davison, 1993; Roizman, 1996) (Fig.1.2).

1.4.2 Capsid

The icosohedral capsid consists of 150 hexameric and 12 pentameric capsomeres. The hexomers occupy the faces and the edges of the capsid, while the pentamers are positioned at the vertices (Wildy *et al.*, 1960; Zhou *et al.*, 1994). The hexomer consists of six molecules of VP5, the major capsid protein, and the pentomer VP5 and 80-100 copies of VP26. A triplex structure made up of VP19 and VP23 co-ordinates the hexons in 3-fold symmetry and a fifth capsid protein VP24 projects into the interior (Rixon, 1993; Zhou *et al.*, 1994). Studies of HSV-1 capsid assembly have become more amenable with the development of an *in vitro* assembly system (Newcomb *et al.*, 1994) and a recombinant baculovirus assembly

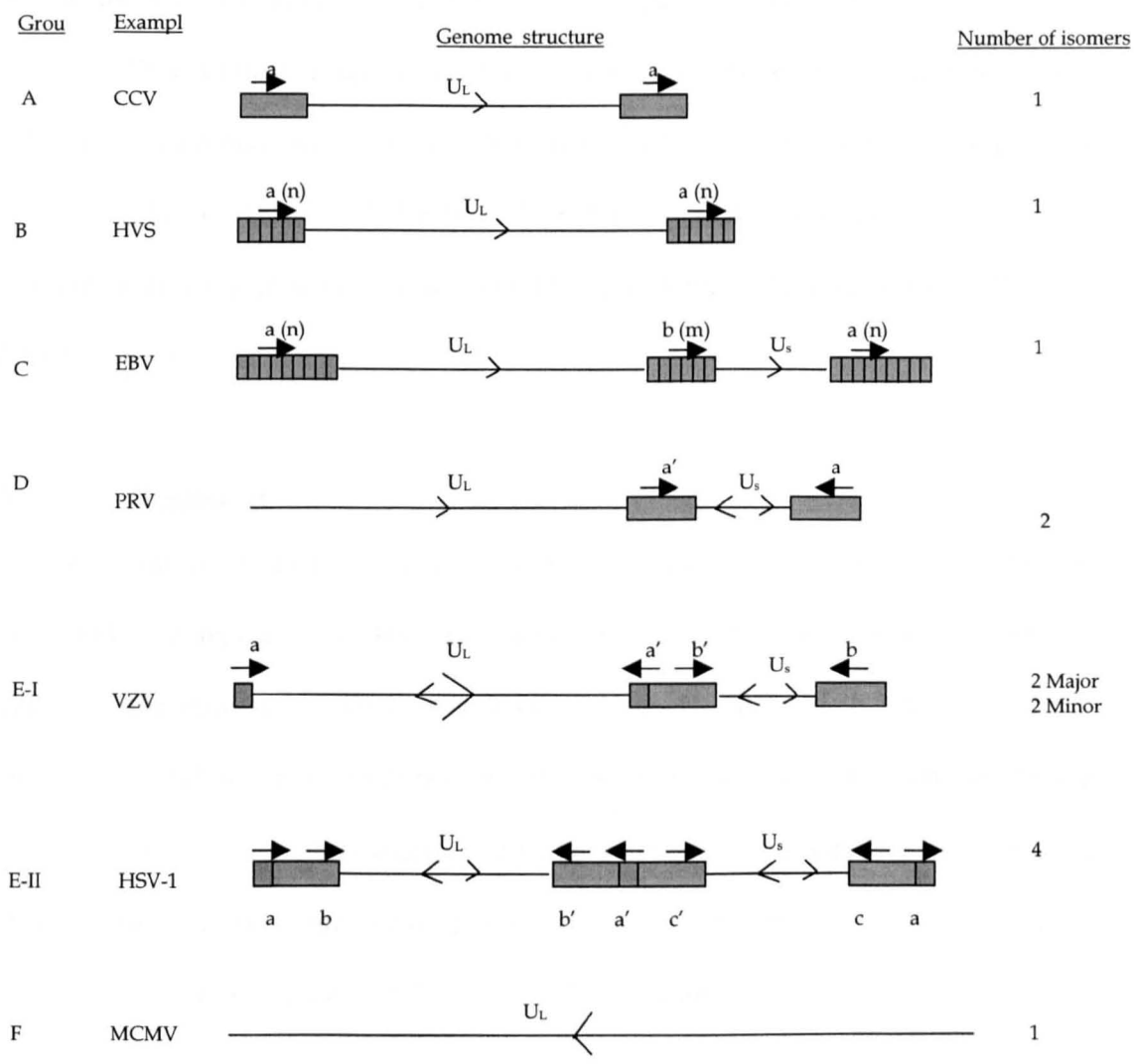


Figure 1.2 Schematic diagram of the sequence arrangments in the six classes of herpesviridae

Single lines represent unique sequences. Repeats of >1kb are represented by open boxes and small reiterated sequences are represented by lined boxes. Arrows above boxes denote whether repeats are direct or inverted. Arrows in the unique sequences indicate their possible orientation.

system in insect cells (Tatman *et al.*, 1994). Three types of capsids A, B, and C can be isolated from HSV-1 infected cells. A and C capsids are similar in protein content but only C capsids contain a genomic equivalent of DNA. The B capsid is composed of seven proteins VP5, VP19c, VP22a, VP21, VP23, VP24, and VP26 (Gibson and Roizman, 1972). A and C capsids have a similar protein content to B capsids but lack the scafolding proteins VP21 and VP22a (Rixon, 1993).

1.4.3 Tegument

The virion tegument is an electron dense material located between the capsid and the envelope and is composed of at least 20 distinct viral proteins (Aurelian *et al.*, 1989). Its structure and the process by which it is acquired is poorly understood. Viral structural proteins known not to be a component of the capsid or envelope are assigned to the tegument (Rixon, 1993). Certain tegument proteins such as *α-trans* inducing factor (α-TIF, VP16 or UL48) and the virion associated host shut-off (vhs or UL41) are involved in initiation of infection (Batterson and Roizman, 1983; Read and Frenkel, 1983).

1.4.4 Envelope

The virion envelope has a trilaminar appearance in electron micrographs (Epstein, 1962) and is acquired from host cell membranes probabely the golgi or golgi derived membranes (van Genderen *et al.*, 1994). The glycoprotein spikes contained in the envelope are approximately 8 nm long and vary in number and relative amount between the different herpesviruses. HSV encodes 12 glycoproteins of which all but gL are found in the viral envelope (McGeoch *et al.*, 1988; Barnett *et al.*, 1992).

The glycoproteins are named gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM and gN (McGeoch *et al.*, 1988; Spears, 1993). Glycoprotein B, gD, gH, gK and gL, are essential for virus replication *in vitro* (Cai *et al.*, 1988; Ligas and Johnson, 1988; Roop *et al.*, 1993; Hutchinson and Johnson, 1995). Six HSV-1 glycoproteins are known to form stable complexes: gB and gC form homo-oligomers and gE, gH, gI and gL form the hetero-oligomers gE/gI and gH/gL (Claesson-Welsh and Spear, 1986; Johnson and Feenstra, 1987; Hutchinson *et al.*, 1992; Handler *et al.*, 1996). In addition, gM and gN form a complex in several herpesviruses (Jons *et al.*, 1998). These glycoproteins play a role in several parts of the viral life cycle namely attachment , penetration, cell to cell spread, cell fusion, envelopment and egress and also in immune invasion. Both gB and gC are thought to have a role in viral adsorption (Herold *et al.*, 1994). Glycoprotein B (Cai *et al.*, 1988), gD (Ligas and Johnson, 1988) and gH/gL (Roop *et al.*, 1993) have been shown to be essential for virus penetration. Glycoprotein K may play a role in both envelopment and subsequent egress of infectious particles (Hutchinson and Johnson, 1995). Glycoprotein B, gD, gH/gL and gE/gI have also shown to be involved in cell-cell spread.

1.5 Human herpesviruses

Eight herpesviruses infect humans as their primary host and cause a wide variety of diseases ranging from minor lesions to viral encephalitis. These viruses may cause severe infections in immunocompromised individuals (Whitley, 1996). The human herpesviruses and the diseases caused are shown in table 1.1.

Herpesvirus	Class	Illness
Herpes simplex virus-1 (HSV-1) (HHV-1)	α	Causes 80-95% of oral lesions (cold sores), 30- 50% of genital lesions, and rarely: conjunctivitis, herpetic whitelaw, keratitis, and encephalitis.
Herpes simplex virus (HSV-2) (HHV-2)	α	Causes 5-20% of oral lesions (cold sores), 50- 70% of genital lesions (life threatening for infected neonates) and rarely: conjunctivitis, herpetic whitelaw, keratitis, and encephalitis.
Varicella zoster virus (VZV) (HHV-3)	α	Primary infection: chicken pox (rash) appears 14-15 days post-infection, accompanied by fever. Reactivation: shingles; lesions appear at the relevant dermatone often accompanied by extreme pain which can last for months after lesions have healed.
Epstein Barr virus (EBV) (HHV-4)	γ	Primary infection in children is often asymptomatic. In older children and adults is infectious mononucleosis (glandular fever). Associated with Burkitt's lymphoma and nasopharyngal carcinoma.
Human cytomegalovirus (HCMV)(HHV-5)	β	Primary infection: enlargement and fusion of macrophages. Often asymptomatic though can be fatal especially in new borns. Infection is problematic in immunocompromised individuals where symptoms include gastro-enteritis and retinitis. A major cause of congenital birth defects.
Human herpesvirus 6 (HHV-6)	β	Infants rash, exanthem subitum.
Human herpesvirus 7 (HHV-7)	β	Febrile illness.
Kaposi's sarcoma associated herpesvirus (KSHV) (HHV-8)	γ	Associated with Kaposi's sarcoma, a vasculated nodular skin lesion.

Table 1.1 Human herpesviruses. The eight herpesviruses known to infect humans, classification and the diseases associated with them.

1.6 Herpes simplex virus (HSV)

Both HSV-1 and HSV-2 belong to the subfamily *alphaherpesvirinae* and the genus simplexvirus. HSV-1 and HSV-2 have 47%-50% sequence homology and their genetic maps are co-linear. Their differences are in restriction endonuclease (RE) cleavage sites, apparent size of viral proteins, antigenic determinants, plaque size and growth in cultured cells (Roizman and Sears, 1996; Roizman et al., 1984). Work in this thesis considers infection with the HSV-1 Glasgow strain 17^+ (Brown *et al.*, 1973).

1.7 Clinical features

This virus is predominantly associated with oral lesions (cold sores) and also with some cases of genital herpes lesions (Kinghorn, 1993). Lifelong latency is established in the sensory ganglion of the PNS. HSV-1 occasionally causes serious diseases such as herpetic whitelaw, eczema, keratoconjunctivitis and encephalitis (Whitley, 1996).

1.8 Genome

The HSV-1 genome is 152 kbp in size, encodes about 84 polypeptides, and has an unusually high G+C content (McGeoch *et al.*, 1985, 1986, 1988; Perry and McGeoch, 1988). It consists of two covalently linked unique regions, the unique long (U_L) and unique short (U_S). The U_L region is 107.9 kbp and the U_S region 13 kbp long. The unique sequences are flanked by inverted repeats, R_L (9 kbp) and R_S (6.5 kbp) (McGeoch *et al.*, 1988). R_L and R_S are not related in sequence apart from the "a" sequence (400 bp) located at the genome termini. One copy of the “a” sequence is located at the S terminus, whereas the L terminus may have more than one copy. The “a” sequence is also present at the L-S junction as an

inverted repeat and again can be present as more than one copy (Wagner and Summers, 1978). The two unique sequences can invert relative to each other via the repeats such that a population of HSV DNA molecules consists of four equimolar isomers (Fig.1.3).

1.9 Lifecycle

The HSV-1 lifecycle consists of both lytic and latent phases.

1.10 Lytic replication

Here the virus enters a host cell, replicates in it and eventually releases progeny virions into the surrounding medium (Roizman and Sears, 1996). This cycle consists of attachment, entry, gene expression, DNA replication, capsid assembly, maturation and egress, respectively, resulting in the death of the host cell. The viral lifecycle is approximately 18-20 h in fully permissive tissue culture cells (Roizman and Sears, 1996). Two types of HSV related particles are produced in infected cells: (i) the infectious virion; and (ii) L-particles which lack the nucleocapsid and viral DNA and are non infectious (Szilagyi and Cunningham, 1991).

1.10.1 Adsorption/penetration

Attachment/entry are the most important stages of viral infection. During attachment, glycoproteins on the virion envelope (1.4.4) interact with specific cellular receptors which leads to binding to the cell surface.

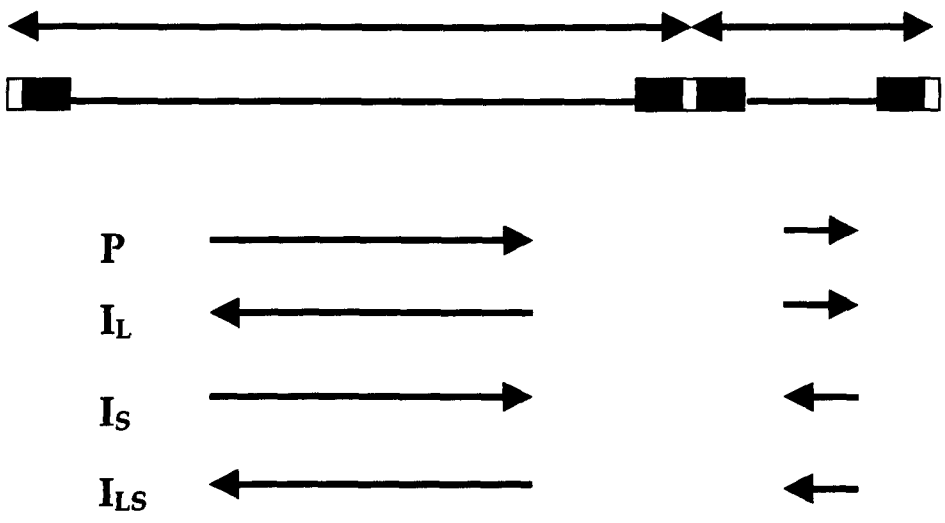


Figure 1.3 Schematic diagram of HSV-1 genome

The unique DNA elements, U_L and Us are covalently linked and flanked by inverted repeats (boxes). The "a" sequence is represented by a white box and is present at each end of the genome and in an inverted orientation at the L-S junction. The L and S regions can invert relative to each other forming four isomers (Hayward *et al.*, 1975). The orientation of the 4 isomers is shown by the arrows.

1.10.2 Gene expression

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: immediately early (IE), early (E), and late (L) (Roizman *et al.*, 1978; Clements *et al.*, 1977).

1.10.3 IE genes

Expression of IE genes can procced without *de novo* viral protein synthesis, and is stimulated by the action of the virion protein α -TIF (also known as Vmw 65 or VP16). Four of the five IE genes products are responsible for the regulated expression of E and L gene. These are ICP22, ICP4, ICPO and ICP27. The last 3 in particular are key regulators. The fifth IE gene, ICP47 is involved in regulating the host immune response (1.11.3).

(i) ICP4

ICP4 is an essential regulator and transactivator gene and is required for expression of E and L genes. It binds to DNA through a strong consensus binding site (Faber and Wilcox, 1986), but subsequent reports have revealed numerous HSV-1 binding sites which do not correspond to the previously identified consensus sequences (Michael and Roizman, 1989). The various forms of ICP4 differ in their affinity of DNA binding (Michael *et al.*, 1988). Dephosphorylated ICP4 is reported to bind to IE promoters, while the phosphorylated form binds to E and L promoters (Papavassiliou *et al.*, 1991). Interaction of ICP4 with TATA binding protein and the transcription complex is further evidence of ICP4 involvement in transactivation of HSV-1 genes (Smith *et al.*, 1993). Binding of ICP4 at its own transcription initiation site leads to autoregulation (Roberts *et al.*, 1988). As consensus ICP4 binding sites

are also found in the promoter of ICPO, ICP4 may also downregulate expression of ICPO by binding to its promoter (Faber and Wilcox, 1986).

(ii) ICPO

ICPO is a non-essential protein but defects in this protein delay expression of E and L genes and impair viral replication (Chen and Silverstein, 1992). From transient expression experiments, ICPO is thought to enhance the activity of ICP4, as well as possibly transactivating gene expression in its own right (Everett, 1984; O'Hare and Hayward, 1985; Quinlan and Knipe, 1985).

(iii) ICP22

This gene appears to be nonessential for HSV-1 infection. Deletion of ICP22 has no effect on viral DNA synthesis. Viral mutants in this gene replicate in Vero and Hep-2 cells as effectively as the parent virus. However in BHK, RAT-1 cell lines and human embryonic lung cells the plating efficiency is reduced and yield is multiplicity dependent. Moreover in these cells E protein synthesis is delayed and the expression of L proteins and the number of capsids detected in the infected cells is reduced (Sears *et al.*, 1985).

(iv) ICP27

ICP27 is an essential IE protein which is required for HSV-1 late gene expression at both the transcriptional and posttranscriptional level.

At the transcriptional level, ICP27 up or down regulates both viral and cellular mRNA transcription either in conjunction with ICP4 or by acting upon it and a physical interaction of these two proteins occurs (Pangiotidis *et al.*, 1997). Other evidence suggests that ICP27 can inhibit the action of proteins which are involved in transcription (Birney *et al.*, 1993).

At the posttranscriptional level, ICP27 has a role in the processing of pre-mRNA and nucleocytoplasmic transport of viral intronless transcripts. ICP27 cause unspliced pre-mRNAs to accumulate in the nucleus and reduces cellular mRNA levels (Hibbard and Sandri-Goldin, 1995). ICP27 shuttles from the nucleus to the cytoplasm (Phelan and Clements, 1997) and has a role in the export of viral mRNAs. Sandri-Goldin (1998) suggested that ICP27 binds and shuttles with all classes of intronless HSV-1 transcripts. Recent evidence suggests that ICP27-mediated viral RNA export requires cellular mRNA export factor, REF and TAP/NXF1 (Koffa *et al.*, 2001).

Co-localization suggests that ICP27 interacts with one or more of the components of the splicing machinery. This is evidenced by immunoprecipitation of ICP27 by anti-SM antibody (Sandri-Goldin and Hibbard, 1996). Recently, it has been demonstrated that ICP27 interacts with the essential splicing factor SAP145 which leads to inhibition of splicing. This inhibition could facilitate the nuclear export of viral mRNA and contribute to host cell shuttoff (Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994; Bryant et al., 2001).

(v) ICP47

This non-essential protein is not involved in the regulation of viral gene expression but down regulates the host immune response (1.11.3).

1.10.4 Induction of E genes

The sequence elements for expression of E genes consist of a TATA box, a cap site (McKnight and Tijan, 1986), and binding sites for cellular factors. For example, the HSV-1 thymidine kinase (TK) gene contains binding sites for SP1 and CCAAT binding protein (CBP), clustered upstream of the TATA box (Pande *et al.*, 1998). ICP4 is almost certainly involved in transactivation of E genes but the mechanism is not clear Viral mutants in ICP4 do not produce any E genes (Dixon and Schaffer, 1980; Watson and Clements, 1980). The binding of ICP4 to E gene promoters requires cellular factors and is determined by both its concentration and state of phosphorylation (Papacassiliou *et al.*, 1991). It has been suggested that ICP4 interacts with cellular factors both enhancing their binding and requiring them for its own stable DNA binding.

1.10.5 Induction of L genes

The control of L gene expression is the least well understood and is likely to vary from gene to gene. Analyses of several genes have suggested that sequences required for efficient expression include the TATA box, and extend into the 5' transcribed non-coding domain (Dennis and Smiley, 1984; Everett and Dunlop, 1984; Johnson *et al.*, 1986; Guzowski and Wagner, 1993; Wagner *et al.*, 1998). Additional sequences downstream from the TATA box

appear to be required for expression of L genes (Greaves and O'Hare, 1989; Wagner *et el.*, 1998).

Embedded in both the promoter and 5' non-coding regions are ICP4 binding sites (Michael *et al.*, 1988). The role of these sites and ICP4 involvement is unclear. In infected cells, ICP4 is required but not sufficient for efficient and correctly regulated expression of L genes. A key observation in understanding expression of L genes has been the observation that deletions in the 5' transcribed non-coding domain of a L gene may cause it to be expressed as an IE gene (Roizman and Sears, 1996). This can be explained in two ways; either the non-coding domain contains a site that allows binding of a transactivating repressor or the region may form a secondary structure which affects expression of L genes. Either way, the data suggests that the 5' transcribed region binds a repressor of some sort. This repressor must preclude expression of L genes under conditions which allow expression of IE and E genes and bind an activator which allows their expression in the absence of the repressor. As L gene expression cannot occur in the absence of viral DNA synthesis, it has been suggested that replication removes the block and/or produces/modifies the transactivating factor. Alternatively removal of the block may be essential to DNA replication.

1.10.6 DNA replication

DNA circulizes in the nucleus and undergoes initial *theta* replication, then DNA replication occurs by a rolling circle mechanism leading to the accumulation of head to tail DNA concatemers at specific sites, termed viral replication sites, in the nucleus which are defined by host cell nuclear architecture (de Bruyn Kops and Knipe., 1994). The DNA replication

mechanism is conserved in all alphaherpesviruses. In HSV-1 a set of seven viral genes are necessary and sufficient for replication (Challberg, 1991).

Viral replication typically starts at about 2 h p.i and peaks between 8-10 h post infection. It is initiated from one of three origins (ori) of replication, one in the middle of U_L and the two others in TR_S and IR_S, via binding of the ori binding protein UL9. The helicase/primase complex (UL5, UL8 and UL52 gene products) subsequently associates with the UL9/ori complex and the hydrolysis of ATP creates an initiation bubble which permits association of the DNA polymerase and DNA binding protein complex (UL30/UL42). DNA synthesis is continuous along one strand and discontinuous along the lagging strand with the major DNA binding protein UL29 maintaining the growing forks.

Other virally encoded enzymes are also involved in nucleic acid metabolism; e.g. thymidine kinase (TK), ribonucleotide reductase (RR), uracil-DNA glycosylase and dUTPase (Roizman and Sears, 1996). None of these are essential as they can be complemented by host cell factors except in non dividing cells such as neurons (1.13).

1.10.7 Transcription

HSV-1 genes are transcribed by the host cell RNA polymerase II (RNA Pol II) (Costanzo *et al.*, 1977). Like the cellular mRNAs, viral mRNAs are capped, methylated and polyadenylated. Methylation is more common in E transcripts than L ones (Hann *et al.*, 1998). Use of alternative polyadenylation sites may be a mechanism of regulating gene expression (McGregor *et al.*, 1996). Unlike cellular mRNAs, most genes lack introns with

only four viral transcripts ICPO, ICP27, ICP47 and UL15 being spliced. Transcripts sharing 5' and particularly 3' termini have been described (Wagner, 1985) and several genes appear to have multiple transcription initiation sites (Murchie and McGeoch, 1982).

1.10.8 Capsid assembly

Following DNA replication, late gene expression and the production of all the necessary components, capsid assembly begins in the cytoplasm and is completed within the nuclear compartment. In the cytoplasm a number of interactions occur. VP5 interacts with preVP22a and VP26 whilst VP19C interacts with VP23 (Homa and Brown, 1997). Protein-protein interactions occur between the viral structural and scaffold proteins leading to the assembly of spherical procapsids (Newcomb *et al.*, 2001). The viral DNA is packaged and cleaved into genome length units directed by signals in the terminal "a" sequence (Stow *et al.*, 1983; Hodge and Stow, 2001). VP22a is expelled and simultaneously the capsid undergoes a structural transition to an icosahedral shape (Rixon, 1993). Infected cells contain three forms of capsids, A, B, and C which can be resolved via sucrose density gradient ultracentrifugation. The mature infectious virion is produced from the C-capsid which contains viral DNA. B-capsids do not contain DNA and are the precursor of C-capsids. VP22a occupies the inner space of the B capsid and functions as a scaffold for assembly of the icosahedral capsid shell. A-capsids do not contain DNA and cannot form infectious particles (Gibson and Roizman, 1972; Rixon, 1993).

1.10.9 Maturation/release

Budding through the inner nuclear membrane and acquiring an envelope, the capsid is released into the perinuclear space (Granzow *et al.*, 2001). Until recently, it was not known whether the virion retains this envelope or acquires a fresh envelope from a cytoplasmic compartment during maturation and egression. In other *alphaherpesviruses* such as pseudorabies virus (PRV), the final envelope is acquired in a cytoplasmic compartment, probably the golgi. Recent evidence suggests a similar situation occurs with HSV. Envelopment occurs as the capsid buds through the outer nuclear membrane, followed by re-envelopment in a post-golgi compartment (Van Genderen *et al.*, 1994; Skepper *et al.*, 2001). The virion is thought to exit via the secretory pathway (Rixon, 1993).

1.11 HSV factors involved in immune modulation

Different viruses have evolved a variety of strategies to modulate the host immune response. HSV-1 has evolved several mechanisms to evade or counteract both the innate and specific host immune response. HSV-1 glycoproteins gC, gE and gI and ICP47 act to protect virions and virus infected cells from several host-immune attack mechanisms.

1.11.1 gC

gC acts as a receptor for complement component C3b and hence is though to protect the virus from complement mediated lysis (Friedman *et al.*, 1984).

1.11.2 gE/I

Glycoprotein E can act as a receptor for the Fc receptor of IgG (Baucke and Spear, 1979). In addition, a complex of gE/gI can also act as a high affinity receptor for the Fc region of IgG. gE forms a heterodimer with gI and both of them are co-precipitated from infected cell extracts using nonimmune rabbit or human serum, or purified nonimmune IgG (Johnson and Feenstra, 1987; Johnson *et al.*, 1988). It has been postulated that HSV gE/I prevents activation of the classical complement pathway by binding to the Fc region of antiviral IgG (Frank and Friedman, 1989).

1.11.3 ICP47

At least one HSV-1 gene, ICP47 acts to disrupt the presentation of antigenic peptides by MHC class I molecules on the surface of infected cells and this is thought to prevent recognition and lysis by cytotoxic T-cells (CTLs). ICP47 binds to a human protein transport associated with antigen processing (TAP) and prevents peptide translocation into the endoplasmic reticulum (York *et al.*, 1994; Fruh *et al.*, 1995; Hill *et al.*, 1995).

Recent work has suggested that HSV-1 has also evolved a mechanism to inhibit or reduce the amount of MHC class II on the surface of infected cells. Preliminary evidence suggests that both the virion host shutoff protein (vhs) and ICP34.5 act to block MHC class II presentation on the surface of infected cells (Trgovcich *et al.*, 2002).

1.12 Latency

HSV-1 and -2 cause two types of infections in humans and experimental animals, lytic (1.10) and latent (reviewed in Roizman, 1996). Replicating in epithelial tissue at the site of primary infection, HSV infects innervating axonal termini of sensory neurons and is transported retrogradly to the nueronal cell body (Stevens and Cook, 1971; Stevens *et al.*, 1987), where it establishes a latent infection in the sensory ganglia that innervate the initial replication site. At least some of the initially infected neurons support virus replication. However, within a few days, no free virus can be detected within the ganglia and latency has been established (Stevens and Cook, 1971). The precise sequence of events is unclear. It seems that in some neurons the virus replicates and destroys the neurons whereas in others the virus undergoes an abortive infection and establishes latency (Stevens *et al.*, 1987). Occular infection leads to latent infection in the trigeminal ganglia (TG) (Kristensson *et al.*, 1978). Latency is established in the appropriate dorsal root ganglia (DRG) following ear, flank, and footpad infection, (Stevens and Cook, 1971; Hill *et al.*, 1975; Harbour *et al.*, 1981). Latency can be separated into three stages: establishment, maintenance and reactivation.

1.12.1 *In vivo* latency models

Experimental HSV-1 infection in the mouse is apparently similar to that in humans. During primary infection, viral replication at the surface is detectable for a 10-12 day period. Within 24 h p.i. virus is transported to the innervating sensory ganglia, where peak titres are achieved around 3 to 4 days p.i, and infectious virus becomes undetectable within the

ganglia by 7 to 9 days p.i. The establishment of latent infections occurs during this replicative phase (Stevens *et al.*, 1987). To investigate latency *in vivo*, mouse footpad (Stevens and Cook, 1971), mouse eye (Kristensson *et al.*, 1978), mouse ear (Harbour *et al.*, 1981), rabbit eye (Nesburn *et al.*, 1977) and guinea pig vagina (Scriba, 1975) models have been developed and utilised.

1.12.2 Establishment

Little is known of the establishment phase since the neurons in which the virus replicates during the first several days after infection obscure the events taking place in the neurons where latency is being established (Stevens *et al.*, 1987). It has been suggested that HSV-1 latency can be established without detectable lytic gene expression (Lachmann *et al.*, 1999) and during latency transcription of the genome, with one exception, is prevented in neurons.

In a lytic infection, transcription of IE genes is stimulated by the action of VP16, a major component of the HSV tegument (reviewed by O'Hare, 1993). VP16 acts through the target sequence TAATGART which is present in at least one copy in all HSV IE promoters. TAATGART is a binding site for a cellular protein, OCT1 (Sturm *et al.*, 1988). VP16 and an additional cellular protein, HCF, bind to the Oct-1/ TAATGARAT complex and stimulate transcription of IE genes. During the establishment of latency it has been suggested that the blockage in virus replication occurs at the level of expression of IE gene products. This hypothesis is supported by the behaviour of *in*1814, a HSV-1 mutant with a mutation in

VP16 that abolishes its interaction with OCT-1 and HCF, resulting in significantly reduced IE gene expression (Ace *et al.*, 1989). Although, this mutant replicates poorly both in tissue culture cells and at the site of inoculation in mice, it establishes latency in at least as many cells as found with wild type HSV-1 (Steiner *et al.*, 1990; Valyi-Nagi *et al.*, 1991, 1992; Ecob-Prince *et al.*, 1993). This suggests that a blockage in IE gene expression causes a predisposition for the virus to establish latency and is in agreement with the hypothesis that the natural block is at the IE expression level. In a wild type infection, failure of transactivation of IE transcription in neurons could result either from a lack of VP16 or from a lack of cellular factors Oct-1 and HCF. One hypothesis is that the virus does not carry VP16 in the tegument into some neuronal nuclei during transport along the axon (Roizman and Sears, 1987; Kristie and Roizman, 1988). Alternatively, it has been proposed that other members of the Oct family, rather than Oct-1, are present in some sensory neurons (He *et al.*, 1989). These proteins can bind to the TAATGART elements but do not interact with VP16. Thus these other Oct proteins might compete with Oct-1 for TAATGART binding and prevent formation of the VP16/Oct-1/HCF complex and thus repress IE transcription.

1.12.3 Maintenance

No replicating virus can be detected in the neuron during the maintenance state. Viral DNA is maintained in an episomal form (Rock and Fraser, 1983; Mellerick and Fraser, 1987) and only a small region of the genome is transcribed to produce LATs (Stevens *et al.*, 1987). Within an individual latently infected neuron the viral gene copy number is variable ranging between 10-1000 per cell. The higher the viral input titre, the more neurons in which latency

is established. and the greater the number of viral genome copies per cell (Sawtell, 1997). Typically, by *in situ* PCR 4.8% of neurons harbour latent HSV DNA. However, only 1.6% of neurons are LAT positive, suggesting that not all latently infected neurons express LAT (Mehta *et al.*, 1995).

1.12.4 Latency associated transcripts (LATs)

A major focus of investigation into genes controlling the establishment, maintenance and reactivation from latency has been the LATs, a family of transcripts arising from the HSV inverted repeats flanking the U_L sequence and therefore present in 2 copies per viral genome (Perng *et al.*, 1996, Stevens *et al.*, 1987) (Fig.1.4). The LAT transcripts overlap the ICPO and ICP34.5 genes in an antisense direction. These transcripts are the only viral transcripts detectable during a HSV latent infection (Stevens *et al.*, 1987; Rock *et al.*, 1987). The LATs consist of abundant 2 and 1.5 kbp transcripts and a less abundant 8.3 kbp transcript. The 8.3 kbp LAT is a cytoplasmic polyadenylated precursor RNA. Derived from the 8.3 kbp product, the 2 /1.5 kbp LATs are nuclear, nonpolyadenylated, stable introns that overlap ICPO. (Fraser *et al.*, 1992; Farrell *et al.*, 1991). The HSV-1 2 kb LAT contains three ORFs (Wechsler *et al.*, 1989). These ORFs are conserved both positionally and in sequence in all HSV-1 strains but in HSV-2 only positionally with no sequence conservation (Coffin *et al.*, 1998; Krause *et al.*, 1991). No protein expression from these ORFs has yet been detected in HSV-1 latently infected neurons (Thomas *et al.*, 1999a). In contrast, in the related alphaherpesvirus bovine herpes virus-1, (BHV-1), LAT expresses a 41 KDa protein in neurons (Hossain *et al.*, 1995).

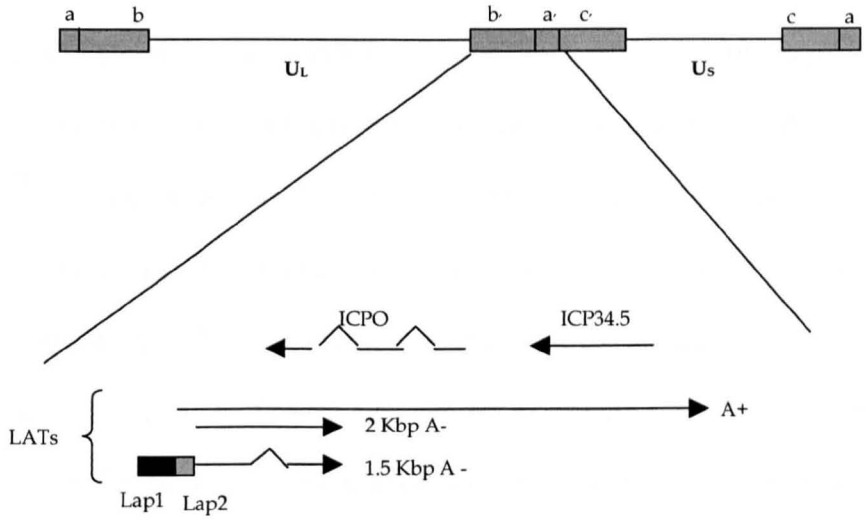


Figure 1.4 Physical map of the internal repeat of HSV-1

(A) Diagram of the HSV-1 genome. (B) Expanded inverted repeat sequence b'-a'-c' lying between 117,000-134,1000 n.p. on the HSV-1 genome. The map shows the locations of sequences specifying the transcripts encoding ICPO, ICP34.5 and the LATs.

However, recent work has demonstrated that constitutive expression of the largest LAT ORF allows replication of wild type HSV-1 in semi permissive neuronal cell lines, complements lacks of ICPO (Thomas *et al.*, 1999a) and overcomes repression of exogenous promoters introduced into the HSV-1 genome which normally occurs in the absence of IE gene expression (Thomas *et al.*, 2002). When an epitope tagged LAT ORF, LATmycHis, is expressed it forms punctuate structures in the infected cell nucleus reminiscent of the structures formed by ICP0. These are associated with the appearance of a phosphorylated form of the protein and are formed adjacent to, or around the edges of, viral replication compartments (Thomas *et al.*, 2002). These properties suggest that the HSV-1 LAT ORF protein is biologically functional and that the tightly regulated expression of this protein may be important *in vivo,* possibly during reactivation (Thomas *et al.*, 2002).

Two LAT promoters have been identified LAP1 and LAP2 (Fig.1.4). LAP1 is a TATA box containing promoter situated at the 5' end of the 8.3 kb LAT 700 bp upstream of the 2 kb intron. *In vitro* the LAP1 TATA box plays an essential role in transcription initiation (Soares *et al.*, 1996; Farrell *et al.*, 1991) and is required for short term expression in latently infected neurons (Margolis *et al.*, 1993). LAP2 is located between LAP1 and the 5' end of the 2 kb LAT (Soares *et al.*, 1996; Goins *et al.*, 1994). LAP2 is required for maintenance of long term expression in neuronal cells (Lachmann and Efstathiou, 1997; Dobson *et al.*, 1995).

The role of the LATs in latency is not known. As the 2 kb LAT is complementary to a part of the ICPO mRNA, it has been suggested that it influences and maintains latency by down regulating ICPO through an antisense mechanism (Farrell *et al.*, 1991; Stevens *et al.*, 1987). In dual transfection assays, expression of the 2 kb LAT has been shown to reduce ICPO transactivation (Farrell *et al.*, 1991). This hypothesis was strenghtened when it was found that ICPO mRNA levels were elevated in BHK cells infected with viruses unable to make the 2 kb LAT (Arthur *et al.*, 1998). However, in contrast Chen *et al* (2002) found that in mouse ganglia latently infected with a LAT deletion mutant the level of ICPO transcripts was not increased. Alternatively transcription of the LAT locus may keep the genome in a transcriptionally competent state during latency such that reactivation is possible (Spivack *et al.*, 1991). Although LATs transcription is not detectable in all latently infected sensory neurons during latency, it is probable that not all neurons reactivate and it may be that LAT negative neurons do not (Preston, 2000). LATs may have a role in efficient establishment of latency (Sawtell and Thompson, 1992; Thompson and Sawtell, 1997).

Although reactivation does not absolutely depend on the presence of LATs (Preston, 2000), in some cases deletion of LATs reduces the capacity of the virus to reactivate following explantation of neurons. Thus LAT may be required for high level reactivation from latency. (Block *et al.*, 1993; Hill *et al.*, 1990; Bloom *et al.*, 1996). The region of LAT associated with decreased reactivation has been mapped to a sequence in the 5' end of LAT (Bloom *et al.*, 1996; Hill *et al* .,1996; Thompson and Sawtell, 1997). Some reports indicate that sequences containing the 2 kbp LAT can protect neurons from apoptosis. This antiapoptotic function of

LAT may be a mechanism to enhance survival of latently infected neurons and hence enhance reactivation (Perng *et al*, 2000, 2002; Inman *et al.*, 2001; Thompson and Sawtell., 2001). Thus, at least one function of LAT may be to promote neuronal survival during the maintenance of a latent infection. Other studies have suggested that viral functions that repress lytic gene expression *in vivo* reside within the LAT domain, although the effectors of these functions have not been identified (Garber *et al.*, 1997; Chen *et al.*, 1997).

Although the exact function of LATs are not known, is not absolutely required for the establishment of latency or reactivation. However, it may be required for optimum establishment of latency and reactivation.

1.12.5 Reactivation

Once HSV latent infection is established in neurons of sensory ganglia, recurrent disease can occur as a consequence of reactivation of latent virus from these cellular reservoirs (Stevens and Cook, 1971). Reactivation occurrs in only a small fraction of the viral DNA containing neurons (Preston, 2000). ICPO is required for both efficient establishment of and reactivation from latency (Halford and Schaffer, 2000) and latent genome copy number strongly correlates with the ability to reactivate *in vivo* (Sawtell *et al.*, 1998).

To investigate the mechanism of reactivation, several *in vitro* and *in vivo* models have been developed. In the most widely used *in vitro* reactivation model, explantation of the HSV

latently infected ganglia leads to reactivation of the virus in culture. This method is more probably a measure of the amount of latent virus than reactivation *per se* (Trousdale *et al.*, 1991). Using several animal models, *in vivo* reactivation models have been developed. These models give a more comprehensive analysis of virus reactivation. Once latency is established, the latent virus can either spontaneously reactivate or be induced to reactivate.

(i) Spontaneous reactivation

Periodic spontaneous shedding of virus occurs after infection of rabbits and guinea pigs. After vaginal inoculation with HSV, spontaneous reactivation periodically occurs in guinea pigs (reviewed by Wagner and Bloom, 1997). Spontaneous reactivation had been reported following HSV ocular infection of rabbits (reviewed by Wagner and Bloom, 1997; Perng *et al.*, 2002). Spontaneous shedding does not occur in mice.

(ii) Induced reactivation

Several different methods have been developed to induce reactivation. In the mouse model, Sawtell and Thompson (1992) have developed a hyperthermic based induced *in vivo* reactivation model. Reactivation has been induced in HSV-1 infected mice following injection of cadmium (Fawl *et al.*, 1996). Reactivation in both rabbit and mouse eye models has been induced by UV light (Coen *et al.*, 1989) or iontophoresis of adrenalin (Willey *et al.*, 1984; Trousdale *et al.*, 1991).

1.12.6 *In vitro* latency models

To attempt to understand the molecular mechanisms of latent HSV infection, several *in vitro* cell culture systems have been developed and utilized. In general, latency is established by infecting cells with virus in the presence of inhibitors of virus replication or at a supraoptimal temperature (O'Neill *et al.*, 1972; Wigdahl *et al.*, 1981, 1982, 1983). Removal of the replication inhibitor or transfer to a lower temperature may result in spontaneous reactivation. Alternatively, superinfection of cells with viruses that express ICPO (Harris *et al.*, 1989) or with HSV-1 temperature sensitive mutants at 38.5^{0}C (Russell *et al.*, 1986) has resulted in reactivation.

An *in vitro* latency system for HSV-2 had been developed. In this system, to suppress viral replication, human foetal lung (HFL) cells were infected with virus at a supraoptimal temperature, 42^{0}C, followed by transfer of the cells to 37^{0}C. Reactivation occurred following superinfection of cells with HSV-1 temperature sensitive mutants at 38.5^{0}C (Russell and Preston, 1986). Using the *in vitro* latency model described above, mutant virus in1814 (1.12.2) was retained in HFL cells following infection at low multiplicity of infection (m.o.i) after incubation at 42^{0}C. It was proposed that lack of VP16 function predisposses HSV to latency, resulting in the retention of the genome similar to that found *in vivo* (Harris and Preston, 1991). Reactivation occurred by superinfection of the monolayers with viruses that express ICPO (Harris *et al.*, 1989).

1.13 HSV pathogenicity

HSV-1 and HSV-2 cause a variety of diseases ranging from benign superficial cutaneous lesions to life threatening encephalitis (Whitley, 1985). A number of genes control HSV-1 pathogenicity. Any alteration in a virus gene which impairs replication *in vitro* will also affect the performance of the virus *in vivo* and so all essential genes could be considered virulence genes. However, here I am only dealing with genes whose deletion can abolish virulence *in vivo* but has a minimal effect in tissue culture. Identification of these genes is important in understanding the fundamental mechanism by which HSV causes disease in humans. The genes involved in HSV-1 pathogenicity can be classified into several broad categories.

One group is glycoproteins which play an important role at the early stages of the virus lifecycle (i.e. adsorption and penetration) and might be expected to also be relevant *in vivo.* However, HSV-1 (and HSV-2) gC^- mutants have been shown to be highly virulent following peripheral or intracerebral inoculation (Dix *et al.*, 1983; Sunstrum *et al.*, 1988). Both HSV-1 strains (Ang) and (KOS) are completely non-neurovirulent following inoculation via the mouse footpad (Kumel *et al.*, 1982; Kaerner *et al.*, 1983; Thompson *et al.*, 1986), but are neurovirulent following intracranial inoculation. Thus there appears to be a defect in neuroinvasiveness. Following passage in the mouse brain, variants of HSV-1 (Ang) were isolated which were neurovirulent following footpad inoculation. The neuroinvasiveness of these isolates was associated with a single base change in gD (Izumi and Stevens, 1990). In HSV-1 (KOS) 2 loci are required for complete restoration of a neuroinvasive phenotype.

One of these is a single base change in gB which significantly affects neurovirulence (Yuhasz and Stevens, 1993).

Although gE/I are non essential for HSV replication in tissue culture cells such as BHK-21, they are required for neuroinvasiveness in mice following peripheral inoculation (Balan *et al.*, 1994) and the gE/I heteroligomer is required for efficient neuron-to-neuron transmission in rats (Dingwell *et al.*, 1995). Meigner *et al* (1988) demonstrated that a gE$^-$ mutant was not significantly impaired in neurovirulence following intracerebral inoculation of mice. Glycoproteins E/I are also important in the pathogenicity of the closely related *alphaherpesvirus* PrV. Deletion of gE and gI impairs spread of PrV compared to the parental strain by affecting transneuronal transfer of PrV (Kritas *et al.*, 1994; Babic *et al.*, 1996).

Another group of genes with a role in pathogenicity are those involved in DNA replication. These genes are essential with their deletion abolishing replication *in vitro*. However, there are some point mutants in these genes which have little effect *in vitro* but abolish replication *in vivo*. Using 3 distinct DNA polymerase mutants in a mouse ear model, Field and Coen (1986) showed that although these mutants exhibited no significant difference in replication kinetics compared to the wild type virus at peripheral sites (ear pinna and flanks), no neurological involvement was observed. Following intracerebral inoculation these DNA polymerase mutants were highly attenuated for pathogenicity (Larder *et al.*, 1986).

Another group is genes involved in nucleotide metabolism e.g TK and ribonucleotide reductase (RR). None of these genes are essential in tissue culture (Cameron *et al.*, 1988;

Efstathiou *et al.*, 1989; Goldstein and Weller, 1988a, b) with cellular enzymes substituting for the virus enzymes in dividing cells. HSV-1 TK- mutants are less virulent than wild type virus and do not replicate in the TG (Field and Wildy, 1978; Tenser *et al.*, 1979, 1981). Their lack of virulence is due to an inability to replicate in non dividing neuronal cells where there is no pool of nucleotide precursors. However, they can establish latency, but are not able to reactivate indicating TK may be required for reactivation from latency (Coen *et al.*, 1989; Efstathiou *et al.*, 1989).

Similarly to TK, RR is non essential in dividing cells but important in virus virulence. A deletion variant of HSV-1 (KOS), ICP6Δ, lacking 90% of the R1 coding sequences showed only a slightly reduced ability to grow on BHK/C13 cells (Goldstein and Weller, 1988a, b). However, this RR mutant showed extreme growth impairment following inoculation in the mouse eye. The mutant replicated poorly in the cornea, did not cause severe ocular disease compared to the wild type and rescuant and only established a low level of virulence (Brandt *et al.*, 1991).

1.14 US11

The US11 gene is located in the Us region of the HSV-1 genome (McGeoch *et al.*, 1985). It is expressed as a γ2 gene (Johnson *et al.*, 1986) and its mRNA is 3' co-terminal with US10 and US12 sharing a common polyadenylation signal (Rixon and McGeoch, 1984, 1985; McGeoch *et al.*, 1985). The protein 21K encoded by this gene is a small basic phosphoprotein that localizes to the nucleolus of infected cells (Puvion-Dultilleul, 1987). It is non essential for virus growth *in vitro* and *in vivo* and the function of US11 during the

HSV-1 lytic cycle is unclear (Longnecker and Roizman, 1986; Nishiyasm *et al.*, 1993). The major properties associated with US11 are RNA binding and the antagonism of the interferon induced, double stranded RNA dependent protein kinase. US11 binds several RNA transcripts separately both *in vitro* and *in vivo* (Roller and Roizman, 1990, 1991; McCormick., *et al* 1999). This protein is incorporated into the virion tegument and is therefore delivered to the host cell upon infection (Roller and Roizman, 1992). Ultrastructure studies have revealed that early in infection 21K co-localizes with viral DNA in the nucleus and perinuclear region which is rich in ribosomes (Puvion-Dultilleul, 1987).

Expression of US11 early in infection was found to be responsible for the suppression of host mediated protein synthesis shutoff in ICP34.5 null mutants (Cassady *et al.*, 1998a; Mulvey *et al.*, 1999) (1.15.3). Inhibition of ds RNA dependent protein kinase R (PKR) autophosphorylation/activation or block of eIF-2α phosphorylation have been suggested to be potential candidates for the mode of US11 action (Cassady *et al.*, 1998a, b; Mulvey *et al.*, 1999; Popper *et al.*, 2000). Several hypotheses have been proposed for the mechanism by which US11 inhibits PKR activation and eIF2α phosphorylation. PKR is activated by dsRNA although the actual sequence of the substrate is unknown (Gunnery and Mathews, 1998). As US11 is a RNA binding protein it has been proposed that US11 binds both PKR and the activating ds RNA thereby preventing PKR dimerization and activation. An alternative hypothesis is that as US11 appears to act upstream of PKR activation it may compete with PKR for binding of dsRNA (Poppers *et al.*, 2000).

Recent evidence suggests that US11 can also inhibit PACT-mediated activation of PKR both *in vitro* and *in vivo*. Binding of the C-terminus domain of US11 to PKR (and not to PACT) is required for inhibition of activation. This domain is the same domain that binds to dsRNA (Peters *et al.*, 2002).

1.15 ICP34.5

1.15.1 Identification:

ICP34.5 has been identified as a HSV virulence gene. Originally, Thompson *et al.* (1983) mapped a neurovirolence locus to the long repeat region of the HSV-1 genome. Subsequently, a gene, ICP34.5, was identified in the long repeat sequence between ICPO and the "a" sequence in HSV-1 strain F (Chou and Roizman, 1986, 1990), in HSV-1 strain 17^+ (Dolan *et al.*, 1992), and in HSV-2 strain HG52 (McGeoch *et al.*, 1991) whose promoter is located in the "a" sequence (Ackermann *et al.*, 1986). As ICP34.5 is located in the long repeat, it is present in two copies per viral genome (Fig.1.5) (Chou and Roizman, 1986, 1990). The virulence function originally identified by Thompson *et al* (1983) was subsequently mapped to ICP34.5 (Taha *et al.*, 1989; Thompson *et al.*, 1989; Chou *et al.*, 1990; MacLean, A. *et al.*, 1991).

1.15.2 Characterization

The ICP34.5 promoter is contained entirely within the terminal "a" sequence, lacks a TATA box and, unlike most HSV-1 genes, contains numerous repetitive G+C rich elements (Chou and Roizman, 1986). Transcription of the ICP34.5 gene is initiated in the DR1 element of

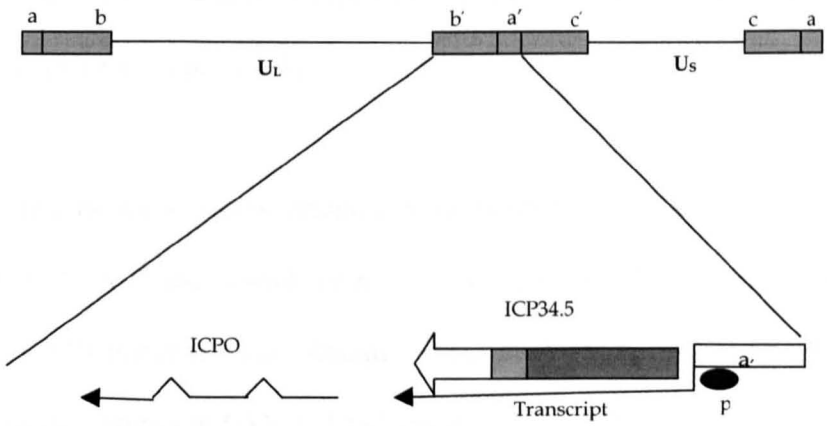


Figure 1.5 Schematic diagram of HSV-1 genome with the location of the ICP34.5 gene

a. The HSV-1 genome. a b and a' b' represent both the terminal and internal inverted repeats flanking the unique sequence of the L component. a c and a' c' represent both the terminal and internal inverted repeats flanking the unique sequence of the S component.

b. An expansion of the internal inverted repeat. Within this region, the 'a' sequence, ICP34.5, and ICPO are shown. The thick arrow is the ICP34.5 ORF with N-basic region (⇦), PAT repeat (□), and C-terminal homology (▭) region highlighted. The promoter and transcript are marked.

the "a" sequence. It is a γ_1 gene and its expression is reduced but not abolished by inhibitors of DNA synthesis (McKay *et al.*, 1993).

The ICP34.5 gene consists of a basic amino acid terminus and a 63 amino acid region with 83% homology to HSV-2 and homology to cellular genes (1.15.3) separated by a DNA repeat encoding a PAT trimer (Proline, Alanine, Threonine) which varies in number between strains being present 10 times in HSV-1 (F) giving a protein of 263 amino acids with a M_r of 39 KDa (Chou and Roizman, 1990) but only five times in HSV-1(17^+) giving a protein of 248 amino acids with a M_r of 37 KDa (Dolan *et al.*, 1992; McKay *et al.*, 1993) (Fig.1.5). Unlike HSV-1, the gene in HSV-2 does not contain the sequence containing the PAT trimer repeat but does contain an intron (McGeoch *et al.*, 1991).

ICP34.5 is distributed in the nucleus, nucleolus, and cytoplasm in transfected or superinfected cells. Deletion analysis revealed that the Arg-rich cluster from amino acids 1 to 16 in ICP34.5 functions as a nucleolar localization signal. The region from amino acids 208 to 236, containing a bipartite basic amino acid cluster, is able to mediate nuclear localization. These results suggest that the ICP34.5 protein continuously shuttles between the nucleus, nucleolus, and cytoplasm, which may be a requirement for the different activities of the ICP34.5 protein in virus infected cells (Cheng *et al.*, 2002).

1.15.3 Function:

ICP34.5 is non essential in most cell types *in vitro* but essential *in vivo* (Chou *et al.*, 1990). Two functions have been attributed to HSV-1 ICP34.5: (i) virulence; and (ii) the prevention of the shut-off of host cell protein synthesis. These two phenotypes will be discussed individually and then correlated.

(i) *In vivo* phenotype

In vivo a number of phenotypes have been attributed to ICP34.5. Following intracranial inoculation, ICP34.5 null mutants are unable to replicate in the CNS of mice and are totally avirulent with a LD_{50} of 10^6 pfu/mouse higher than wild type or rescued virus (Chou *et al.*, 1990; MacLean, A. *et al.*, 1991; McKie *et al.*, 1994). Detailed analysis of replication characteristics in the CNS have revealed that while some cells allow limited gene expression, the only cell type which allows productive replication are ependymal cells and this terminates after 14 days (Markovitz *et al.*, 1997; Kesari *et al.*, 1998).

ICP34.5 negative mutants establish latency in the CNS as determined by the presence of LAT positive neurons (Kesari *et al.*, 1996, 1998). Following peripheral inoculation in the eye, the virus does not appear to replicate in the cornea or TG and although establishing latency in TG does so at a significantly lower frequency than wild type. Based on the level of LAT expression in the TG, the number of LAT+ve cells and the absence of detectable HSV DNA by PCR, the decrease in amount of latent virus is 10 and possibly 100 fold. This is probably due to lack of replication in both the cornea and TG (Spivack *et al.*, 1995). Virus can be reactivated either *in vitro* following explantation or *in vivo* for example

following ionotrophesis of adrenalin (Perng *et al.*, 1995, 1996). Following footpad or vaginal inoculation, virus may undergo limited replication at the initial site of inoculation (Roberston *et al.*,1992; Whitely *et al.*, 1993; Spivack *et al.*, 1995). No replication takes place in the DRG and latency is established inefficiently although not as inefficiently as in the TG and can be reactivated *in vitro* following explantation (Spivack *et al.*, 1995). Initial work suggested that ICP34.5 is a neurovirulence gene (Chou *et al.*, 1990; MacLean, A. *et al.*, 1991). However, the lack of replication in the cornea, the possible lack of replication in the footpad, and lack of pathogenicity in SCID mice, suggested that the defect may be a lack of replication *in vivo per se* (Valyi-Nagy *et al.*, 1994; Randazzo *et al.*, 1996). Thus ICP34.5 should be regarded as a virulence gene.

One anomalous result is an ICP34.5 null mutant called 17termA which is a mutant in HSV-1 (17^+) with a stop codon after the N-terminal 30 amino acids (Bolovan *et al.*, 1994) and is essentially ICP34.5 null. However this mutant instead of being avirulent, showed only a 25-100 fold reduction in virulence compared to wild type (Bolovan *et al.*, 1994). This might suggest a partial function mapping to the N-terminus of ICP34.5. However the HSV-1 (F) mutant, R4009, with an identical mutation was completely avirulent (Chou *et al.*, 1990) as was a strain 17^+ mutant, 1771, with a stop codon immediately after the initiating methionine codon (McKie *et al*, 1994; Spivack *et al.*, 1995). The anomaly of 17termA still retaining some virulence has yet to be resolved.

(ii) *In vitro* phenotype

In tissue culture, ICP34.5 null mutants replicate normally in many non neuronal cell types such as Vero and BHK 21/C13 cells, but are restricted in SK-N-SH neuroblastoma cells, human foreskin fibroblast (HFF), murine 10T1/2 cells, stationary phase primary mouse embryo and mouse embryo fibroblast (3T6) cells (Chou *et al.*, 1990, 1994; Chou and Roizman, 1992; Bolovan *et al.*, 1994; Brown *et al.*, 1994a). In general, in nonpermissive cells the growth defect of ICP34.5 null mutants is greater when they are in a stationary phase (Brown *et al.*, 1994a). One explanation is that nonpermissive cells infected with ICP34.5 null mutants undergo a total shutoff of protein synthesis. Therefore in these cells ICP34.5 provides a mechanism for cell survival which enables the virus to sustain its replication cycle and produce infectious virus (Chou and Roizman, 1992; Chou *et al.*, 1995; He *et al.*, 1997a) (see below).

Another explanation for the role of ICP34.5 *in vitro* is that failure to express ICP34.5 results in a defect in virus maturation and egress from the nuclei of infected cells. Brown *et al.* (1994a) demonstrated that the growth of ICP34.5 null mutants was impaired in 3T6 cells with the impairment amplified in the stationary state. These workers demonstrated that the impairment in growth was due to a defect in envelopment and egress of virus particles with an accumulation of unenvelopped capsids in the nucleus of infected 3T6 cells with little or no effect on the behaviour of the virus in BHK cells (Brown *et al.*, 1994b).

(iii) Shutoff of host protein synthesis

The cellular response to virus infection is a complex process and includes induction of interferons (IFNs). IFNs are key components of the host innate immunity to virus infection and act through several pathways to block virus growth and virulence (Stark *et al.*, 1998). Cells infected with a variety of both DNA and RNA viruses synthesize double stranded RNA which induces an IFN response (Katze, 1995). PKR is induced by IFNs and is a major mediator of the cellular response to stress such as virus infection (Fig 1.6). PKR becomes activated through autophophorylation and dimerization (Merrick, 1992; Wu *et al.*, 1998) and phosphorylates eIF-2α. This leads to the loss of functional eIF-2α which results in inhibition of protein synthesis (Katze, 1993). This is the primary mechanism by which PKR prevents viral replication by total shuttoff of protein synthesis (Mathews, 1996).

In HSV-1 infected cells, transcription of the complementary DNA strands results in the formation of double stranded RNA, induction of IFN and activation of the PKR pathway which should result in premature shutoff of host protein synthesis. However, HSV encodes a gene ICP34.5 which blocks the shutoff of host protein synthesis (Chou and Roizman, 1994). It has been suggested that some cell lines such as Vero cells might be defective in the IFN-induced mechanism targeted by ICP34.5 (Mossman and Smiley, 2002), explaining their ability to support replication of ICP34.5 negative viruses.

In HSV-1 infected cells PKR is activated and eIF-2α is phosphorylated. However, a cytoplasmic complex containing protein phosphatase 1α (PP1α) and ICP34.5 is formed and PP1α is redirected to dephosphorylate eIF-2α (He *et al.*, 1997a, 1998) and thus prevent

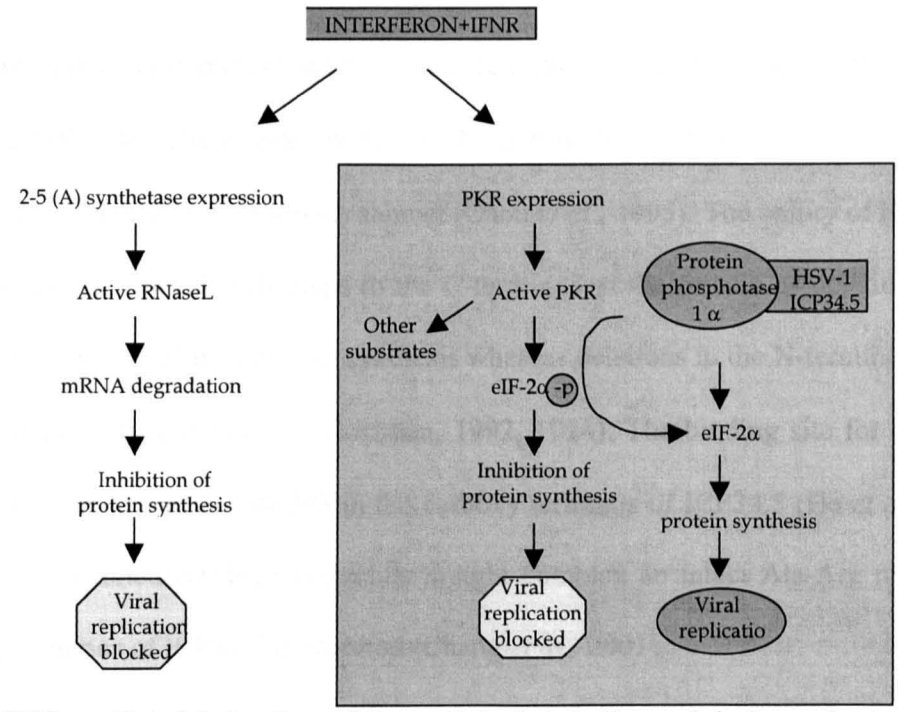


Figure 1.6 IFN-mediated induction of the antiviral state through independent pathways by RNase L and PKR

IFN binds to its receptor and activates two pathways. On the left the RNase L pathway and on the right the PKR pathway. In right side panel, IFN induces expression of PKR which is activated through double stranded RNA binding.

One of the activities of PKR is to phosphorylate eIF-2α leading to cessation of protein synthesis and blocking of viral replication. To evade this antiviral mechanism, HSV-1 ICP34.5 binds to protein phosphatase1α and dephosphorylates eIF-2α, lifting the block on viral replication.

In left side panel, IFNs interacting with their receptors lead to expression of 2-5A synthetase, which, after double-stranded RNA binding, produces 2', 5'-oligodenylates that lead to RNase L activation and blockage of viral replication (Leib *et al.*, 2000)

premature shutoff of host protein synthesis induced after the onset of viral DNA synthesis (Chou *et al.*, 1995). In cells infected with ICP34.5 null mutants, PKR is activated, eIF-2α is phosphorylated and protein synthesis is shutoff (Chou *et al.*, 1995). The ability of ICP34.5 to prevent protein synthesis shutoff maps to the C-terminus of the protein as deletions in this region preclude shutoff of host protein synthesis whereas deletions in the N-terminal have no effect on this phenotype (Chou and Roizman, 1992, 1994). The binding site for PP1α has been mapped to amino acids 190-203 in the carboxy terminus of ICP34.5 (He *et al.*, 1998). In addition, for a functional high molecular weight complex an intact Ala-Arg motif from the carboxy terminus of ICP34.5 is required (Cheng *et al.*, 2001).

The carboxy terminus of ICP34.5 has homology to cellular genes involved in response to cellular stress and DNA damage These are MyD116 (Lord *et al.*, 1990; Barnett and McGeoch, 1991) and GADD34 (Fornace *et al.*, 1989). The carboxy terminus of MyD116 also interacts with protein phosphatase 1a (PP1α) both *in vitro* and in the yeast-2-hybrid system (Chou and Roizman, 1994; He *et al.*, 1997a). It is likely that GADD34 will also have a similar function to interact with and redirect PP1α to dephosphorylate eIF-2α to enable continued protein synthesis in conditions of cellular stress (He *et al.*, 1997a). Substitution of the ICP34.5 carboxy terminus by the corresponding domain of MyD116 in the context of the viral genome restored the ability of an ICP34.5 null mutant to preclude host protein synthesis shutoff in both neuroblastoma cells and human foreskin fibroblasts (He *et al.*, 1996).

Both ICP34.5 and MyD116 interact with proliferating cell nuclear antigen through the 63-amino acid conserved domain (Brown *et al.*, 1997). The carboxy terminal domain of ICP34.5 also shows significant homology to an African swine fever virus (ASF) gene 23-NL (NL-S) which has been shown to have a role in ASF virulence (Sussman *et al.*, 1992; Zsak *et al.*, 1996).

Second-site compensatory ICP34.5 null mutants have been isolated by Mohr and Gluzman (1996) which exhibit restored ability to prevent host shutoff of protein synthesis and to grow in some neoplastic neuronal cell lines. In these mutants the deletions were present in the α gene US12 such that the γ2 gene US11 (Johnson *et al.*, 1986) was now expressed as an α gene (Fig.1.7). This was confirmed by the construction of targeted deletion mutants where US11 was expressed under the US12 α promoter (1.14) (Cassady *et al.*, 1998a, b; He *et al.*, 1997b). However, these mutants are still nonneurovirulent (Mohr *et al.*, 2001). Interestingly, although US11 is a structural tegument protein (Roller and Roizman, 1992) and therefore will be delivered early in infection, *de novo* expression is required to prevent protein synthesis shutoff. Thus either there is not enough US11 in the virion or it is not in the correct processed form to prevent shutoff of protein synthesis (Mulvey *et al.*, 1999; Mohr *et al.*, 2001; Cassady *et al.*, 1998a, b).

In these revertants the US11 RNA binding protein prevents shutoff of host protein synthesis by inactivating the PKR pathway. The 68 amino acid carboxy terminus fragment of US11 contains a novel proline rich basic RNA binding domain which is sufficient to inhibit activation of the kinase activity of the cellular PKR (Poppers *et al.*, 2000). A 30 amino acid

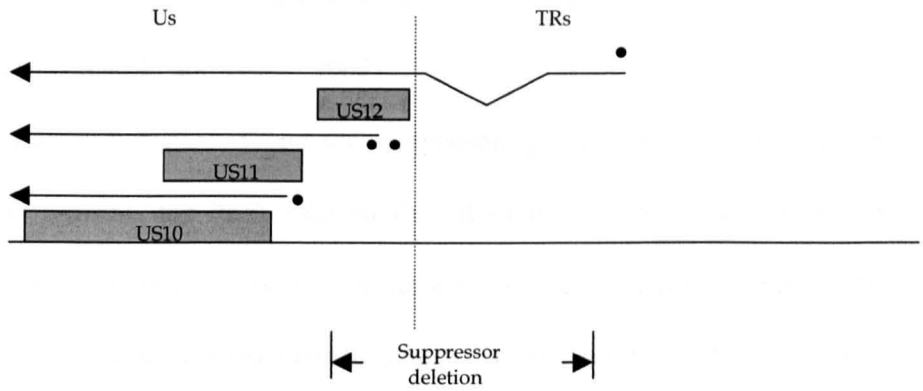


Figure 1.7 Map of the junction region where Us joins the terminal repeat (TRs)

A diagram illustrating the Us/TRs junction region (dashed line). The US 10, 11 and 12 ORFs in Us are shown. ORFs are denoted as heavy boxes. The 3' terminal mRNAs appear as arrows terminating at their common polyadenylation site. The cis-elements which direct transcription of the various open reading frames are denoted as solid circles (•) above their respective transcript. The region deleted in the suppressor viruses is shown below (▶◀).

domain within this carboxy terminus of US11 interacts with PKR in the ribosomes of infected cell (Cassady and Gross, 2002).

Recently a second class of second site compensatory ICP34.5 null mutants has been isolated which also prevents the PKR induced shutoff of protein synthesis. In these mutants, the mutation although shown to occur outside the US10-12 domain, has not yet been mapped. In this class of mutants neurovirulence is partially restored (Cassady *et al.*, 2002).

(iv) Relevance of shutoff of host protein synthesis to virulence

ICP34.5 clearly has two functions: a role in allowing replication *in vivo*; and preventing host protein synthesis shutoff in some cell types *in vitro.* The question arises as to whether the lack of replication and hence avirulence *in vivo* is due to the host protein synthesis shutoff phenotype. There is evidence both in favour and against this hypothesis.

Against is the fact that the second site US11 revertants whilst regaining the host shutoff phenotype do not regain any virulence (Mohr *et al.*, 2001). In addition, the recombinant virus where the MyD116 carboxy terminus has been substituted for the ICP34.5 carboxy terminus whilst being fully functional for the host shutoff phenotype, is completely avirulent (Andreansky *et al.*, 1996).

However, in favour of the linkage is the observation that second site revertants outside US11 do regain some virulence (Cassady *et al.*, 2002). Most importantly is transgenic mice data which strongly suggest that there is linkage between inhibition of PKR function and virulence. A HSV-1 ICP34.5 mutant grows to normal levels and shows wild type virulence

in mice lacking both type 1 and type 2 IFN receptors whereas the same mutant has a 10^4 reduction in replication and neurovirulence in normal mice (Leib *et al.*, 1999). This was shown to be due to the PKR response as an ICP34.5 deletion mutant had wild type replication characteristics and virulence in transgenic mice from which PKR had been deleted (Leib *et al.*, 2000).

1.16 L/STs, ORFs O and P

1.16.1 L/STs

A set of L/S junction-spanning transcripts (L/STs) with their upstream regulatory sequences has been described by two groups (Bohenzky *et al.*, 1993; Yeh and Schaffer, 1993). The L/STs are a series of 5'-coterminal polyadenylated RNAs ranging in size from 2.3 to >9.0 kb, are transcribed from the same DNA strand as the latency-associated transcripts (LATs) and overlap the 3' terminal 2.3 kb of the 8.3 kb LAT (Fig.1.8) (Yeh and Schaffer,1993). The L/STs exhibit a novel pattern of regulation since they are not detected during normal infection but are overproduced during infection with a virus in which ICP4 is inactive. A strong binding site for ICP4 is found at the 5' end of the L/STs (Yeh and Schaffer, 1993; Lagunoff and Roizman, 1995; Randall *et al.*, 1997). Mutagenesis of this site also results in overproduction of L/STs during normal infection, indicating that ICP4 represses transcription of L/STs (Yeh and Schaffer, 1993, Lagunoff and Roizman, 1995; Yeh and Schaffer, 1998).

The HSV-1 unspliced 8.3 kb LAT has been shown to contain at least 16 ORFs greater than 50 amino acids in length (Lagunoff and Roizman, 1994). These ORFs have been designated

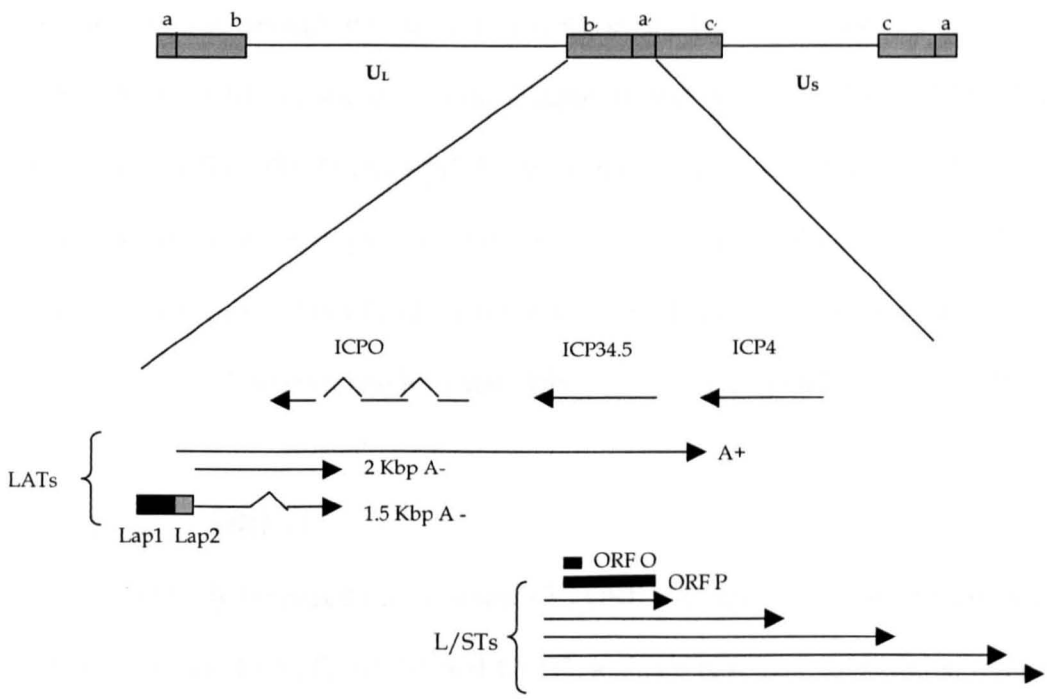


Figure 1.8 Physical map of the internal repeat of HSV-1 DNA

(A) Diagram of the HSV-1 genome. (B) Expanded inverted repeat sequence b'-a'-c'lying between n.p.117,000-134,1000 on the HSV-1 genome. The map shows the locations of sequences specifying the transcripts encoding ICPO, ICP34.5 and ICP4 and sequences specifying the LATs. ORFs including ORF O and ORF P are hatched boxes. L/STs transcripts are indicated as arrows (⟶).

by the letters A through P (Lagunoff and Roizman, 1994). At least two of these 16 ORFs, ORF O and ORF P, are expressed (Lagunoff and Roizman, 1994, 1995; Randall and Roizman, 1997). ORF O and ORF P are located in the 3' domain of LAT, almost entirely antisense to the ICP34.5 gene and are also contained at 5' end of the L/STs. Both ORFs O and P are expressed from L/STs and not from the LAT (Bohenzky *et al.*, 1993; Yeh and Schaffer, 1993; Lagunoff and Roizman, 1994, 1995; Randall and Roizman, 1997).

1.16.2 ORF O

It was originally proposed that in strain 17^{+}, ORF O is approximately 300 codons in length, whilst in strains KOS, F, MH10, and CVG2, a single base pair substitution terminates ORF O after approximately 160 codons (Yeh and Schaffer, 1998). ORF O overlaps ORF P but in a different reading frame. These workers assumed that the initiating methionine codon of ORF O is located in the TATA box of the L/STs. However, for a number of reasons, Randall *et al* (1997) suggested that this methionine is not the initiating methionine. Firstly ORF O appears to be expressed from the L/STs and secondly mutation of the ORF P initiating methionine also prevents ORF O protein expression in strain F infected cells at 39.5°C, the nonpermissive temperature for ICP4. Thus ORF O, like ORF P, is downregulated by ICP4 (Randall *et al.*, 1997). Analysis of cells infected with a derepressed ORF P failed to detect a separate RNA corresponding to ORF O (Randall *et al.*, 1997). Detailed analyses of the products encoded by the ORFs showed that the translation of ORF O begins at the ORF P initiating methionine codon and then shifts into the ORF O reading frame before amino acid 35 of ORF P. Based on this information, ORF O actually consists

of 245 amino acids in strain 17+ and 105 amino acids in strains KOS, F, MH10 and CVG2 (Randall *et al.*, 1997).

In vitro, ORF O protein specifically binds to ICP4 inhibiting its binding to its cognate site (Randall *et al.*, 1997).

1.16.3 ORF P

The first identified and largest ORF in the L/STs is ORF P, which encodes the ORF P protein. ORF P is expressed at detectable levels only under conditions which allows expression of the L/STs (Lagunoff and Roizman, 1994; Yeh and Schaffer, 1993). The most striking feature of ORF P is that it is nearly completely antisense to ICP34.5. Only 8 codons of ORF P are not antisense to ICP34.5, and only 23 codons of ICP34.5 are not antisense to ORF P (Lagunoff and Roizman, 1994). ORF P expressed by HSV-1 (F) is predicted to contain 248 amino acids. It contains a short amino terminal domain followed by an amino acid trimer of Ala-Gly-Val repeated 10 times and a long carboxy terminal domain. The protein in HSV-1 (17^+) contains 233 amino acids with the amino acid trimer of Ala-Gly-Val repeated only 5 times. Similarly to ICP34.5, the variation in the length of the protein between different strains is because of the variation in the number of repeats of this trimer (Lagunoff and Roizman, 1994).

Expression of L/STs and their protein products, ORF P and ORF O has not been detected during productive infection by Lagunoff *et al.* (1996). However, it has been demonstrated in our laboratory (McKie and MacLean, personal communication) that in BHK/C13 cells RL1

and L/ST transcripts and ICP34.5 and ORF P are simultaneously expressed in the presence of functional ICP4 during a productive infection by HSV-1 (17^+). Work in this thesis confirms simultaneous expression of ICP34.5 and ORF P during productive infection.

As most ICP34.5 null mutants also affect ORF P due to the extensive overlap of their sequences, it was difficult to assign a role to ORF P. Therefore, to investigate the role of ORF P in the HSV-1 lifecycle and any possible effects of ORF P on the ICP34.5 gene, a number of mutants have been constructed and characterized. The following mutants are ORF P derepressed mutants which produce L/STs and ORF P during productive infection.

Lagunoff *et al.* (1996) constructed a HSV-1(F) ORF P derepressed mutant, ORF P++, in which the ICP4 binding site in the L/ST promoter was mutagenized to allow expression of the L/STs and the ORF P gene throughout the lytic cycle of the mutant virus. ICP34.5 was not detected and the level of its RNA was decreased in infected cells. This mutant was highly attenuated upon inoculation into mice by the intracerebral or ocular route. It could not be recovered following explantation from the TG. However, protein synthesis was not prematurely shutoff, possibly as only a low level of ICP34.5 is necessary for this phenotype (Chou and Roizman, 1994). It had been suggested that, in the ORF P++ mutant, either overproduction of ORF P blocks the expression of ICP34.5 or derepression of L/STs transcription has a negative effect on the transcription of the antisense ICP34.5 RNA (Lagunoff *et al.*, 1996).

A second HSV-1 (F) mutant R7546 suggested that the reduction in the accumulation of ICP34.5 mRNA and protein in cells infected with ORF P++ is due to the effect of L/STs

antisense transcription rather than a function of the ORF P protein (Randall and Roizman 1997). In R7546, L/STs transcription was again derepressed but the initiating ORF P methionine codon was mutagenized and ORF P protein was not produced. The phenotype of R7546 was identical to ORF P++.

A similar mutant to ORF P++, L/ST4BS with the ICP4 binding site mutated was constructed in HSV-1 strain KOS by Yeh and Schaffer (1998). In L/ST4BS, L/STs and ORF P were expressed during a productive infection. Similar to ORF P++, this mutant was also highly attenuated following intracranial inoculation of juvenile mice. However, replication of this mutant was efficient in the mouse eye and TG. In Vero cells as well as the L/STs, both ICP34.5 and LATs transcripts were abundant. However, in mouse neuroblastoma cells (NB41A3) ICP34.5 and LAT transcripts were barely detected. In contrast to ORF P++ in SK-N-SH cells, L/ST4BS induced premature shutoff of protein synthesis in NB41A3 cells. The reason for the difference in results between these two mutants is unclear but might reflect either the slightly different mutation in the ICP4 binding site or a strain difference.

Both Yeh and Schaffer (1998) and Randall *et al* (2000) have constructed mutants which do not express ORF P but have no effect on the ICP34.5 coding sequence. A HSV-1 strain KOS mutant containing a single base pair alteration in ORF P at codon 38 results in termination of ORF P translation and expression of a truncated ORF P protein (Yeh and Schaffer, 1998). L/ST-n38 exhibited no premature shutoff of infected cell proteins and had no observable phenotype relative to wild type virus *in vitro and in vivo*. This mutation induced an Arg-to-Thr change at amino acid of 38 of ORF O. If ORF O protein is involved

in any of the functions measured, this substitution had no detectable effect on these functions (Yeh and Schaffer, 1998).

Similarly, Randall *et al.* (2000) constructed an HSV-1 (F) mutant, R7538(P-/O-), in which the ORF P initiating methionine codon, which also serves as the initiating methionine codon for ORF O, was mutated. The R7538 (P-/O-) mutant failed to express ORF O and ORF P proteins but expressed a wild-type ICP34.5 protein. Similar to L/STn-38, the R7538 (P-/O-) showed similar behaviour to that of the wild type following infection of SK-N-SH and Vero cells, and infection of mice by the intracerebral or ocular routes.

The ability of L/STs, ORF O and ORF P to be expressed in the absence of viral protein synthesis and the complete repression of this gene by ICP4 suggested that ORF O and P are pre-α genes, i.e. expressed under condition in which the α genes are not expressed (Bohenzkey *et al.*, 1993; Lagunoff and Roizman, 1995). Their ability to be expressed in the absence of protein synthesis is disputed by Yeh and Schaffer (1993).

Interaction of ORF P with a number of proteins involved in splicing has been demonstrated. Using immunoflourescense microscopy, ORF P has been shown to colocalise with SM antigens and SC35 in the nuclei of infected cells, interact with SM components by GST pulldown assay and interact with P32 in a yeast-two hybrid screen (Brunei and Roizman, 1996). In cells infected with a virus carrying a derepressed ORF P, it is rapidly posttranslationally modified and ORF P appear to inhibit the synthesis of the spliced immediate early transcripts of the ICP0 and 22 genes early (8 h) but not late (18 h) in

infection, while the levels of two immediate early proteins synthesized from intronless mRNAs, ICP4 and ICP27, are unchanged (Brunei and Roizman, 1996; Randall and Roizman, 1997). However, in Vero and NB41A3 cells infected with the ORF P null mutant L/ST- *n*38 no difference has been detected in the levels of ICP0 transcripts at early times (6 h) p.i. compared with wild-type virus-infected cells (Yeh and Schaffer, 1998).

ORF O protein inhibits the function of ICP4 (Randall *et al.*, 1997). ORF P protein reduces the accumulation of ICPO and ICP22 (Randall and Roizman, 1997). Thus ORF O and P appear to inhibit the synthesis or function of three key immediate early proteins, ICPO, ICP22, and ICP4 which are required for viral replication and lytic gene expression. The L/STs and ORF P derepressed mutants ORF P++ and L/ST-4BS establish latency. However, although L/ST-n38 and R7538(P-/O-) reactivate from latency, the amount of latent R7538 (P-/O-) DNA is eightfold less than that of its rescuant. Thus ORF O and ORF P whilst not essential for the establishment of latency in mice may play a role in determining the quantity of latent virus maintained in sensory neurons (Randall *et al.*, 2000). In contrast, Yeh and Schaffer (1998) suggest that ORF P plays no major role in neurovirulence or in latency and reactivation in mice. To date, there is no evidence of expression of ORFs O and P in neurons harbouring latent HSV genomes (Preston, 2000). Thus, the function of ORF O/P remains to be determined.

1.17 Gene therapy/delivery

The introduction of genes into cells for a theraputic purpose such as treatment of many inherited genetic diseases and other diseases and use of a gene instead of ordinary drugs is referred to as gene therapy. The gene has to be efficiently delivered to the appropriate cells and gene expression must be at the right level. The ideal vector would be non toxic, immunogenic or mutagenic in the patient, and easily produced in high concentration and purity at a low price. To date, two approaches viral and non-viral vectors have been developed to deliver genes. For the advantages and disadvantages of each approach see table 1.2.

1.18 Viral vectors

Some of the characteristics important in viruses for gene delivery are: delivery of the gene of interest (GI) into cells, often of a very specific cell type; easy removal of a viral gene/genes; and later replacement by the GI(s). The recombinant vectors are packaged into virus particles and infection of cells by the particles leads to expression of the GI. To date, the following virus vectors have been used for gene therapy: retroviruses, adenoviruses, adenoassociated viruses (AAV) and herpes simplex virus-1 (HSV).

1.18.1 Retroviruses

Effective retroviral vectors have been created by deleting essential genes. All essential genes (gag, pol, and env) are removed and a foreign gene and selectable marker are inserted into the vector which retains the packaging signals. Retroviruses are 7-9 kb in length and at most 9 kb can be packaged into these vectors. This plasmid is transfected into a packaging cell

Vectors	Advantages	Disadvantages
Retroviruses	-Integrate into the genome -All viral genes removed -Relatively safe	-Random integration -Only dividing cells transduced -Low titres obtained *in vitro* -Size limitation
Adenovirus	-Large insert (35 kbp) -High titres obtained *in vitro* -Efficient transduction into different cell types -Transduce dividing/ non-dividing cells	-Toxicity -Immunological response -Transient expression -Prior exposure
Adeno associated virus	-Non pathogenic -Transduce dividing/ non-dividing cells -Long term expression -Safe -Stable expression -Integrates into known location	-Small genome -High multiplicity of virus for transducing some cell types -Toxicity associated with helper virus
Herpes simplex virus	-Large insert -Lifelong latent state -Transduce dividing/non-dividing cells -Amplicon: Efficient delivery Low toxicity -Defective: Infects neurones High titres *in vitro*	-Hard to obtain long term expression -Amplicon: Low titres obtained *in vitro* Hard to separate from helper virus Low packing efficiency -Recombinant: Toxicity Transient expression
Non-viral vectors	-Easy to manipulate -Lack of viral components -Size -Stable on storage -Safe, lack of immune recognition	-Rely on cellular transport into nucleus -Lack tissue targeting -Transient gene expression -Inefficient delivery

Table 1.2 Advantages and disadvantages of different vectors in gene therapy (Kay et al., 1997).

line which contains and expresses a recombinant retroviral backbone expressing gag, pol, and env proteins which are essential for retroviral replication but does not contain the packaging signal. It is essential that the retroviral backbone and vector do not have any homology to prevent the production of wild type retroviruses (Logg *et al.*, 2002). The packaged vector is able to enter the nucleus and recombine into the chromosome of dividing cells (Miller, 1992). These vectors are not able to infect nondividing cells and hence are inappropriate for gene transfer to neuronal tissue *in vivo*. However, newer retrovirus vectors based on lentiviruses have been developed which also infect non dividing cells (Chang *et al.*,1999).

1.18.2 Adenoviruses

Adenoviruses have a large 35 kbp DNA genome and have been studied for their potential use in gene therapy. These viruses usually replicate as an episomal element in the nucleus and rarely, if ever, integrate into the host genome (Danthinne and Imperiale, 2000; Shalev *et al.*, 2001). Deletion of E1-E4 creates a adenovirus vector with a large capacity for foreign genes. The major limitation in the development of adenovirus is that despite the deletion of E1, leaky expression of late genes occurs leading to a host immune response (Yang *et al.*, 1994). However, these vectors can potentially be used for cancer therapy and vaccination where avoiding an immune response is not so important (Danthinne and Imperiale, 2000).

1.18.3 Adenoassociated viruses (AAV)

AAV is a small ssDNA defective parvovirus (Fink *et al.*, 1996). The AAV genome integrates site specifically into the short arm of chromosome 19 and as the vector does not

contain any wild type viral genes, is less immunogenic (Dong *et al.*, 1996). This virus will reside latently in the host genome until the cell is superinfected by a helper virus (Ponnazhagan *et al.*, 2001). However, due to its small genome size, only 1-2 kb can be inserted (Dong *et al.*, 1996). Integration can lead to prolonged expression from an AAV vector (Xiao *et al.*, 1996).

1.18.4 HSV-1

Two different types of HSV-1 vectors, defective and amplicon based have been developed. Amplicon based vectors consists of a bacterial plasmid that contains a copy of a HSV replication origin, a HSV packing sequence and the GI. 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 fact defective virions, containing multiple copies of the amplicon sequences and are able to infect mammalian cells (Table1.2) (Wang *et al.*, 2002). One problem is the difficulty in removing the helper virus.

Defective HSV-1 vectors are constructed by flanking the GI and usually a reporter gene with HSV-1 DNA sequences and recombining them back into the mutant virus at the desired site (Latchman, 2001). Such viruses are replication defective as they are deleted in an essential gene and are therefore produced in a complementing cell line. However, due to the toxicity of the remaining genes such viruses could not be used for gene therapy. To overcome this and create vectors with minimal toxicity, several genes, especially IE genes, are removed. For example deletion of IE genes ICP4, ICP27, ICP22, and ICPO will create a vector with

minimum toxicity (Krisky *et al.*, 1998; Lilley *et al.*, 2001). However, such a vector may be difficult to grow. An alternative approach is to delete only the essential ICP4 and ICP27 and mutagenise Vmw65 to downregulate expression of the other IE genes (see section 1.10.3; Thomas *et al.*, 1999b).

1.19 Non viral vectors

Non viral vectors, either naked plasmid DNA or packaged in liposomes can be used for transferring genes into cells. These vectors can be manipulated easily with standard molecular biology techniques and contain no packaging sequences required for viral vectors (Smith *et al.*, 1996). Foreign genes up to 48 kbp can be inserted into these vectors and hence it is possible to transfer large genes/regulatory sequences or a few smaller genes at the same time into the cell (Cotten *et al.*, 1990). Non viral vectors are also less immunogenic (Wells *et al.*, 2000). However, non viral vectors use the cellular machinery to transfer DNA into the cell and the nucleus which is possibly why transfection efficiency and hence protein expression are low (Smith *et al.*, 1996).

1.20 Cancer therapy using viral vectors

Although advances have been made in the treatment of many cancers by conventional means such as surgery, radiation and chemotherapy, this is not true for all tumour types. For example, in the case of malignant glioma, the lifespan of patients has not been significantly enhanced (Kirn *et al.*, 2001). The resistance of many cancers to conventional therapies has led to the search for novel strategies. One approach is based upon gene therapy with the introduction of "tumour killing" genes into tumour cells by means of disabled (usually

retroviral) vectors (Walther and Stein, 2000). Alternatively, the inherent cytotoxicity, spread from cell to cell and inherent tumour selectively of some viruses led to the hypothesis that replication competent viruses could be used for cancer treatment. Originally a number of wild type viruses such as vesicular stomatitis virus (VSV), Newcastle disease virus (NDV) and reovirus (Ahlert and Schirrmacher, 1990; Hashiro *et al.*, 1977; Stojdi *et al.*, 2000) were used to treat cancer but this approach was abandoned due to lack of tumour selective toxicity (Southam *et al.*, 1960).

More recently, recombinant DNA technology has led to the use of genetically engineered viruses for this antitumour approach. This is achieved by deleting viral genes necessary for efficient replication in normal cells but which are expendable in tumour cells, to generate tumour specific replication competent viruses. The use of tumour specific promoters and modification of the viral envelope to lead to selective uptake by tumour cells may ultimately increase the specifity of such replication competent viruses. Replication competent viruses can also be used to deliver "cytotoxic genes" to cells to increase their tumour killing potential. The first selectively replication competent genetically engineered virus for cancer therapy was tested in animal models in the early 1990s (Martuza *et al.*, 1991). Two viruses, HSV and adenovirus have been extensively used in this approach and several mutants are in clinical trials.

1.20.1 Adenoviruses

In normal cells both the adenovirus E1A and E1B 55K proteins are involved in binding and inactivating the cellular proteins pRB and p53, respectively, and thus allowing virus

replication to take place by forcing cells into S phase (Nevins and Vogt, 1996). Hence, in normal cells mutants in these viral genes will be unable to replicate. However, due to the defect in the control of cell proliferation, these two viral proteins are not required for adenovirus replication in many tumour cells. An E1A deletion mutant has been shown to replicate to wild type level in all cancer cells tested (Heise *et al.*, 2000) and one such mutant is now in phase I clinical trials. An E1B 55 K deletion mutant (ONYX-015) is able to replicate in p53 negative tumour cells (Bischoff *et al.*, 1996) and is now undergoing phase III clinical trials for head and neck tumours and phase I trials for several other tumour types (Nemunaitis *et al.*, 2000). ONYX-015 appears especially effective when given in combination with the chemotherapeutic agent cisplatin (Khuri *et al.*, 2000).

1.20.2 HSV

HSV encodes a large number of genes such as those involved in nucleotide metabolism, necessary for replication in nondividing neuronal cells but not in dividing cells including tumours. Mutants in these genes are usually highly attenuated (Efstathiou *et al.*, 1989). Early HSV tumour studies deleted genes involved in nucleotide metabolism, for example TK and RR (Cameron *et al.*, 1988). However, these mutants still exhibit some residual neuropathogenicity and although succesful at killing the implanted tumours in animal models would not be suitable for use in humans (Martuza *et al.*, 1991). Currently, research is based upon the development of viruses deleted in ICP34.5 which seem unable to replicate in any normal tissues *in vivo* (1.15.3) but retain the ability to replicate in tumour cells. Two HSV mutants G2O7 (Markert *et al.*, 2000) and 1716 (Rampling, 2000) are in phase I clinical trials for cancer therapy in patients with malignant glioma. In 1716, both copies of ICP34.5

are deleted whereas in G207 as well as both copies of ICP34.5 being deleted, RR has also been inactivated by the insertion of *lacZ* (Mineta *et al.*, 1995). No toxicity has been attributed to either of these viruses.

Several new HSV deletion mutants based upon ICP34.5 negative viruses are approaching the stage of clinical trials. The most promising candidate as well as having a direct cytopathic effect on the tumour will also invoke an antitumour immune response by expressing the IL-12 gene. In animal models, such an ICP34.5 negative mutant expressing murine IL-12 led to significantly longer survival times compared to treatment with the parental virus lacking the IL-12 gene (Parker *et al.*, 2000).

1.20.3 Aims

This project consisted of two main aims: i) *in vitro* characterization of the role of two HSV-1 overlapping antisense genes ORF P and ICP34.5; and ii) identification of both cellular and viral proteins which interact with ORF P.

i) To investigate the role of ICP34.5 and ORF P in the HSV-1 lifecycle, a number of HSV-1 recombinants were constructed and characterized *in vitro*. All recombinants were made in the background of the HSV-1 ICP34.5/ORF P null mutant, 1716 and express ICP34.5 or ORF P separately in nonessential loci.

ii) To investigate both cellular and viral proteins which interact with ORF P, GST pulldowns were carried out with a GST-ORF P fusion protein against infected cell extracts.

2.1 Materials

2.1.1 Bacteria

The *E.coli* strains used in this thesis were: i) BL21 [BF$^-$ dcm ompT hsds (rB-mB-) gal] (Stratagene); ii) C+ [BF$^-$ dcm ompT hsds (rB-mB-) gal] (Stratagene); iii) C41 [BF$^-$ dcm ompT hsds (rB-mB-) gal] (Stratagene); iv) Novablue [recAendALacIq] (Novagen); and v) XL-2blue, [recA1 end A1 gyrA96thi-1 hsdR17 supE44 relA1lac [F'proAB lacI^{q}ZΔM15 Tn10 (Tet^r) Amy Cam^r]] (Stratagene).

2.1.2 Bacteriophage

CE6 λ phage (Novagen) was used to express T7 RNA polymerase and induce expression of GST-ORF P in the Novablue *E.coli* strain (Studier and Moffatt, 1986).

2.1.3 Bacterial culture media

All strains were grown in L-broth (170mM NaCl, 10g/L bactopeptone, 5g/L yeast extract) or 2YT broth (85mM NaCl, 16g/L bactopeptone, 10g/L yeast extract). L-broth/ agar plates were made with 1.5% (w/v) agar in L-broth. Where necessary, media and L-broth/agar plates were supplemented with the appropriate antibiotic, e.g. 100ug/ml ampicillin.

2.1.4 Plasmids

The plasmids used in this thesis were: i) p35-ORF P (Holman, 2000), in which gD/ORF P was inserted into the HSV-1 UL43/43.5 genes in p35 (n.p. 91610-96751, MacLean, C. *et al.*, 1991) at the NsiI site (n.p. 94711) in both orientations; ii) pAT5.1 which contains a HSV-1 *Bam*HI/*Eco*RV fragment (n.p. 136289-139258) spanning the US5 gene cloned

into pAT153 (supplied by Dr A. MacLean); iii) GST (Pharmacia); GST-ORF P (supplied by Dr. A. MacLean) in which ORF P was inserted in frame at the 3' end of GST; v) GST-ICP27 (Bryant, 2000) in which ICP27 was inserted in frame at the 3' end of GST.

2.1.5 Tissue culture media

The following tissue culture media were used throughout this thesis.

ETC10: Eagle's medium (Life technology) supplemented with 10% (v/v) newborn calf serum (Life technology), 10% (v/v) tryptose phosphate broth (Busby *et al.*, 1964), 100 u/ml penicillin and 100mg/ml streptomycin (Life technology).

DMEM10: Dulbecco's modified Eagle's medium (Life technology) supplemented with 10% (v/v) foetal calf serum, 100 u/ml penicillin and 100mg/ml streptomycin (Life technology).

Emet/5C2: Eagle's medium containing one-fifth the normal concentration of methionine and 2% (v/v) newborn calf serum was used for radiolabelling cells.

EMC10: Eagle's medium containing 1.5% (w/v) carboxymethyl cellulose and 10% (v/v) newborn calf serum was used to overlay titrations to stop virus spread. If the cells were confluent, the percentage of calf serum was reduced to 5% (v/v) or 2% (v/v).

2.1.6 Cells

Baby hamster kidney 21 clone 13 (BHK) cells (Macpherson and Stoker, 1962) were grown in ETC10 medium. Human neuroblastoma (SK-N-SH) cells and mouse embryo fibroblast 3T6 cells (European Tissue Culture Collection) were grown in DMEM10.

2.1.7 Viruses

The wild type virus used was HSV-1 strain 17^+ (Brown *et al.*, 1973). The HSV-1 strain 17^+ deletion variant 1716 (MacLean, A. *et al.*, 1991) was used as the parental virus for making recombinant viruses and as an ICP34.5 negative control in many experiments. TsK, a HSV-1 (17+) mutant with a *ts* lesion in ICP4 (Preston, 1979) which results in overproduction of ORF P at the NPT was also used.

2.1.8 Antisera

The following antisera were used throughout this thesis: a polyclonal antiserum, 137, against a GST-ICP34.5 fusion protein (Brown *et al.*, 1997); two rabbit polyclonal antisera, 128 and 129, against a GST-ORF P fusion protein (provided by Dr. A. MacLean); an antipeptide serum against US11 (Johnson *et al.*, 1986); and a monoclonal antibody against GST (Sigma). Protein A-HRP (Sigma) and anti mouse IgG HRP (Sigma) congugated sera were used as secondary antibodies. Antibodies used to detect ORF P interactions with both cellular and viral proteins are shown in table 2.1.

2.1.9 Radiochemicals

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

[^{35}S]-methionine	[>1000 Ci/mmol]
[^{32}P]-dCTP	[3000 Ci/mmol]

Primary antibody	Type	Function	References
α CPSF (25 KDa)	Mouse	3' cleavage and polyadenylation of mRNA	(Maneley, 1995)
hnRNP K	Rabbit	Interacts with a number of cellular proteins involved in transcription, translation and signal transduction	(Bustello *et al.*, 1995)
CK II β sununit	Mouse	phosphorylates and modulates the function of a number of viral proteins	(Sanz-Ezquerro *et al.*, 1998)
Thymidine kinase	Mouse	Deoxypyrimidine kinase (phosphorylation of thimidine)	(Evans *et al.*, 1998
P32	Mouse	Involved in splicing	(Krainer, 1990)
SC35 [1]	Mouse	Splicing factor	(Sandri-Goldin et al., 1995)
hnRNP J	Mouse	Multifunctional protein involved in posttranscriptional regulation	(Bustello *et al.*, 1995)
α ASF	Mouse	Recognition and cleavage of 5' splice sites	(Krainer *et al.*, 1991)
ICP27	Rabbit	Multifunctional protein involved in posttranscriptional regulation	(Bryant *et al.*, 2000
α CFI (25 KDa)	Rabbit	3' cleavage and polyadenylation of mRNA	(Takagaki *et al.*, 1989)
α CFI (68 KDa)	Rabbit	3' cleavage and polyadenylation of mRNA	(Takagaki *et al.*, 1989)
α CPSF (30 KDa)	Rabbit	3' cleavage and polyadenylation of mRNA	(Maneley, 1995)

Table 2.1: Cellular and viral proteins whose *in vitro* interaction with ORF P was investigated. Identified proteins interacting with ORF P indicated [1].

2.1.10 Enzymes

Restriction enzymes were purchased from Boehringer Mannheim and New England Laboratories.

2.1.11 Membranes

Hybond-N (Amersham) was used for Southern and Northern blotting. Either Hybond-ECL (Amersham) or Immobilin-P (Millipore) was used for Western blotting.

2.1.12 Plasmid harvesting reagents

TE buffer:	10mM Tris HCl, 1mM EDTA, pH 7.5
Solution I	50mM glucose, 10mM EDTA, 25mM Tris HCl, pH 8 and lysozyme (4 mg/ml)
Solution II	0.2M NaOH and 1% (w/v) SDS
Solution III	3M KAc, pH 4.8

2.1.13 Tissue Culture Reagents

HEBS:	130mM NaCl, 4.9mM KCl, 1.6mM $NaHPO_4$, 5.5mM D-glucose, 21mM HEPES, pH 7.05
NTE buffer:	10mM Tris HCl, 10mM NaCl, 1mM EDTA, HCl pH 7.05
RSB	10mM KCl, 1.5mM $MgCl_2$, 10mM Tris, pH 7.6
PBS A	170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH 7.2
Trypsin	0.25% (w/v) trypsin in tris/saline containing (0.002%) phenol red, adjusted to pH 7.5 with $NaHCO_3$
Versene	0.6mM EDTA in PBS A, 0.002% phenol red

2.1.14 Agarose gel reagents

TBE: 89mM Tris, 89mM boric acid, 2mM EDTA.

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

2.1.15 Southern blotting reagents

Gel Soak I: 200mM NaOH, 600mM NaCl

Gel Soak II: 1M Tris HCl, 600mM NaCl, pH 8

20xSSC: 0.3M Na_3 citrate, 3M NaCl

Hybridization buffer: 0.5M NaP, 7% (w/v) SDS, pH 7.4

Washing solution: 2x SSC, 0.1% (w/v) SDS

2.1.16 Northern blotting reagents

10X MOPS 0.2M MOPS, 80mM sodium acetate, 10mM EDTA, pH 8

Denhardt's solution 1% (w/v) BSA, 1% (w/v) Ficoll, 1% (w/v) PVP

Northern blot prehybridization solution:

50% (v/v) formamide, 5X SSC, 5X Denhardt's, 50mM Na_2HPO_4, 0.1% (w/v) SDS, 100 µg/ml salmon sperm DNA, pH 7.0

Northern blot hybridization solution:

50% (v/v) formamide, 5X SSC, 2X Denhardt's, 5% (w/v) dextran sulphate, 0.5% (w/v) SDS, 250 µg/ml salmon sperm DNA, pH 7.0

RNA loading buffer	15% (w/v) Ficoll, 10mM Sodium phosphate, 1mM EDTA, 0.25% (w/v) bromophenol blue, pH 7.0

2.1.17 Small scale nuclear and cytoplasmic extracts

Buffer A	50mM Tris HCl, 5mM $MgCl_2$, 10% (v/v) NP40, pH 7.5
Buffer B	20mM Tris HCl, 420mM NaCl, 1.5mM $MgCl_2$, 0.5mM PMSF, (v/v) 25% glycerol 0.1% (v/v) 5mM β-mercaotoethanol, NP40, pH 8
Hypotonic lysis buffer	10mM Tris HCl, 10mM KCl, 2.5mM $MgCl_2$, pH 7.5

2.1.18 SDS-PAGE and Western blot reagents

BM:	3×BM: 1 ml SGB, 25% (w/v) SDS, 0.5 ml β-mercaptoethanol, 1ml glycerol, 0.25% (w/v) bromo phenolblue
RGB:	375mM Tris HCl, 0.1% (w/v) SDS, pH 8.9
SGB:	0.1M Tris HCl, 0.1% (w/v) SDS, pH 6.7
Acrylamide (single)	30% (w/v) acrylamide [29.25% (w/v) acrylamide, 0.75% (w/v) N,N'-methyllene bisacrylamide]
Acrylamide (gradient)	30% (w/v) acrylamide [28.5% (w/v) acrylamide, 1.5% (w/v) N,N'-methyllene bisacrylamide]
Coomassie stain	50% (v/v) methanol, 7% (v/v) acetic acid, 0.02% (w/v) Coomassie brilliant blue
Destain	5% (v/v) methanol, 7% (v/v) acetic acid
HEPES extraction buffer	50mM Hepes, 50mM NaCl, 0.1% (v/v) NP40, pH 7.5

Towbin buffer:	20% (w/v) methanol, 25mM Tris HCl, 192mM glycine, pH 8.3
Protein gel running buffer	0.05M Tris, 0.05M glycine, 0.1% (w/v) SDS

2.1.19 Immunoprecipitation buffer (Zweig's buffer)

0.1M Tris HCl, 10% (w/v) glycerol, 0.5% (w/v) NP40, 0.5% (w/v) deoxycholate, pH 8.0

2.1.20 Pull-down reagents

50mM Tris HCl, 0.5mM NaCl, 1mM EDTA, 0.5% (v/v) NP40 and Boehringer Mannheim protease inhibitors, pH 8.0

2.1.21 Chemicals

Chemicals used were analytical grade and mostly supplied from BDH chemicals, U.K or Sigma Chemicals Co. APS and TEMED were supplied from Bio-Rad Laboratories. Ampicillin (penbritin) was supplied from Beecham Research.

2.1.22 Autoradiography

Autoradiograph grade XS-1 film was supplied by Kodak. Autoradiographs were exposed for the appropriate time and developed in a Kodak-X-omat model ME-3.

2.1.23 Miscellaneous equipment

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

2.2 Methods

2.2.1 Small scale isolation of plasmid DNA

A single plasmid containing bacterial colony from a L-broth agar petri dish containing 100 μg/ml ampicillin was picked with an autoclaved toothpick and placed in a universal bottle containing 5 ml L-broth and 100μg/ml ampicillin. The bottle was placed in a 37^0 C shaker overnight. 1.5 ml of the culture was centrifuged at low speed for 1 min. The cell pellet was resuspended in 100 μl of solution I by vortexing and incubated at RT for 5 min. 200 μl of solution II was added, mixed by inversion several times and incubated at RT for 5 min. 150 μl of solution III was added, mixed by inversion, incubated at RT for 5 min and centrifuged at high speed for 5 min. The supernatant containing plasmid DNA was transferred to a fresh tube and mixed with 450 μl of phenol:chloroform (1:1). The samples were vortexed and centrifuged at 13,000 rpm for 3 min at RT. The aqueous layer was transferred into a fresh tube, mixed with 1 ml EtOH and centrifuged at 13,000 rpm for 5 min at RT. The DNA pellet was washed with 70% EtOH, dried for 1 h at RT, resuspended in 100 μl dH_2O containing 50 ug/ul RNase A and stored at -20^0C.

2.2.2 Large scale isolation of plasmid DNA

A 500 ml plasmid containing E. *coli* culture was grown in a 37^0C shaker overnight in L-broth containing 100 μg/ml ampicillin. Cells were pelleted by centrifugation at 7,000 rpm for 10 min at RT. The supernatant was discarded and the pellet resuspended in 10 ml Solution I by vortexing and incubated at RT for 5 min. 15 ml of solution II was added, mixed by inverting several times and incubated at RT for 5 min. 20 ml of solution III was added, mixed by inversion, incubated at RT for 5 min and centrifuged at 3,000 rpm for 15 min. The pellet was discarded and the supernatant containing plasmid DNA was transferred to a fresh Falcon tube, mixed with 45 ml of phenol and centrifuged at

3,000 rpm for 15 min. The aqueous layer was transferred into a fresh Falcon tube and the phenol extraction repeated followed by a chloroform extraction. The aqueous layer was transferred into a fresh tube, mixed with two volumes of EtOH by vortexing and centrifuged at 3,000 rpm for 20 min at RT. The pellet was washed with 70% EtOH and dried overnight at RT. The pellet was resuspended in 1ml dH_2O containing 50 ug/ul RNase A. This solution was considered as plasmid DNA and kept at -20^0C.

2.2.3 Restriction endonuclease digestion

Plasmid and viral DNA was routinely digested by different restriction endonucleases, using the buffer and temperature (usually 37^0C) recommended by the manufacturer. Enzyme activity was stopped either by phenol extraction or by adding RE stop to the mixture. Digestion was visualized by electrophoresis through an agarose gel.

2.2.4 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 at a concentration of 0.5μg/ml was added to the gel prior to pouring. The solution was poured into a sealed tray which held a comb and placed in an electrophoresis kit. Once the agar was cool, the comb was removed and the samples pipetted into individual wells. Electrophoresis of the samples was carried out by running the gel overnight and the gel visualized and photographed under shortwave U.V light.

2.2.5 Purification of restriction enzyme fragments from agarose gels

DNA was digested with the appropriate restriction enzyme and electrophoresed through an agarose gel contanining 0.5 μg/ml ethidium bromide until the fragment to be isolated

was well resolved. The gel was visualised under long wave UV light and the desired fragment excised using a sterile scapel. The gel slice was added to a filter tube (Costar) and centrifuged at 13,000 rpm for 15 min to elute the DNA. Residual DNA was eluted by adding an equal volume of 0.3M NaAc and recentrifuging. DNA was then phenol:cloroform extracted and EtOH precipitated.

2.2.6 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. The upper aqueous layer containing DNA was pipetted into a fresh tube and two volumes EtOH and 0.1 volume 3M NaAc added, vortexed and placed on dry ice for 20 min. The solution was centrifuged for 5 min at 13,000 rpm at RT. The supernatant was discarded, the DNA pellet left to dry and the pellet resuspended in dH_2O containing 50µg/ml RNase A.

2.2.7 Ligation of plasmid DNA

Different molar ratios of plasmid DNA to vector were added to a total volume of 14 µl. 4 µl 5x ligation buffer was added, the solution mixed and centrifuged to collect at the bottom of the tube. Lastly 2 µl T4 DNA ligase (3u/µl) was added to the solution which was mixed well and placed in a water bath at 16^0C overnight. The following day this was used in a transformation reaction.

2.2.8 Transformation of bacterial cells

5 ml L-broth or 2YT broth were inoculated with *E.coli* and grown overnight at 37^0C in a shaking incubator. 100 ml of fresh media were inoculated with 1 ml of the overnight culture and grown for approximately 3 h until an OD_{600} of 0.6-1.0 had been reached. The

cells were pelleted by centrifugation at 2,000 rpm for 15 min at 4^0C and the bacterial pellet resuspended in 50 ml 50mM $CaCl_2$. The bacteria were pelleted as before, resuspended in 10 ml 50mM $CaCl_2$ and incubated on ice for 30 min-24 h and used for transformation.

Alternatively, Ultracompetent *E.coli* (Strategene) were thawed on ice from -70^0C and 20 µl aliquoted into an eppendorf tube. 1 µg of plasmid DNA or 1µl of plasmid DNA solution was added, mixed and the tube placed on ice for 30 min followed by heat shocking in a 42^0C water bath for exactly 90 sec followed by placing on ice for 2 min. 80 µl 2YT broth was added and the reaction tube incubated for 1 h at 37^0C. After 1 h the mixture was pipetted and spread onto a L-broth agar plate containing the appropriate antibiotic (usually amp at 100 µg/ml). The 100 µl mixture was adsorbed on the agar for 15 min at RT before the plates were inverted and incubated in a 37^0C incubator for 16 h.

2.2.9 Growth and passaging of cells

T-175 flasks or roller bottles with confluent monolayers were opened in a category 2 microbiological safety hood, the supernatant decanted and 10 ml or 20 ml, respectively, of a trypsin/EDTA (0.25% v/v) solution poured over the monolayer and decanted. This was repeated and the flask or roller bottle left for 2 to 3 min. The cells were shaken into 10 or 20 mls media for further use. For use in experiments the cells were plated onto 130 mm ($2x10^8$ cells), 60 mm ($5x10^6$ cells) and 35 mm ($2x10^6$ cells) petridishes or Linbro wells ($5x10^5$ cells).

For passage, BHK cells, seeded at a 1 in 10 dilution, were grown in 100 ml ETC10 in 850 cm^2 roller bottles with 5% CO_2 at 37°C for 3 to 4 days. Confluent cells ($1x10^8$/roller bottle) were harvested as described previously.

3T6 cells seeded at a 1 in 4 dilution, were grown in 50 ml DMEM10 in large flasks (T-175) with 5% CO_2 at 37°C for 3 to 4 days. Confluent cells ($3x10^7$/T-175 flask) were harvested as described previously.

Human neuroblastoma (SK-N-SH) cells at a 1 in 4 dilution were grown in 50 ml DMEM10 in large flasks (T-175) with 5% CO_2 at 37°C for 3 to 4 days. Confluent cells ($3x10^7$/T-175 flask) were harvested as described previously.

2.2.10 Cryopreservation of cells

Confluent cell monolayers of SK-N-SH and 3T6 cells were harvested from T175 flasks as described above, resuspended in 15 ml media and pelleted in Falcon tubes at 3,000 rpm at 4°C for 10 min. Cells were resuspended in 5 ml of DMEM10 containing 10% (v/v) DMSO, aliquoted into 1 ml, frozen overnight at -70^{0}C and moved to a liquid nitrogen freezer (-140^{0}C) for long term storage.

2.2.11 Growth of HSV stocks

Roller bottles containing a fresh monolayer of BHK cell ($1x10^8$ cells/bottle) were infected with 0.01 pfu/cell of HSV-1 in 20 ml of ETC10 and incubated at 31^{0}C for 4 days or until c.p.e. was complete, when the cells were shaken into the medium and the cell suspension poured into a 250 ml plastic Falcon tube and the cells pelleted by centrifugation at 2,000 rpm for 10 min at 4^{0}C. The supernatant and cell pellet were

divided into 2 individual stocks. The cell pellet was resuspended in 5 ml medium/roller bottle, pipetted into a sterile universal bottle, thoroughly sonicated for 5 min in a sonibath and centrifuged at 2,000 rpm for 10 min at 4°C. The process was repeated, the pellet discarded and the two supernatants combined and aliquoted into 1.5 ml sterile cryovials, stored at -70°C and considered as cell associated virus (CA). The supernatant was poured into a 250 ml sterile centrifuge bottle and spun at 12,000 rpm for 2 h at 4°C. The supernatant was discarded and the virus pellet resuspended in 5 ml fresh ETC10 /roller bottle and sonicated until homogeneous. This was aliquoted into 1.5 ml sterile cryovials, stored at –70°C and considered as supernatant virus (SV).

2.2.12 Sterility checks on virus stocks

BHI plates containing 10% (v/v) horse blood (blood agar) were used to check for yeast or bacterial contamination in viral stocks. A small aliquot of a viral stock was streaked onto the medium plates which were sealed with parafilm and incubated at 37°C for 24-48 h. If there was no growth by 48 h, stocks were considered sterile.

2.2.13 Multicycle replication kinetics

35 mm petri dishes were seeded with $2x10^6$ cells/plate in 2 ml ETC10 and grown overnight at 37°C. Growth media was poured off and 100 ul of virus (0.0001 pfu/cell) added. The plates were returned to 37°C for 1 h. After 1 h the petridishes were washed with 2 ml PBSA/10% CS and overlaid with 2 ml ETC10. This was regarded as timepoint "O" h. Petri dishes were incubated at the appropriate temperature and harvested at the designated time points (usually 0, 6, 12, 24, 48, 72 and 96 h) by scraping the cell monolayer into the medium and transferring the suspension to a sterile bijou bottle

(Harland and Brown, 1985). The samples were sonicated and stored at -70^{0}C until the experiment was complete. Titration of samples was carried out as described below.

2.2.14 Virus titration

Virus stocks were serially diluted 10-fold in PBS 10% CS and 0.1 ml aliquots added to fresh monolayers of BHK cells on 60 mm petridishes from which the media had been removed. The petri dishes were incubated at 37°C for 1 h to allow adsorption to the cells, before overlaying with 4 ml EMC10. The petridishes were incubated at 37^{0}C for 48 to 72 h. The EMC10 was poured off each petri dish and the monolayer fixed and stained with Giemsa stain at RT for 1 h. After rinsing the petridishes with tap water and allowing them to dry, plaques were counted using a dissection microscope and virus titres calculated as pfu/ml.

2.2.15 Preparation of HSV virion DNA

Ten roller bottles containing a fresh monolayer of BHK cells (1x10^8 cells/bottle) were infected with 0.01 pfu/cell of HSV-1 in 20 ml ETC10. These were incubated at 31^{0}C 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 pelleted by centrifugation at 2,000 rpm for 10 min at 4^{0}C. The supernatant was kept on ice while nuclei were extracted from the cell pellet by treatment with 0.5% (v/v) NP40 in RSB buffer followed by centrifugation at 2,000 rpm for 10 min at 4^{0}C. The supernatant was kept on ice and the above process repeated before pooling the three supernatants and centrifuging at 12,000 rpm for 2 h at 4^{0}C. The supernatant was discarded and the virus pellet lysed by resuspension in NTE buffer and the addition of EDTA and SDS to a final concentration of 10 mM and 2% (w/v), respectively. Viral DNA was extracted 3 times with saturated

phenol and once with chloroform, 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 0.67 tube volume 70% (v/v) EtOH by gentle inversion and centrifuged as described above. The supernatant was discarded and the pellet air dried for 15 min at RT before redissoving in dH_2O containing 50 μg/ml RNase A.

2.2.16 Transfection of viral DNA

The growth medium was removed from 60 mm petri dishes containing $4x10^6$ cells and a solution containing HEBS (with 10μg/ml calf thymus DNA), 130mM $CaCl_2$ and HSV DNA (ranging from 0.2 to 2.0 μg) was added to each monolayer and incubated at 37^0C for 40 min, overlayed with ETC10 and incubated at 37^0C for 4 h. The medium was removed, the monolayers washed 1x with ETC10 and 1 ml HEBS (25% (v/v) DMSO) was added and incubated again at RT for 4 min. The medium was removed quickly, washed 2x with ETC10, overlayed with 4 ml ETC10 and incubated for 3 days at 37^0C until c.p.e was complete. The amount of HSV DNA giving the maximum level of c.p.e, was used to determine the amount of HSV DNA for use in marker rescue.

For marker rescue the same procedure was carried out but with a linearized cloned HSV fragment containing the desired mutation present in the transfection mixture at varying molar ratios, usually 1:1 to 50:1. Again when c.p.e was complete, after 3-4 days, infected plates were harvested and titrated.

2.2.17 Plaque purification

Serial 10-fold dilutions from either a transfection mixture or semipurified virus were made in PBS A/10%CS. 100 µl from each of the 10^2 to 10^6 dilutions were plated onto fresh BHK monolayers in 60 mm petri dishes from which the media had been removed. After 1 h adsorption at 37°C, the petri dishes were overlayed with 5 ml EMC10 and incubation continued at 37°C for a further 48 to 72 h. Using a 100 µl Gilson pipette, plaques from the monolayer with the fewest well separated plaques were picked, resuspended in 500 µl PBS A/10%CS and sonicated in a sonibath until homogeneous. This procedure was either repeated or a Southern blot performed to check the purity of the recombinant virus.

Occasionally, after plaque purification, recombinant viruses were further purified by limited dilution cloning. Here virus was 10 fold serialy diluted and each dilution was plated onto a 96 multiwell dish ($5x10^4$ cells). Wells from a plate where less than 33% of wells were infected were regarded as originating from a single particle and virus stocks prepared and the DNA profile analysed.

2.2.18 X-gal staining.

Recombinant viruses expressing ß-galactosidase were plaque purified by staining with X-gal. BHK cell monolayers were infected with virus and overlayed as described previously using EMC2 containing 150 µg/ml X-gal. Recombinant plaques were isolated on the basis of their blue staining

2.2.19 Infected cell DNA preparation

Linbro plates with BHK cell monolayers were infected at a moi of 5 pfu/cell at 31^{0}C. After 48 h the supernatant was removed and cells lysed by incubating with 2 ml lysis buffer (0.6% (w/v) SDS, 10 mM EDTA and 10 mM Tris HCl, pH 7.4) containig 500µg/ml protease for 4 h at 37^{0}C. Infected cell DNA was extracted twice with an equal volume of phenol and once with chloroform, precipitated by the addition of 2 volumes of EtOH, the pellet washed with 70% EtOH and air dried for 30 min before redissolving in dH_2O containing 50 µg/ml RNase A.

2.2.20 Southern blotting and hybridization

Purified DNA was digested with appropriate restriction enzymes 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 20x SSC for 1 h at RT.

A pack of 'Hi-dri' towels was stacked on the bench followed by 3 sheets of dry Watmann 3 MM paper. On top were 3 sheets of pre-soaked 3 MM paper and 1 sheet of nitro cellulose membrane (Hybond N) in 20 x SSC. The gel was placed on top, a glass plate and heavy weight placed over the top and 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 crosslink the DNA to the membrane. The membrane was allowed to dry, placed in a glass bottle and hybridization buffer and radiolabelled probe DNA 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, air dried, covered with cling film and exposed to XS1 film.

2.2.21 Radiolabelling DNA

Approximately, twenty five ng template DNA was made up to a final volume of 11 ul with dH_2O, 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 placed on ice while 4 µl of 5x High Prime reaction buffer containing the appropriate amount of enzyme (Boehringer Mannheim) and 2 µl (20µCi) [α^{32}P]dCTP] were added and the tube incubated for 30 min at 37°C. The reaction was stopped by placing the tube in a boiling water bath for 5 min. After boiling, the radiolabelled probe was used with the blot as described in the previous section.

For Northern blotting, the radiolabelled probe was purified from the unincorporated nucleotides by adding to a Mini quick spin RNA column (Roche) and spinning at 6 K for 4 min. The eluate was the purified probe.

2.2.22 Isolation of total RNA

Total cellular RNA was prepared by Trizol (Life technology) extraction according to the manufacturer's protocol. The cell monolayer was washed twice with PBS A and harvested in 5 ml Trizol by passing the cell lysate several times using a plastic pipette. The lysate was incubated 5 min at RT in a 10 ml Starsted tube. 1 ml chloroform was added, incubated 2-3 min at RT, spun at 2000 rpm for 15 min and the RNA containing aqueous upper layer transfered to a fresh tube. 2.5 ml of isopropanol was added and incubated 10 min at RT, followed by centrifugation at 2,000 rpm for 15 min. The

supernatant was discarded and pellet was kept in 75% EtOH at -70^{0}C until use. At this time the EtOH was removed by spinning at 2,000 rpm for 15 min. The pellet was air dried for 5-10 min at RT, dissolved in 100 µl dH_2O containing 0.5% (w/v) SDS at 60^{0}C for 10 min and stored at -20^{0}C.

For Northern blotting, the sample was further purified. 100 µl of phenol:chloroform (5:1) was added to the sample, shaken and spun at 2,000 rpm for 10 min. The top layer was transfered into a fresh tube, mixed with chloroform and spun again as before. The top layer was transfered into a fresh tube and treated with DNase solution (22 µl DEPEC dH_2O, 3 µl DNase I, 10 µl 10xbuffer) at 37^{0}C for 1 h. The sample was mixed with 100 µl of phenol:chloroform and precipitated at 12,000 for 10 min, kept at -70^{0}C and checked by loading on a gel containing 2.2% (v/v) formaldehyde, 1x MOPS, 1.2% (w/v) agarose and electrophoresised in 1x MOPS buffer at 140 mV until the dye front had migrated two thirds of the gel.

2.2.23 Northern blotting

10 ug of total RNA was dried under vacuum and resuspended in 15 ul of a buffer containing 50% (v/v) formamide, 16.6% (v/v) formaldehyde and 1x MOPS and incubated at 37^{0}C for 10 min. 3 ul RNA loading buffer was added, the sample boiled for 2 min and chilled on ice. The RNA samples were electrophoresed as described previously. The marker lane was removed and stained in 5 ug/ml ethidium bromide for 20 min, washed twice in dH_2O and left to destain overnight in 500 ml dH_2O.

The gel was washed three times with 2x SSC and the transfer set up. For this, a 14 x 9 cm horizontal gel slab tray to be used as a support was placed upside down in about 2 cm of

2x SSC in a tray. To form the wick to allow the 2x SSC to travel into the tower, two pieces of 14 x 18 cm 3 MM Whatman filter paper were pre-wet in 2 x SSC and laid over the support so the overhanging edges dipped into 2 x SSC. This was overlayed by four 14 x 9 cm 3 MM papers prewet in 2 x SSC. The gel slab was placed on the filter paper and a Hybond-N membrane, cut to the same size as the gel, placed on top. The tower was completed by the addition of 2 pieces of 3 MM papper cut to the same size as the gel and a stack of paper towels. A glass plate was placed on the top and the tower weighted down. At all times care was taken to ensure the removal of air bubbles. Transfer was left to occur overnight at RT.

To prepare the membrane for probing, the tower was disassembled and the nitrocellulose membrane left to air dry. The RNA was crosslinked to the membrane for 2 min in a UV cross linker. To probe the blot for specific RNAs, a column purified random primed probe was used. For this, the membrane was prehybridized for 4 h at 42^0C in 5ml of pre-hybridization buffer and at the end replaced by 2 ml hybridization buffer. The [^{32}P]-labelled DNA probe was denatured for 5 min at 100^0C, chilled on ice, added to the hybridization buffer and incubated with the membrane overnight at 42^0C. The blot was washed at 65^0C, 1x with 2 x SSC/0.1% (w/v) SDS and 1x with 1 x SSC/0.1% (w/v) SDS and monitored for radioactivity. If the blot was still fairly radioactive it was washed a further 1x with 0.5 x SSC/0.1% (w/v) SDS and 1x with 0.1 x SSC/0.1% (w/v) SDS at 65^0C. The blot was sealed in a heat sealable bag and exposed to X-Omat XS1 film at least overnight at -70^0C.

2.2.24 Extraction of infected cell proteins

60 mm plates containing $4x10^6$ cells were infected with 20 pfu/cell and incubated for the appropriate time (usually overnight) at 37°C. Samples were harvested by removing the media, washing the infected cell monolayers twice with cold PBS A and the monolayers extracted in 500 µl boiling mixture (BM/3). For immunoprecipitation experiments, the same procedure was carried out but the monolayers were harvested with 500 ul Zweig's buffer.

2.2.25 SDS-polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyse both viral and cellular proteins. Slab gels were cast vertically in a sandwich consisting of 2 glass plates separated by 1.5 mm perspex spacers. Either a single concentration gel ranging from 7.5% to 12.5% (w/v) acrylamide or a 5-12.5% (w/v) acrylamide gradient gel was used. The resolving gel was prepared using RGB with 0.05% (w/v) APS and 0.04% (v/v) TEMED. A 1 ml layer of butan-2-ol was poured on top to exclude air and enable polymerisation of the gel. Prior to adding the stacking gel, the butan-2-ol was removed by rinsing the surface of the running gel with dH_2O. The stacking gel was composed of 5% (w/v) acrylamide crosslinked with the same ratio of N,N-methylene-bisacrylamide used in the resolving gel, mixed with SGB, 0.05% (w/v) APS and 0.04% (v/v) TEMED. This was poured on top of the resolving gel, a comb installed and allowed to solidify for 30 min. Gels were electrophoresed either for 3 to 4 h at 50 mA, or overnight at 10 mA (Marsden *et al.*, 1978) and if appropriate prepared for Western blotting.

In addition to Western blotting, gels were used for analysis of protein expression both by Commassie blue staining and autoradiography. Gels were stained for 1 h at RT, destained

3x for 30 min, and if appropriate enhanced in 50 ml of En^{3}Hance (New England Nuclear) for 15-30 min, dried under a vacuum for 1.5 h at 80^{0}C and exposed to X-omat film at –70°C.

2.2.26 Western blotting

Samples were separated by SDS-PAGE as described above. Hybond-ECL membrane (Amersham) or Immunoblot (Millipore) and two 3 MM sheets were cut to the same size as the gel and presoaked in Towbin buffer. The proteins in the gel were transferred to the membrane for at least 3 h at 250 mA. After transfer, the membrane was blocked in PBS A/T (PBS A and 0.05% (v/v) Tween 20) containing 5% (w/v) Marvel milk for 1 h at RT. The primary antibody was diluted to the desired concentration in 25 ml PBS A/T/Marvel and incubated at either 37^{0}C for 2 h, RT for 2 h or 4^{0}C overnight. The membrane was washed 3 times in PBS A/T for 10 min at RT. The membrane was incubated in the appropriate HRP conjugated secondary antibody, diluted 1:1000 in PBS A/T/Marvel at RT for 1 h followed by 3 washes in PBS A/T for 10 min. A detection reagent (ECL) was added to the membrane for 1 min and the membrane exposed to XS-1 film for the appropriate amount of time.

2.2.27 Immunoprecipitation

Cell monolayers in 60 mm plates were infected with virus at a moi of 20. At the appropriate time(s) monolayers were harvested by washing with PBS A twice, adding 500 µl Zweig's buffer to each plate and incubating for 60 min at 4^{0}C. The extract from each plate was transferred into a bijou, sonicated for 3 min and spun for 3 min at 13,000 rpm. The supernatant was pipetted into a fresh eppendorf tube and either used immediately or stored at –70°C. 200-500 ul of the supernatant (cell extract) was mixed

with the appropriate volume of antibody and incubated at either 4°C overnight or 37°C for 2 h. 75 µl 50% (v/v), protein-A-sepharose in Zweig's buffer was added and the sample incubated on an end-over-end mixer for 45 min at 4°C. After that, the sample was centrifuged at 7,000 rpm for 1 min, the supernatant discarded and the pellet washed 3-4 times with Zweig's buffer. After the final wash, the pellet was harvested in 50 µl boiling mixture and analysed by SDS-PAGE.

2.2.28 Nuclear and cytoplasmic fractionation

Initially, the same method was used to extract nuclear and cytoplasmic fractions. However, due to contamination of nuclear with cytoplasmic material, different methods were subsequently used. Confluent monolayers in either 60, 90 or 130 mm plates were infected with virus at a m.o.i. of 20 pfu/cell at 37°C for 16 h. Cells were washed twice with PBS A and harvested by scraping the monolayer into PBS A.

To extract the cytoplasmic fraction, the monolayer was scraped into 1 ml PBS A. The cell suspension was poured into an eppendorf tube, briefly vortexed and spun at 6,000 rpm for 1 min at 4°C. The cell pellet was resuspended in 100µl buffer A containing 0.5% (v/v) NP40, incubated on ice for 15 min and homogenized on ice in a Dounce homogenizer. After incubation the samples were centrifuged at 6,000 rpm for 15 sec at 4°C. The supernatant was kept on ice while the pellet was resuspended with 100 µl buffer A containing 0.5% (v/v) NP40, incubated on ice for 2-3 min, spun at 6,000 rpm for 15 sec and the supernatant mixed with previous one and used as the cytoplasmic fraction.

To extract the nuclear fraction, the monolayer was scraped into 10 ml PBS A. The cell suspension was poured into a 15 ml Falcon tube, briefly vortexed and spun at 3,000 rpm for 5 min at 4^0C. The cell pellet was resuspended in 4 ml hypotonic lysis buffer (HLB) and incubated on ice for 15 min. After incubation the samples were centrifuged at 3,000 rpm for 5 min at 4^0C. The supernatant was left on ice while the pellet was resuspended with 1 ml supernatant and homogenized on ice in a Dounce homogenizer. The homogenized solution was mixed with the rest of supernatant in a 15 ml Falcon tube, underlayed with 1 ml HLB+10% (w/v) sucrose and respun at 3600 rpm for 5 min at 4 ^{0}C. The supernatant was discarded and the pellet containing the nuclei resuspended in 100 µl boiling mix. Samples were boiled for 10 min, analysed by SDS-PAGE and Western blotted.

2.2.29 Analysis of virus and host cell protein synthesis shutoff

Cells were either mock infected or exposed to 20 pfu/ml virus for 1 h at 37^0C. Growth media was added and petridishes incubated for 14 h at 37^0C. At 14 h pi, the media was poured off, the cells washed twice with 2 ml Emet/5C2 and overlayed with Emet/5C2 containing 50 uCi/ ml [^{35}S]-methionine for 2 h. At 16 h pi cells were washed twice with PBS A, harvested in boiling mixture, analysed by SDS-PAGE and autoradiography.

2.2.30 GST fusion protein growth and purification

A single freshly transformed colony was grown overnight in 5 ml 2YT containing 100ug/ml ampicillin. This culture was diluted 1/100 in 2YT containing 100ug/ml ampicillin and grown in a shaking 37^0C incubator to reach an OD_{600} of 0.6-0.1. To induce expression of the GST fusion protein, 0.2mM IPTG was added and the cultures placed back in the incubator for either 2 h at 37^0C, 4 h at 31^0C or overnight at RT. After

induction, the culture was centrifuged at 13,000 rpm at 4^0C for 5 min, the supernatant discarded and the pellet resuspended in 300 µl ice cold PBS A. Cells were lysed using a soniprobe and centrifuged at 13,000 rpm for 5 min at 4^0C to remove the cell debris. The supernatant was transferred to a fresh ependorf tube, 50 µl of 50% (v/v) glutathione agarose beads added to the supernatant and mixed end-over-end for 1-3 h at 4^0C. Samples were centrifuged at 13,000 rpm for 1 min at 4^0C, the supernatant discarded and 1 ml of PBS A added to the pellet, vortexed, and centrifuged for 1 min at RT. Washing was repeated 3 times and the pellet harvested in boiling mixture and analysed by SDS-PAGE or stored at 4^0C for use in a pulldown assay (2.2.32).

To induce expression of GST fusion proteins in Novablue strain, bacterial cultures were grown as described above but after reaching an OD_{600} of 0.6 to 1.0, superinfected with 2-4x10^9 pfu/ml (5-10 moi) of λCE6 phage at 37^0C for 3 h. After induction, the same procedure as above was carried out to harvest expressed protein.

2.2.31 Cell extract preparation for GST pulldown

For pulldown assays, soluble protein extracts were prepared from either labelled or unlabelled infected or mock infected cell extracts. For labelled extracts, proteins were labelled with 100µCi/ml [^{35}S]-methionine in E-met/5. Cell monolayers were washed twice with PBS A, 1.3 ml HEBS extraction buffer added for 30-60 min at 4^0C and cells harvested by scraping. The cells were lysed by sonication for 2 min in a sonibath and cell debris pelleted by centrifugation at 13,000 rpm for 2 min. The pellet was discarded and soluble protein extracts were stored at –70^0C.

2.2.32 Pulldown assay

Freshly prepared glutathione agarose beads with bound GST fusion proteins were mixed with 300 or 400 u l labelled or unlabelled cell protein extract and incubated for at least 3 h at 4^0C with continuous end-over-end mixing. The beads were harvested by centrifugation at 13,000 rpm for 1 min and washed 3 times in 1 m l HEBS extraction buffer containing varying amounts of NaCl (0.5-500mM). The beads were harvested in boiling mixture and either stored at -20^0C or boiled for 5 min, analysed by SDS-PAGE Gels were either fixed, dried and autoradiographed or used for Western blotting.

3.0 Construction and purification of HSV-1 recombinant viruses

3.1 Introduction

Using a HSV-1/HSV-2 intertypic recombinant virus, RE6, Thompson *et al.* (1983) mapped a HSV-1 neurovirulence locus to a region in the long repeat sequences. A number of genes are encoded by the HSV-1 long repeat between ICPO and the 'a' sequence (see chapter 1.15.1). In 1986, Chou and Roizman demonstrated that in HSV-1 strain F, the "a" sequence contains the promoter and transcription initiation site of a gene which was subsequently identified by Ackerman *et al.* (1986). Using an antiserum directed against a synthetic peptide containing a Proline-Alanine-Threonine (PAT) repeat from within the putative open reading frame (ORF), these workers identified that this ORF expresses a protein named ICP34.5. Dolan *et al* (1992) subsequently named this ORF in HSV-1 strain 17^+ RL1. 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* (Brown *et al.*, 1994a; MacLean, A. *et al.*, 1991; Thompson *et al.*, 1989; Chou *et al.*, 1990). Thus ICP34.5 appear to encodes the virulence locus originally identified by Thompson *et al* in 1983. The experiments could not discount the possibility that the later identified ORF O and P were responsible for the phenotype observed.

In 1994, Lagunoff and Roizman demonstrated that there are 16 ORFs (ORFs A-P) located in the opposite direction to RL1 within the LAT transcript in the long repeat sequences in strain F. Two of these ORFs (O and P), map almost entirely antisense to RL1. However, both ORF O and P are not expressed from LAT but from their own promoter and associated RNA internal to and 3' coterminal with LAT. This transcript is known as the L/ST transcript (Yeh and Schaffer, 1993). Binding of ICP4 to a consensus ICP4 binding site, ATCGTC, upstream of the ORF O/P transcription initiation site completely represses this transcript during

productive infection (Lagunoff and Roizman, 1995). Using a virus with a mutation in the ICP4 binding site of the putative ORF O/P promoter which causes repression of ORF O and P transcription, Lagunoff and Roizman in 1995 demonstrated that these two ORFs express proteins.

ORFs O and P are not expressed during productive infection in Vero cells by HSV-1 strain F at its permissive temprature of 37^0C, but are detectable following infection of F at its non-permissive temperature of 39.5^0C as ICP4 is temperature sensitive in this strain (Lagunoff and Roizman, 1994; Lagunoff *et al.*, 1996). Similarly, infection with a HSV-1 strain 17^+ mutant (*ts*K) with a temperature sensitive (*ts*) lesion in ICP4 results in overexpression of ORF P at the non permissive temperature (NPT) of 38.5^0C. This virus is used as a control for expression of ORF P (chapter 4). The functions of ORFs O and P are not well underestood and are discussed in chapter 1.

A HSV-1 strain 17^+ spontaneous deletion mutant, 1716, which has 759 bp of the long repeat region deleted, has been isolated and characterized (MacLean, A. *et al.*, 1991). In 1716 both copies of ICP34.5 and ORFs O and P in the inverted repeat sequences are deleted. The mutant showed major differences in behaviour both *in vivo* and *in vitro* compared to 17^+ (see 1.15.3). To assign the phenotypes of this mutant to either ICP34.5 or ORF O and P, a number of recombinant viruses based on 1716 were constructed. In these mutants either only ICP34.5 or ORF P were inserted singly within 1716 or both were inserted independently. In most cases the site of insertion was in the non essential UL43/43.5 locus at the 5' end of

UL43. The site of insertion was at a unique *Nsi*I site which had been modified to include *Spe*I and *Bgl*II sites in a plasmid, p35 (pacI) (Fig.3.1).

3.2 1622

3.2.1 Introduction

This recombinant virus was constructed by Holman (2000) in the background of 1716 and expresses both ICP34.5 and *LacZ* in the UL43/43.5 locus (Fig.3.2). In this study 1622 was used for two reasons. Firstly, to analyse the role of ICP34.5 *in vitro* in the context of a virus which does not express ORF P. Secondly, to construct a further mutant, 1625, in the 1622 background (section 3.5).

3.2.2 Isolation and purification

This mutant was constructed and purified by Holman (2000). Its purification was based on its ability to express β-galactosidase that turns its substrate, X-Gal, blue. To check its purity, further plaque purification (section 2.2.17) was carried out. After several rounds of plaque purification, the DNA of some of the best isolated blue plaques was analysed by Southern blotting.

DNA was digested with *Bam*HI and Southern blotted with a fragment spanning the site of insertion (p35). The structure of 1622 is shown in Fig.3.2b and 3.3a and the resultant Southern blot in Fig.3.3b. Insertion of ICP34.5/*LacZ* introduces a new *Bam*HI site into UL43/43.5 of 1622 (Fig 3.3a). Following digestion with *Bam*HI, 17^{+} (and 1716) with no insertion gives a 6.6 kbp band (Fig.3.3b, lane1) whilst 1622 gives 2 bands of 4.5 and 7.8. kbp. As this figure shows, 1622 had been succesfully purified (Fig.3.3b, lanes 2 and 3).

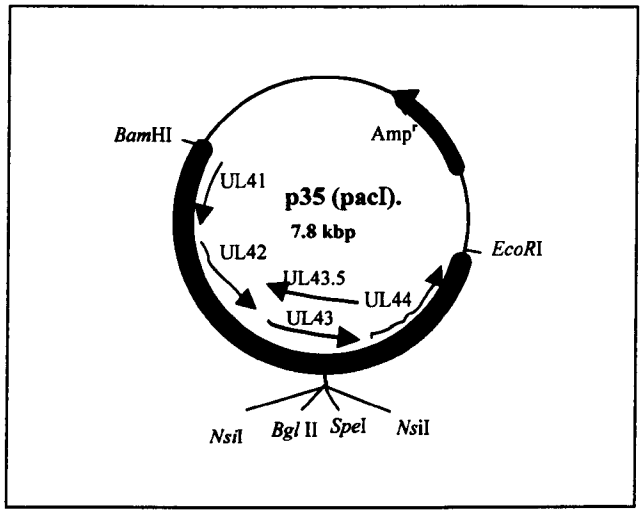


Figure 3.1 Structure of p35 (pacI)

p35 (pacI) containing a 5.1kbp (n.p 91610-n.p. 96751) *Bam*HI/*Eco*RI fragment spanning HSV-1 genes UL41 to UL44. *Spe*I and *Bgl*II sites were inserted into the unique *Nsi*I at the 5' end of UL43. The HSV-1 genes with their orientations are indicated.

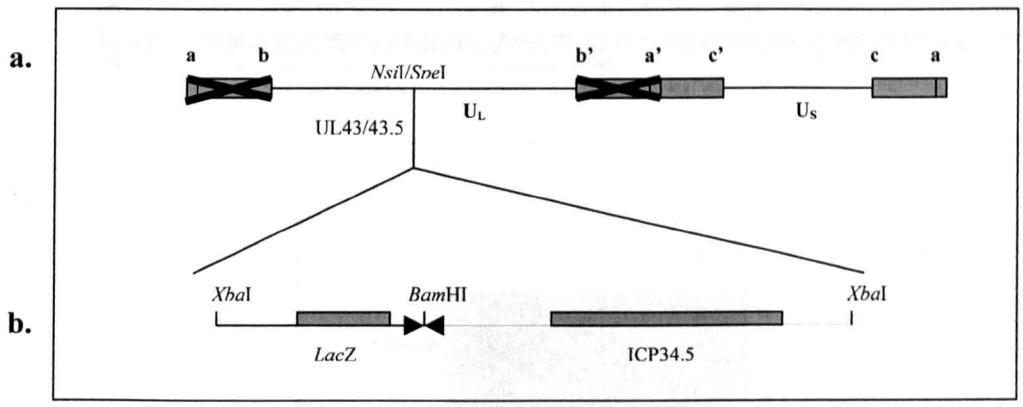


Figure 3.2 Structure of 1622

a. This figure shows the structure of 1716. "a" is a direct repeat sequence present at both termini and in inverted orientation "a" in the internal repeat. "b" refer to the long and "c" to the short repeat. "X" indicates the deletion of ICP34.5/ORF P. The position of the *Nsi*I site plus additional *Spe*I site in UL43/43.5 is shown.

b. This line shows the structure of 1622. ICP34.5/*LacZ* containing an internal BamHI site was inserted into UL43/43.5 with ICP34.5 in the UL43.5 and *LacZ* in the UL43 orientation. The arrow heads indicate the orientation of transcription.

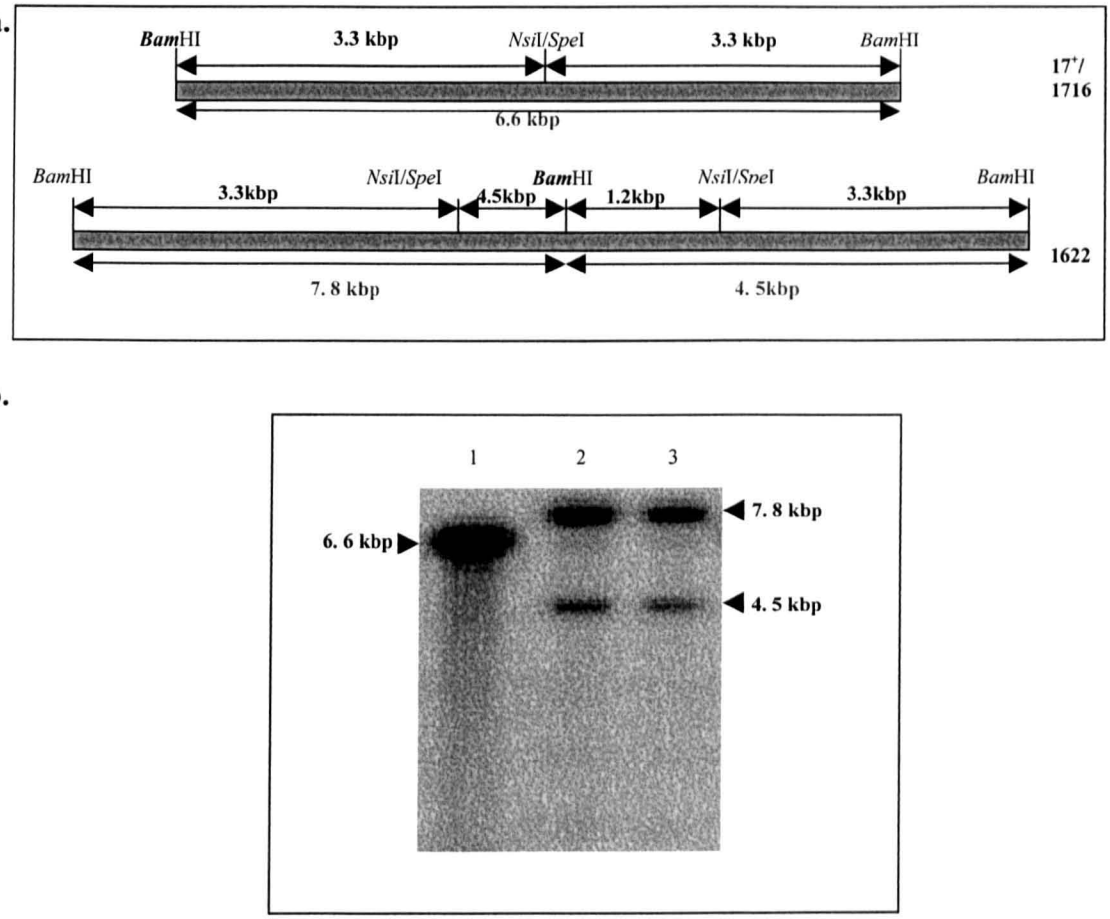


Figure 3.3 Purification of 1622

a. A linear diagram illustrating the *Bam*HI fragment spanning UL43 in wild type and recombinant viruses, the site of insertion at the *Nsi*I/*Spe*I sites, and sizes of *Bam*HI fragments following digestion.

b. A Southern blot of HSV DNA digested with *Bam*HI and probed with [^{32}P]dCTP labelled p35 and exposed to an autoradiograph for 24h at -70^{0}C . Lane 1: 17^{+}; lane 2: the original 1622; Lane 3: purified 1622. The sizes of the bands are marked and determined from a 1 kbp ladder (not shown). Hybridising bands are indicated (▶).

Eventually, one completely purified plaque was isolated and used to prepare a viral stock. Previously, Holman (2000) had determined the orientation of ICP34.5/*Lac*Z and shown that ICP34.5 was transcribed in the UL43.5 orientation and *Lac*Z in the UL43 orientation.

3.3 1624

3.3.1 Introduction

In order to examine the role of ORFs O and P in the replication of HSV-1 *in vitro*, two recombinant viruses, 1624, and 1624.5 (Fig.3.4) were constructed, using 1716 as parent. The ORF O and P genes were reintroduced under the gD promoter in a novel location in UL43/43.5 in 1624 and 1624.5. ORFs O and P should be expressed with the early/late kinetics of gD since the ICP4 binding sequence is not present. In 1624, ORFs O and P are transcribed in the same orientation as UL43 (Holman, 2000) and in 1624.5 the same orientation as UL43.5. These mutants should express only ORFs O and P, and not ICP34.5. Thus, the effect of expression of only ORFs O and P or any synergistic effect of ORFs O and P and ICP34.5 could be elucidated.

3.3.2 Insertion of gDORF P in the UL43/43.5 locus

For insertion of ORF P into UL43/43.5, several stages of construction were carried out. Firstly, two primers complementary to the N and C termini of ORF P with terminal *Eco*RI and *Bam*HI sites respectively were synthesised and a 705 bp ORF P fragment generated (Ross Reid, personal communication). This fragment was inserted into the *Eco*RI/*Bam*HI sites of pGEX 5X1 to generate pGEX/ORF P. Using pGEX/ORF P the ORF P gene was isolated by *Sma*I/*Bam*HI digestion (Fig.3.5a) and ORF P inserted into the compatible *Sma*I/

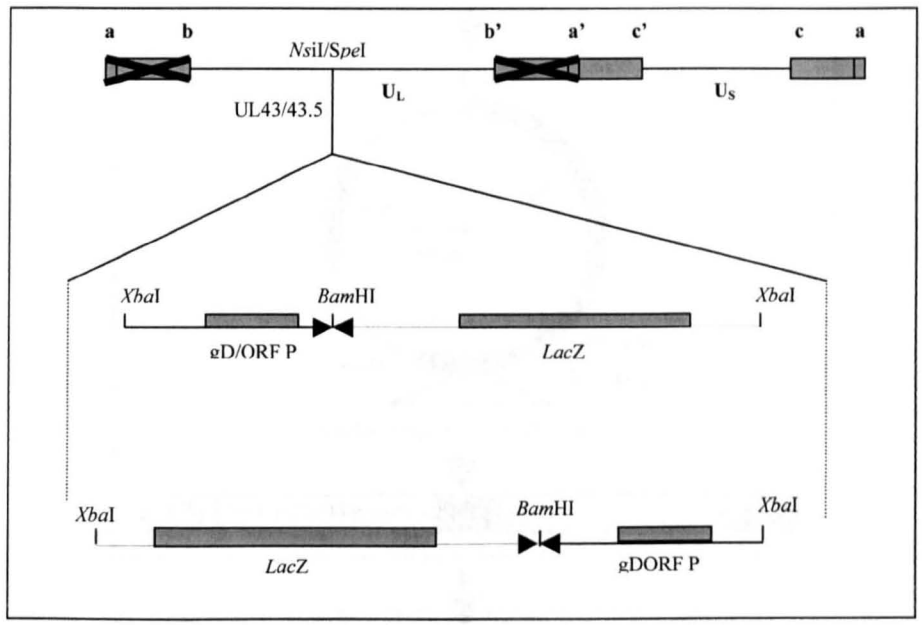


Figure 3.4 Structure of 1624/1624.5

a. This line shows the structure of 1716. "a" is a direct repeat sequence present at both termini and in inverted orientation"a'" in the internal repeat. "b" refer to the long and "c" to the short repeat. "X" indicates the deletion of ICP34.5/ORF P. The position of the *Nsi*I site plus additional *Spe*I site in UL43/43.5 is shown.

b. This line shows the structure of 1624. gDORF P/*LacZ* containing an internal *Bam*HI site was inserted into UL43/43.5 with gDORF P in the UL43 and *LacZ* in the UL43.5 orientation. The arrow heads indicate the orientation of transcription.

c. This line shows the structure of 1624.5. gDORF P/*LacZ* containing an internal *Bam*HI site was inserted into UL43/43.5 with gDORF P in the UL43.5 and *LacZ* in the UL43 orientation. The arrow heads indicate the orientation of transcription.

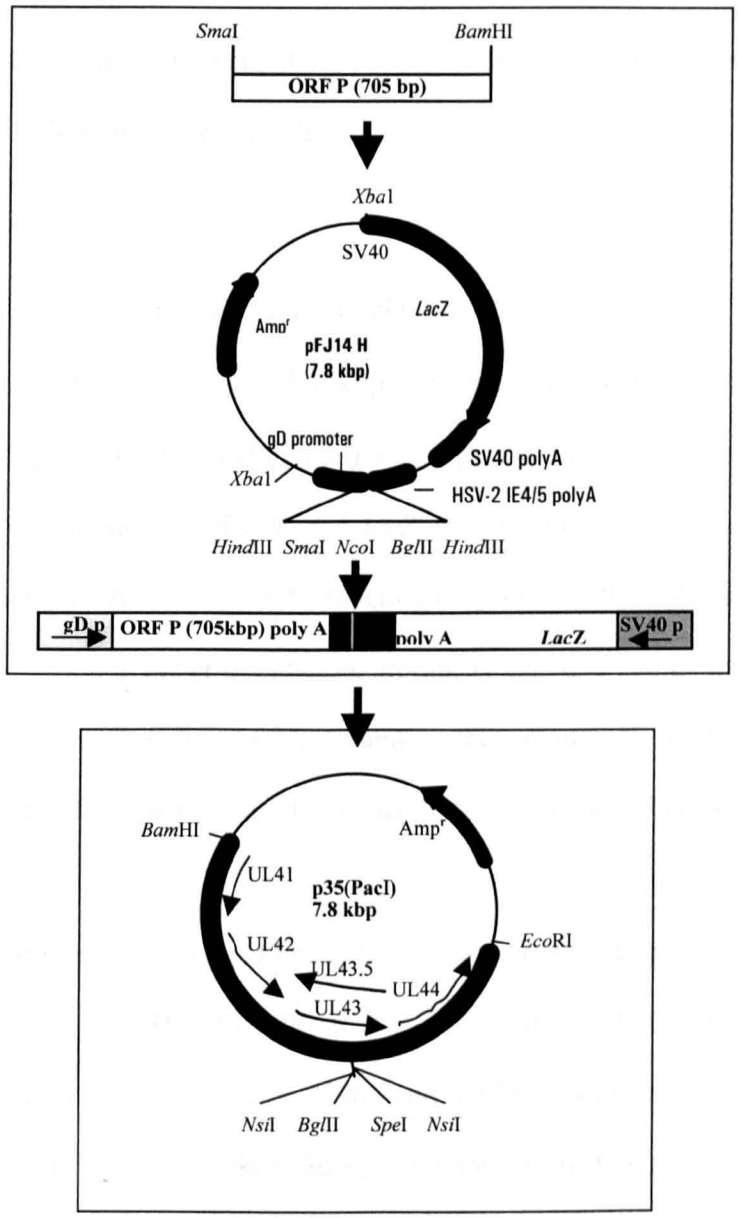


Figure 3.5 Subcloning of gDORFP into p35 (pacI) .

a. Diagram of the PCR generated 705bp fragment spaning ORF P isolated from pGEX5X1. There is a *Sma*I site at the N terminal and a *Bam*HI site at the C terminal.

b. ORF P is inserted into the *Sma*I/*Bgl*II sites of pF14H under the gD promoter.

c. A *Xba*I fragment containing gDORF P and *LacZ* is isolated from pF14H/ORF P.

d. This fragment is inserted into the *Spe*I site of p35 (PacI).

*Bgl*II sites in pFJ14H under the HSV-1 gD promoter and upstream of the HSV-2 immediate early IE5 poly A sequence (Holman, 2000; Fig.3.5b). This plasmid also contains *LacZ* under the control of a SV40 promoter and a SV40 poly A sequence in the opposite orientation to the ORF P gene.

pFJ14H was digested with *Xba*I and the gDORF P/*LacZ* containing fragment isolated (Fig.3.5c). P35 (pacI) containing a 5.1 kbp *Bam*HI/*Eco*RI fragment (n.p 91610-96751) spanning HSV-1 genes UL41-44 (Fig.3.5d) was digested with *Spe*I (in the linker) and the *Xba*I fragment containing gD/ORF P and *lacZ* inserted into the compatible *Spe*I site in UL43/43.5 (MacLean, A. *et al.*, 1991; McGregor *et al.*, 1999; Holman, 2000). Ligation could result in insertion in either orientation, arbitrarily called A and B. In the orientation A, ORF P is in the same direction as UL43 and in the orientation B, the same as UL43.5 (Fig.3.6a, b). These plasmids were used to generate 1624 and 1624.5 respectively.

To determine the orientation of gD/ORF P and *LacZ*, both plasmids were digested with *Eco*RI/*Bam*HI and *Bam*HI/*Bgl*II. In the A orientation, following digestion with *Eco*RI/*Bam*HI, bands of 5.95, 3.7, 2.7, 800bp and 450bp should be generated and in the B orientation bands of 7.35, 2.7, 2.2kbp, 800bp, and 450 bp. In Fig.3.6c, lanes 1 and 3 these bands can be seen confirming the structure of these plasmids. On *Bam*HI/*Bgl*II digestion, in the A orientation bands of 9.0, 3.3, and 1.2 kbp and in the B orientation, bands of 5.7, 4.5, and 3.3 kbp should be generated. In Fig.3.6c, lanes 3 and 4 these bands can again be seen confirming the structure of these plasmids.

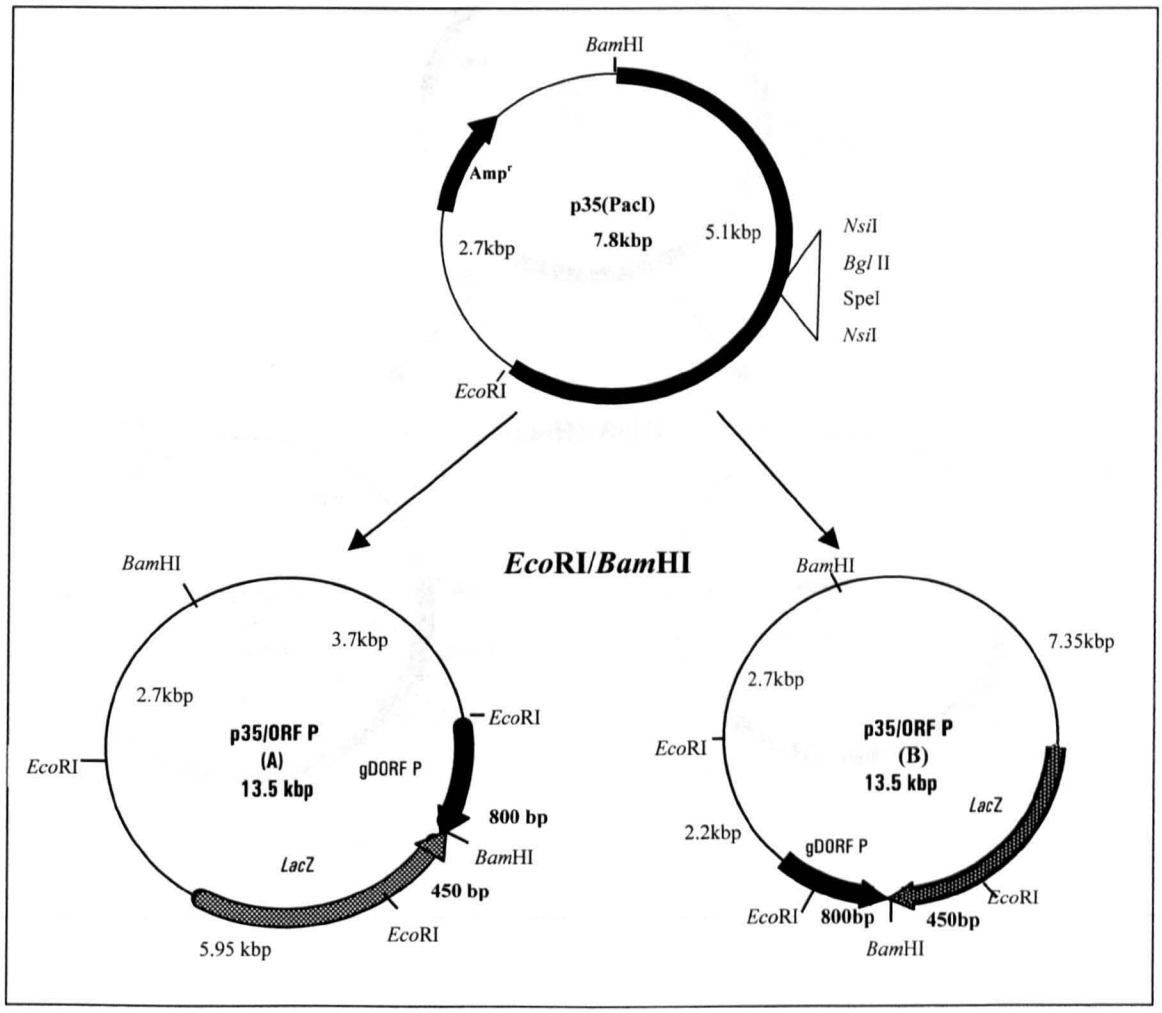


Figure 3.6a Orientation of gDORF P/ *LacZ* in p35/ORF P

i) p35 (PacI) containing a 5.1kbp *Bam*HI-*Eco*RI HSV-1 fragment spanning genes UL41-UL44 (in bold). The multicloning site in UL43 containing *Nsi*I/*Bgl*II/*Spe*I sites is shown

ii) The 5.7 kbp gDORF P (bold) /*LacZ* (stiple) fragment is inserted into the *Spe*I site in p35 in either orientation. The *Bam*HI/*Eco*RI sites and resultant fragments used to determine the orientation are marked. The arrow heads indicate the orientation of transcription.

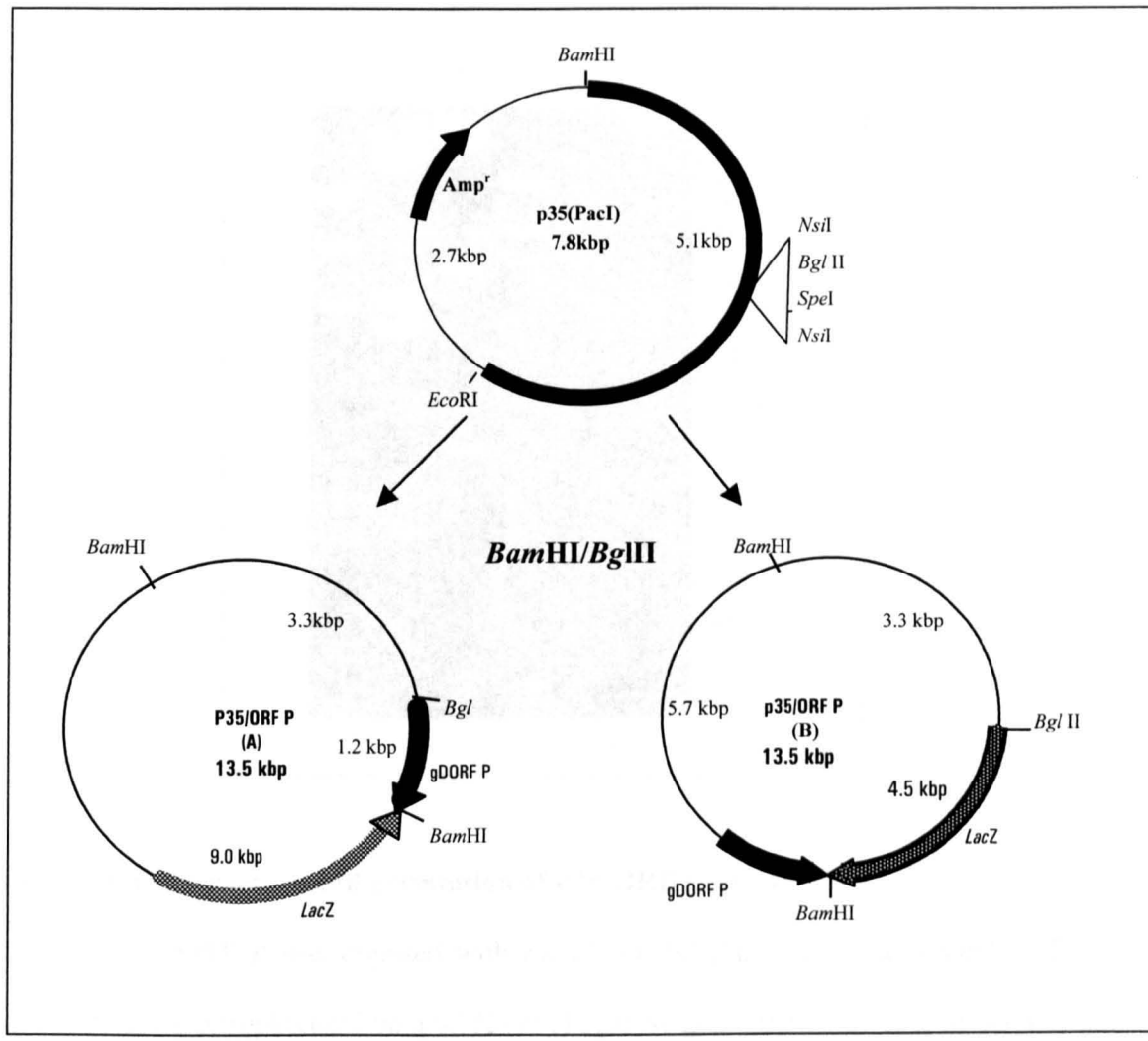


Figure 3.6b Orientation of gDORF P/ *LacZ* in p35/ORF P

i) p35 (PacI) containing a 5.1kbp *Bam*HI-EcoRI HSV-1 fragment spanning genes UL41-UL44 (in bold). The multicloning site in UL43 containing *Nsi*I/*Bgl*II/*Spe*I sites is shown.

ii) The 5.7 kbp gDORF P/*LacZ* (stiple) fragment is inserted into the *Spe*I site in p35 (PacI) in either orientation. The *Bam*HI/*Bgl*II sites and resultant fragments used to determine the orientation are marked. The arrow heads indicate the orientation of transcription.

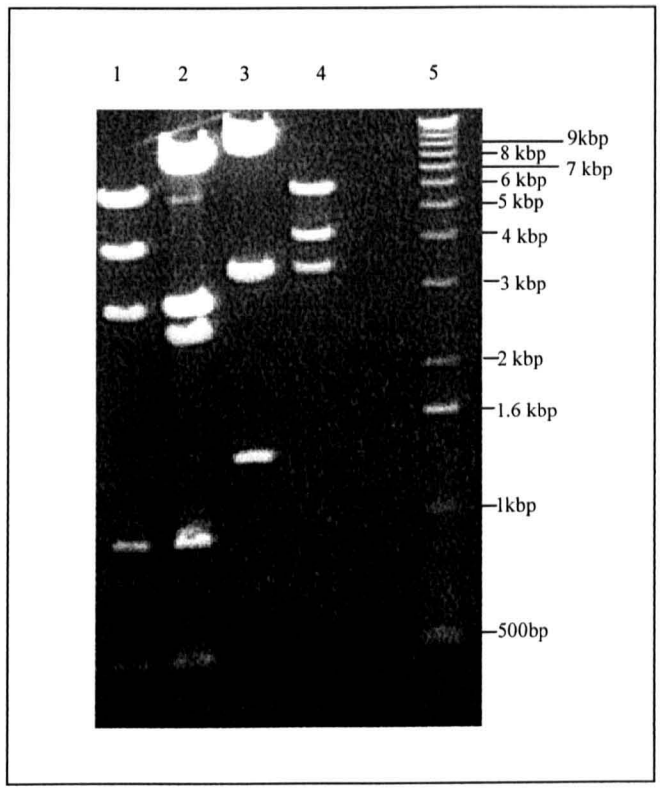


Figure 3.6c The structure and orientation of P35/ORF P (A, B)

DNA from p35/ORF P was digested with *Eco*RI/*Bam*HI (lanes 1, 2) or *Bam*HI/*Bgl*II (lanes 3, 4) and electrophoresed on a 0.7% (w/v) agarose gel. Orientation A is in lanes 1, 3 and orientation B in lanes 2, 4. A 1 kbp ladder is shown in lane 5. The maps of these plasmids are shown in figure 3a (*Eco*RI/*Bam*HI) and figure 3b (*Bam*HI/*Bgl*II).

A transfection with 1716 DNA and p35/ORF P (A) (Fig.3.7) had previously been performed by Holman (2000), but no purification had been carried out. Purification of 1624 was based on its ability to express *β-galactosidase* and turn its substrate, X-Gal, blue. After several rounds of plaque purification (section 2.2.17), the DNA of some of the best isolated blue plaques was analysed by Southern blotting.

DNA was digested with *Bam*HI and Southern blotted with a fragment spanning the site of insertion (p35) . The structure of the recombinant virus, compared with its parent, 1716, is shown in Fig.3.8a and the resultant Southern blot in Fig.3.8b. Insertion of gDORF P/*LacZ* introduces a new *Bam*HI site into UL43/43.5 of 1624 (Fig.3.8a). Following digestion with *Bam*HI, 17^+ (and 1716) containing no insertion give a 6.6 kbp band (Fig.3.8b, lane 1) whilst 1624 with an insertion containing a *Bam*HI site should give 2 bands of 4.5 and 7.8. kbp (Fig.3.8b, lane 3). As this figure shows, during purification the wild type 6.6 kbp band disappears and is replaced by two bands of 4.5 and 7.8 kbp, indicating the purity of 1624. The profile of DNA from the original transfection to generate 1624 is shown in lane 2. Only the 6.6 kbp is seen, indicating a low percentage of 1624 is present in the transfection mixture. Eventually, one completely purified plaque was isolated and used to prepare a viral stock.

3.4 1624.5

3.4.1 Introduction:

Holman (2000) had previously initiated construction of 1624 in which ORF P sequence inserted in the UL43 orientation (Fig.3.4b). After purification and during characterization, growth of the 1624 mutant in BHK cells was impaired compared to the growth of 17^+, 1716

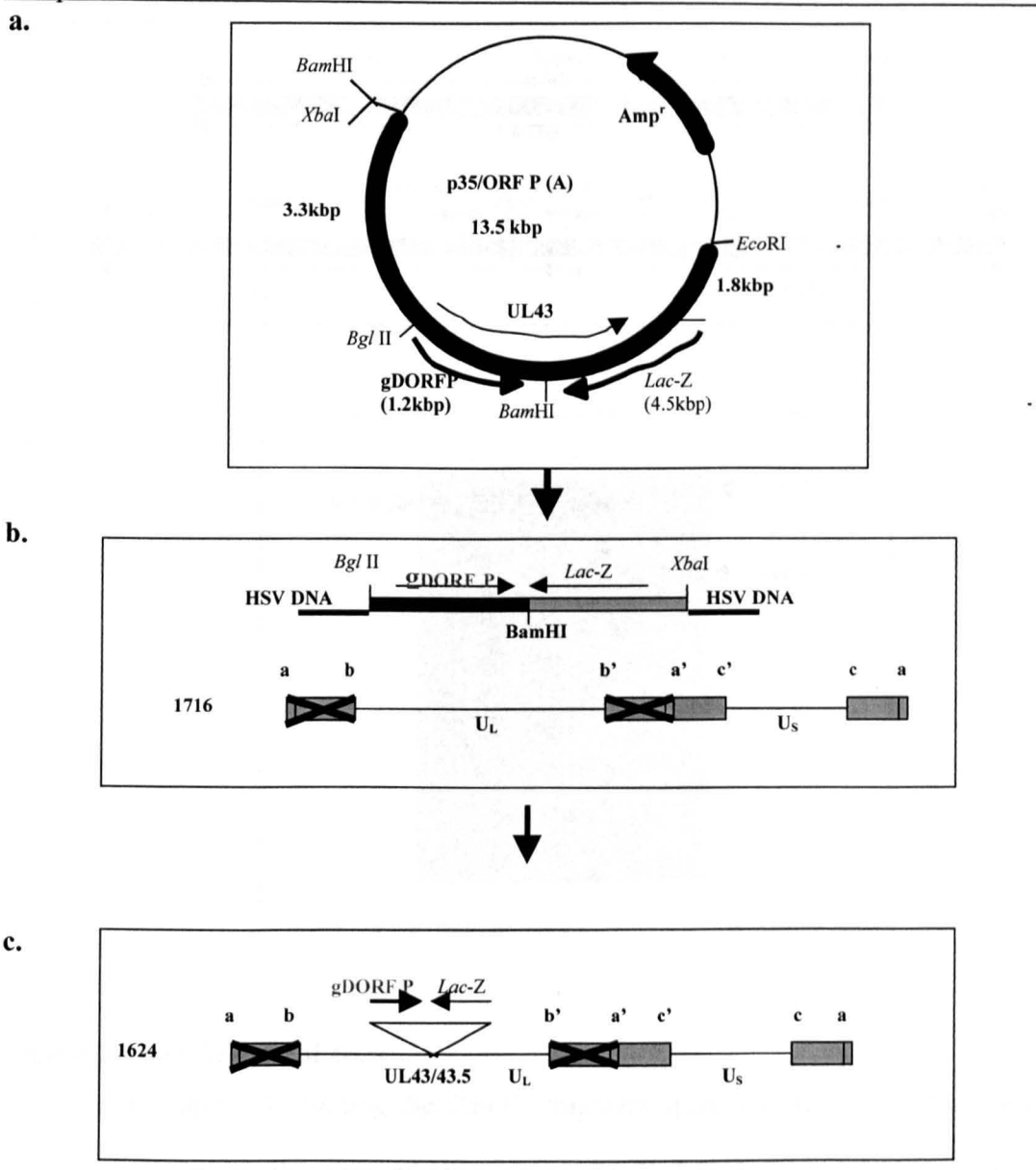
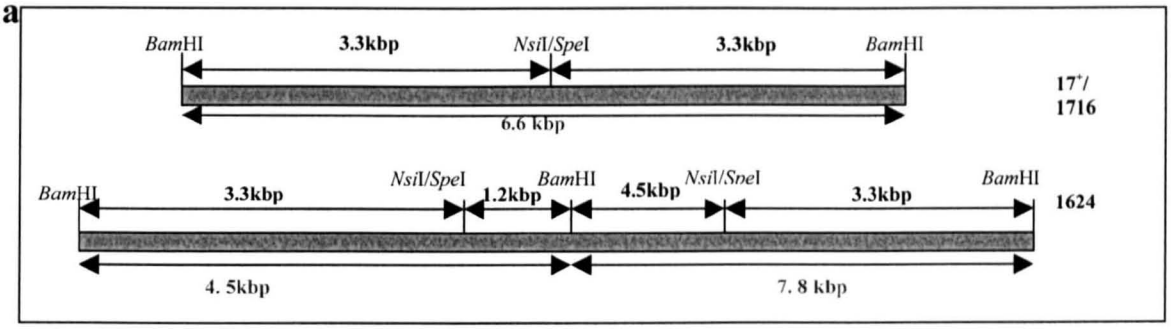


Figure 3.7 Construction of 1624

a. p35/ORFP (A) containing gDORF P /*LacZ* with ORF P in the same orientation as UL43 and *LacZ* in the same orientation as UL43.5 was digested with *Xba*I to isolate gDORF P/*LacZ*. The arrow heads indicate the orientation of transcription.

b. gDORF P/*LacZ* with flanking HSV-1 DNA was recombined with 1716.

c. The structure of 1624 is shown. The arrow heads indicate the orientation of transcription.



b.

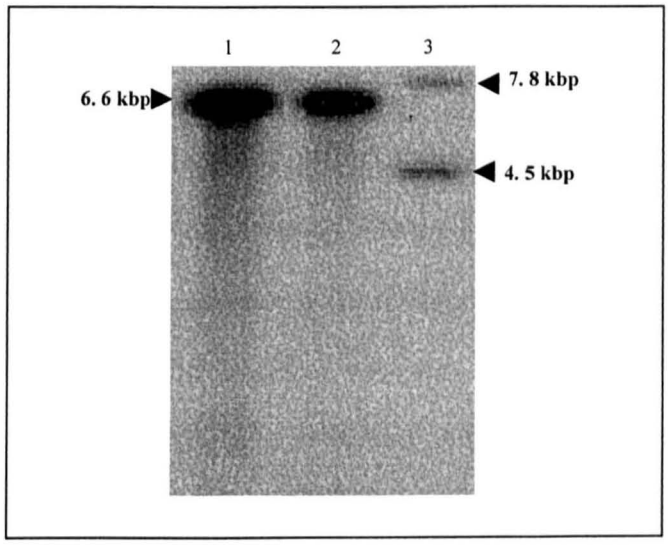


Figure 3.8 purification of 1624

a. A linear diagram illustrating the *Bam*HI fragment spanning UL43 in wild type and recombinant viruses, the sites of insertion at the *Nsi*I/*Spe*I site, and size of *Bam*HI fragments following digestion.

b. A Southern blot of HSV DNA digested with *Bam*HI and probed with [^{32}P]dCTP labelled p35 and exposed to an autoradiograph for 48h at -70^{0}C. Lane 1: 17^{+}; lane 2: the original 1624 transfection; lane 3: purified 1624. The sizes of the bands are marked and determined from a 1 kbp ladder (not shown). Hybridizing bands are indicated (▶).

and 1622. In addition, 1624 seemed to express at best only low levels of ORF P. Based on Holman's (2000) preliminary finding that expression in the UL43.5 orientation is stronger than expression in the UL43 orientation, we decided to construct a mutant, based on 1716, that ORF P sequence inserted in the UL43.5 orientation and named this mutant 1624.5 (Fig.3.4c).

3.4.2 Construction:

Fig.3.9 shows the strategy for construction of 1624.5. P35/ORF P (B) in which gD/ORF P is inserted in the same orientation as UL43.5 was used to construct this mutant (Fig.3.9a). This plasmid was digested with *Xba*I to generate a linearized fragment containing gD ORF P/ *Lac*Z with flanking HSV DNA. This fragment was recombined with 1716 (Fig.3.9b) to generate 1624.5 in which gDORF P/*LacZ* was inserted in UL43/43.5 of 1716 with ORF P being expressed in the UL43.5 orientation (Fig.3.9c).

3.4.3 Isolation and purification:

1624.5 was isolated on the basis of its ability to express β-galactosidase and turn its chromatogenic substrate, X-Gal, blue. 1624.5 was purified from its parent as described in section 2.2.17 through several rounds of plaque purification. After several rounds of plaque purification, the DNA of some of the best isolated blue plaques was analysed by Southern blotting.

DNA was digested with *Bam*HI and Southern blotted with a fragment spanning the inserted fragment (p35). The structure of the recombinant virus, compared to its parent, 1716, is shown in Fig.3.10a and the resultant Southern blot in Fig.3.10b. Insertion of gDORF P/*LacZ*

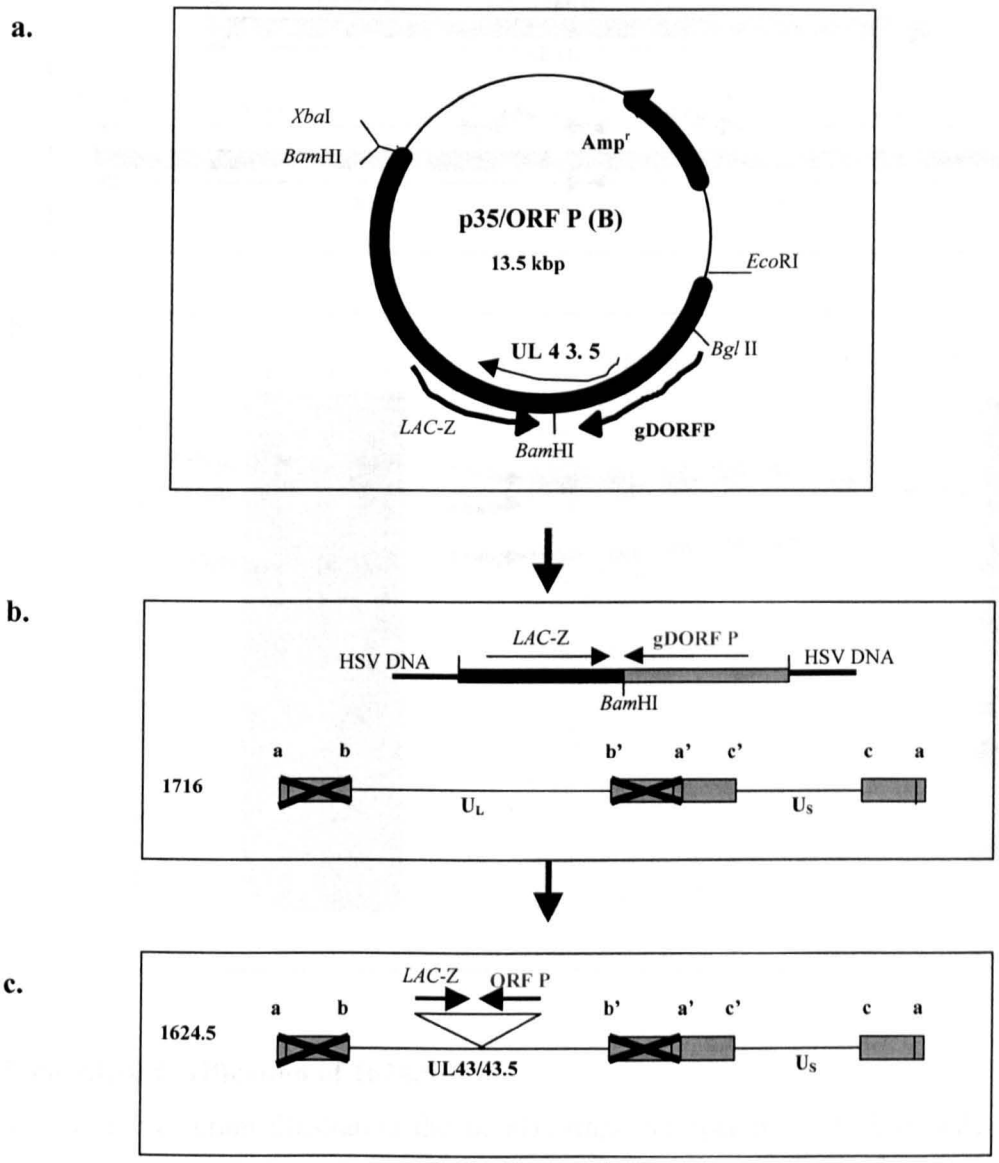


Figure 3.9 Construction of 1624.5

a. p35/ORFP (B) containing gDORF P /*Lac*Z with ORF P in the same orientation as UL43.5 and *Lac*Z in the same orientation as UL43, was digested with *Xba*I to isolate gDORF P/*Lac*Z. The arrow heads indicate the orientation of transcription.

b. gDORF P/*Lac*Z with flanking HSV-1 DNA was recombined with 1716.

c. The structure of 1624.5 is shown. The arrow heads indicate the orientation of transcription.

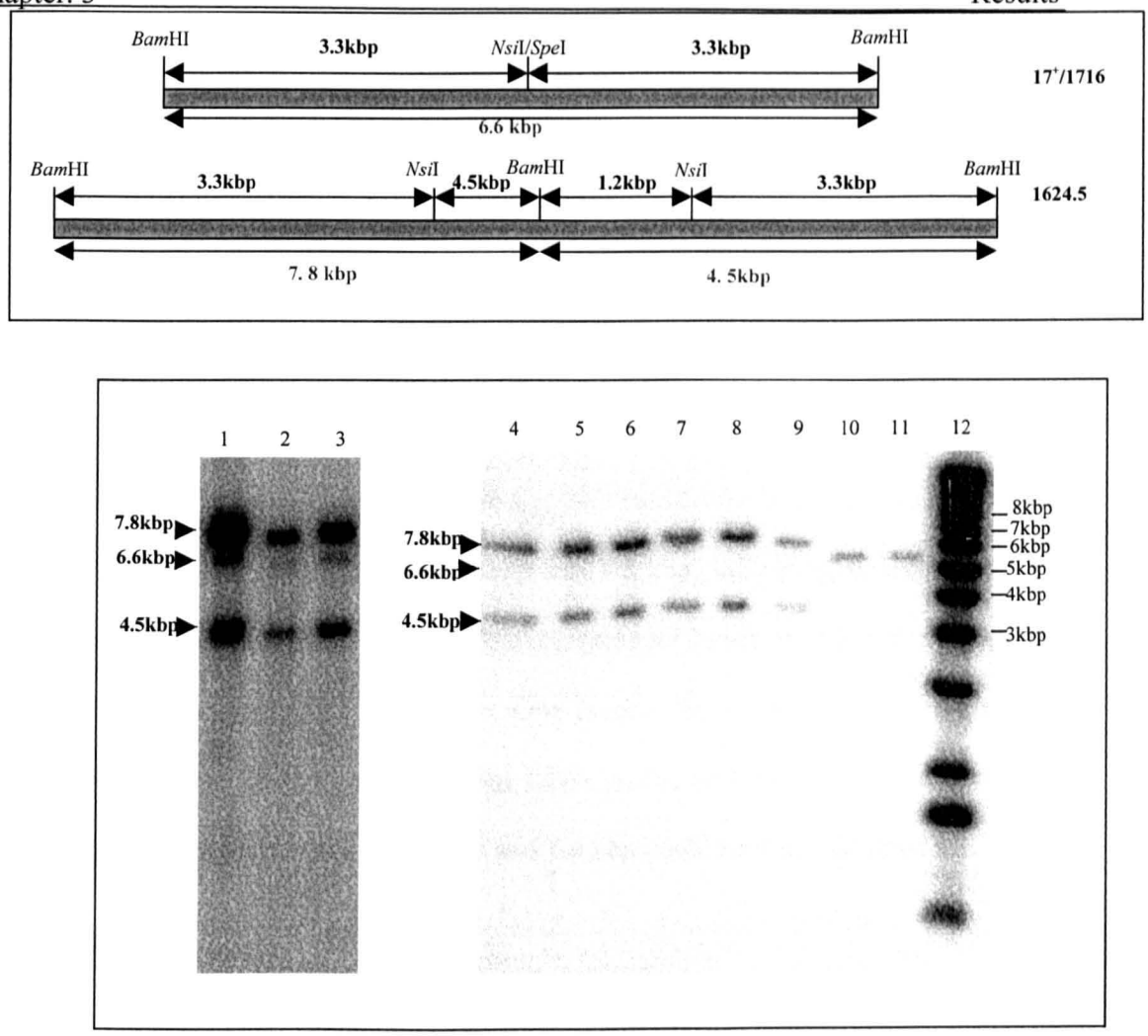


Figure3.10 Purification of 1624.5

a. A linear diagram illustrating the *Bam*HI fragment spanning UL43 in wild type and recombinant viruses, the sites of insertion at the *Nsi*I/*Spe*I site, and size of *Bam*HI fragments following digestion.

b. A Southern blot of HSV DNA digested with *Bam*HI and probed with [^{32}P]dCTP lablled p35(pacI) and exposed to an autoradiograph for 24h at -70^{0}C . Lanes 1-3: semipurified 1624.5; lanes 4-8: purified 1624.5; Lane 9: 1624.5 stock; Lane 10: 1716; lane 11: 17^{+}; and lane 12: 1 kbp DNA ladder with the sizes marked. Hybridizing bands are indicated (►).

introduces a new *Bam*HI site into the UL43/43.5 locus of the recombinant virus (Fig.3.10a). Following digestion with *Bam*HI, 17^+ (and 1716) with no insert should give a 6.6 kbp band (Fig.3.10) while 1624.5 with an insertion containing a *Bam*HI site should give 2 bands of 7.8 and 4.5 kbp (Fig.3.10).

On Southern blotting, 17^+ (Fig.3.10b, lane 11) and 1716 (lane 10) gave a 6.6 kbp band as expected. The initial plaques (lanes 1-3) gave a profile containing both the wild type band of 6.6 kbp and the recombinant bands of 4.5 kbp and 7.8 kbp, indicating a mixture of virus. Upon further purification (lanes, 4-8), only the recombinant bands of 4.5 and 7.8 kbp could be detected indicating that 1624.5 was pure. One plaque was chosen as the prototype and used to prepare a large scale virus stock. The DNA profile of this stock was analysed and again only the recombinant bands of 4.5 kbp and 7.8 kbp could be detected (lane 9).

3.5 1625

3.5.1 Introduction:

This mutant based on 1622 expresses both ICP34.5/*LacZ* and ORF P independently in the UL43/43.5 and US5 loci respectively (Fig.3.11).

3.5.2 Construction:

To construct a plasmid to generate 1625, p35ORF P (B) and pAT5.1, which contains *Bam*HI/*Eco*RV HSV-1 sequences (n.p.136289-139258) spanning US5, were used. A 1.2 kbp *Bgl*II/*Bam*HI fragment containing gD/ORF P was digested from p35/ORFP (B) (Fig.3.12a, 3.13a) and purified on a 0.8% gel (Fig.3.13b). PAT5.1 was linearized by *Bgl*II (Fig.3.12a,

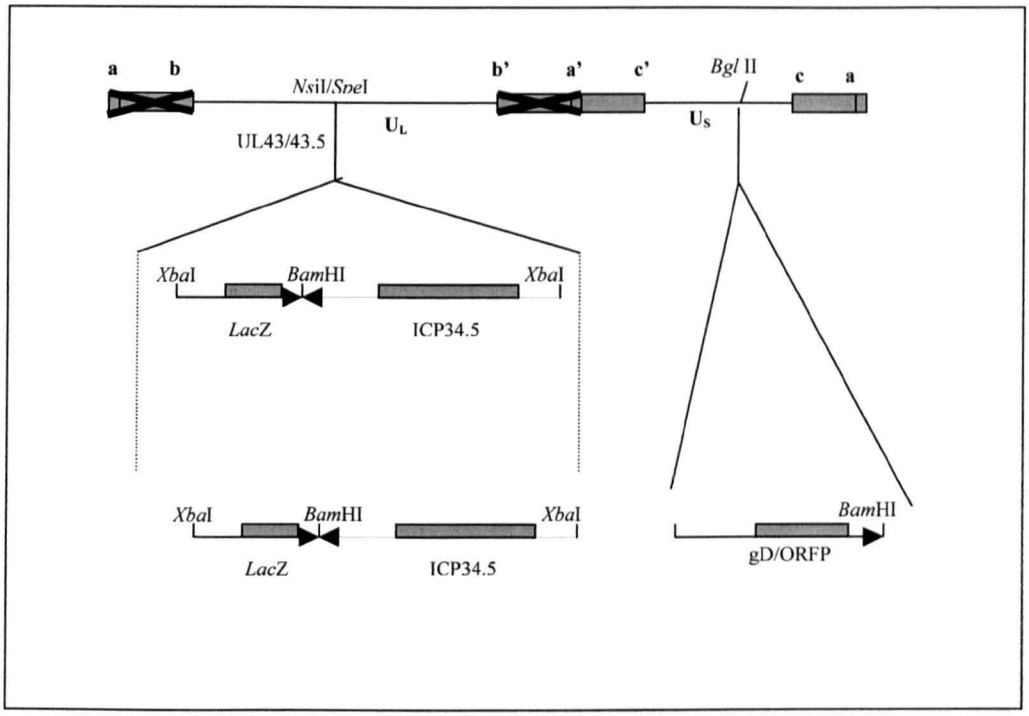


Figure 3.11 Structure of 1625

a. This figure shows the structure of 1716. "a" is a direct repeat sequence present at both termini and in inverted orientation "a' " in the internal repeat. "b" refers to the long and "c" to the short repeat. "X" indicates the deletion of ICP34.5/ORF P. The position of the *Nsi*I site plus additional *Spe*I site in UL43/43.5 is shown.

b. This line shows the structure of 1622. ICP34.5/*LacZ* containing an internal *Bam*HI site was inserted into UL43/43.5. The arrow heads indicate the orientation of transcription

c. This line shows the structure of 1625. ICP34.5/*LacZ* containing an internal *Bam*HI site was inserted into UL43/43.45 and gD/ORF P was inserted into US5 orientaion. The arrow heads indicate the orientation of transcription.

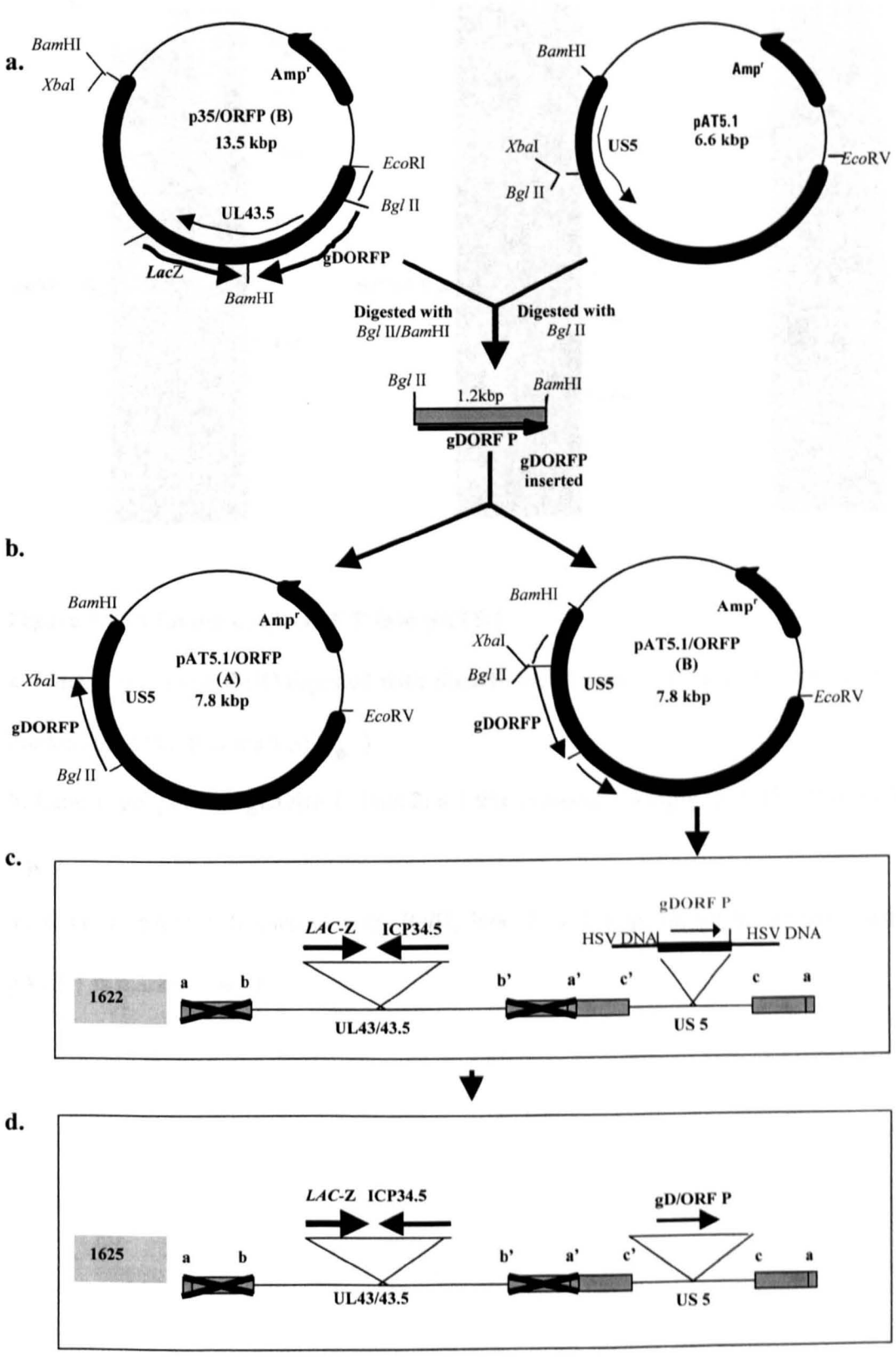
Figure 3.12 Construction of 1625

a. p35/ORFP (B) containing gD/ORF P in the same orientation as UL43.5 was digested with *Bgl*II/*Bam*HI to isolate gD/ORFP. pAT5.1 was digested with *Bgl*II.

b. gD/ORFP was inserted in this plasmid to generate pAT5.1/ORF P (A+B).

c. Linearized pAT5.1/ORF P (B) was recombined with 1622. In this recombination, gD/ORF P was inserted into US5, in the US5 orientation.

d. The structure of 1625 is shown.


a.
BamHI
XbaI
Amp^r
p35/ORFP (B)
13.5 kbp
EcoRI
Bgl II
UL43.5
LacZ
gDORFP
BamHI
BamHI
Amp^r
pAT5.1
6.6 kbp
XbaI
US5
Bgl II
EcoRV
Digested with
Bgl II/BamHI
Digested with
Bgl II
Bgl II
1.2kbp
BamHI
gDORF P
gDORFP
inserted
b.
BamHI
Amp^r
pAT5.1/ORFP
(A)
7.8 kbp
XbaI
US5
gDORFP
Bgl II
EcoRV
BamHI
Amp^r
pAT5.1/ORFP
(B)
7.8 kbp
XbaI
Bgl II
US5
gDORFP
EcoRV
c.
gDORF P
LAC-Z
ICP34.5
HSV DNA
HSV DNA
1622
a
b
b'
a'
c'
c
a
UL43/43.5
US 5
d.
LAC-Z ICP34.5
gD/ORF P
1625
a
b
b'
a'
c'
c
a
UL43/43.5
US 5

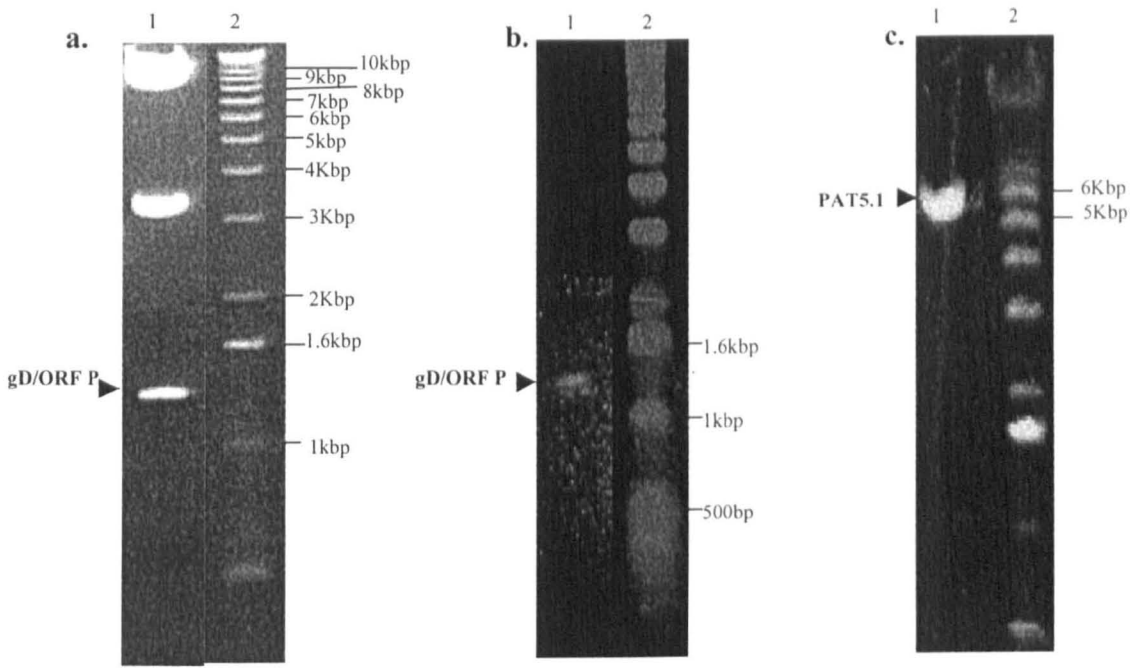


Figure 3.13 Cloning of gDORF P into pAT5.1

a. Lane 1: p35/ORF P (B) digested with *Bam*HI/ *Bgl*II. lane 2: a 1 kbp molecular weight marker. gD/ORF P is marked (▶).

b. Lane 1: gel purified gDORF P; lane 2: a 1 kbp molecular weight. gD/ORF P is marked (▶).

c. Lane 1: pAT5.1 linearized with *Bgl*II; lane 2: a 1 kbp molecular weight marker. pAT5.1 is marked (▶).

3.13c) and gD/ORF P was inserted into the *Bgl*II site of pAT5.1 (Fig.3.12a) to generate pAT5.1/gDORF P (Fig.3.12b). Insertion of the 1.2 kbp gD/ORFP fragment into US5 generates a 7.8 kbp plasmid in which gD/ORFP can insert in either orientation, A or B (Fig.3.12b). To construct 1625 we used the B orientation of pAT5.1/gDORF P plasmid where gDORF P is in the same orientation as US5 (Fig.3.13c, d). In the A orientation, gDORF P is in the opposite orientation to US5. Firstly to check for insertion of gDORF P, plasmid DNA was digested with *Xba*I (Fig.3.14). In Fig.3.14 lane 3, pAT5.1 with no insertion gives a linear band of 6.6kbp. In lanes 1 and 2, pAT5.1 with insertion of gDORF P give a 7.8kbp band. To check the orientation of gDORF P in pAT5.1, plasmid DNA was digested with *Bgl*II/*Xba*I (lanes 4-6). In the A orientation digestion of this plasmid with *Bgl*II/ *Xba*I generates 2 bands of 6.6+1.2 kbp (lane 5) whereas in the B orientation, the same digestion generates only 1 band of 7.8 kbp (lane 4). pAT5.1 with no insertion gives a linear band of 6.6kbp (lane 6).

3.5.3 Isolation and purification

The parent of 1625 is 1622 (Fig.3.11). This mutant expresses both ICP34.5 and the marker gene, *Lac*Z, in the HSV-1 UL43/43.5 locus with only gD/ORFP being inserted into US5. To isolate 1625, 1622 DNA was cotransfected with linearized pAT5.1/ORF P (B), progeny virus harvested and 10 fold serial dilutions of the transfection mixture containing both 1622 and recombinant plated out. Individual plaques were isolated and DNA prepared and analysed by Southern blotting. DNA of individual plaques were digested with *Bam*HI, separated on a 0.8% agarose gel, Southern blotted and probed with pAT5.1/gDORF P.

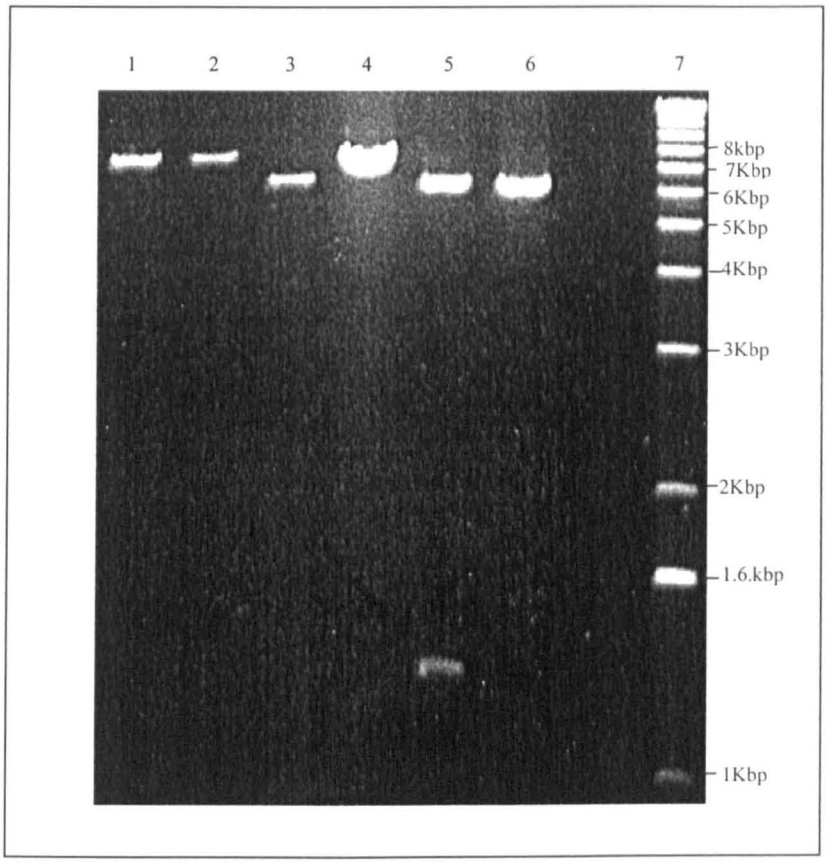


Figure 3.14 Insertion and orientation of gDORF P in pAT5.1

Lanes 1-3: *Xba*I digestion ; lanes 4-6: *Xba*I/*Bgl*II digestion. Lanes 1, 4: pAT5.1/ORF P (B); lanes 2, 5: PAT5.1/ORF P (A) ; lanes 3, 6: pAT5.1; lane 7 shows a 1 kbp molecular weight marker.

In 1622 (Fig.3.15a) the wild type 6.6 kbp band spanning US5 will be obtained, whereas in recombinant 1625, a 7.8 kbp band will be detected (Fig.3.15a). In the DNA from one plaque, both the 6.6 kbp and 7.8 kbp bands were detected (Fig.3.15b, lane1), indicating a mixture of 1622 and 1625. This plaque was taken through several rounds more purification and the DNA profile again analysed by Southern blotting. However we were unable to obtain greater than 90% purity (Fig.3.15b, lane 2) with 90% of the 7.8 kbp band but 10% contamination with the 6.6 kbp band. Rigorous attempts to further purify 1625 including 2 rounds of limited dilution cloning failed to produce a virus greater than 90% pure, indicating possible instability in the DNA structure. The frequency of 1622 contamination did not increase when a virus stock was prepared (Fig.3.15b, lane 3). In lanes 1-4, a band of 4.5 kbp is seen due to crosshybridization between the pAT5.1/ORF P probe and ICP34.5 in UL43.

Figure 3.16 shows the sequence of the ICP34.5/ORF P genes, positions of the two ORFs, the promoters and the restriction sites used to cut the ORFs.

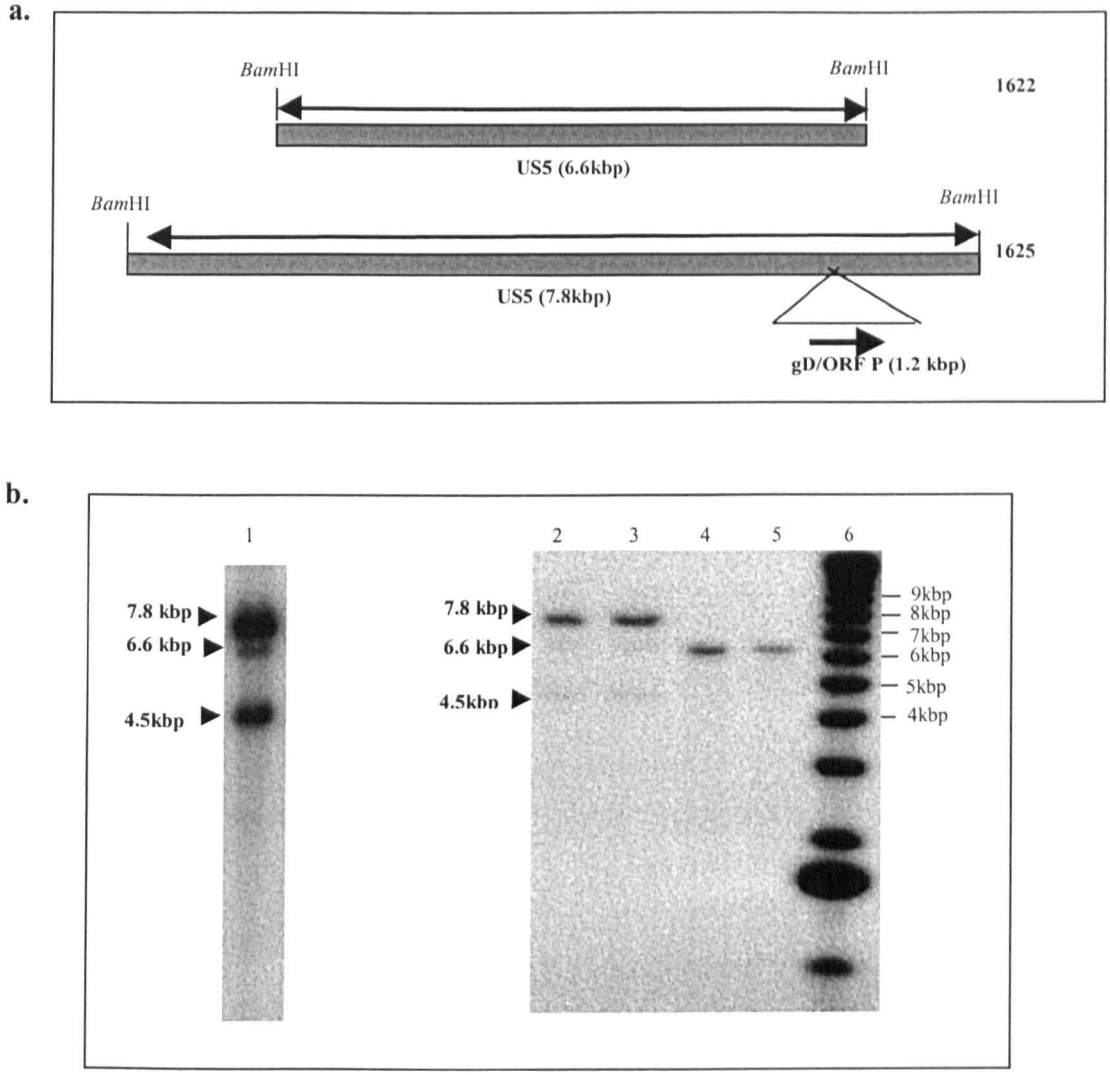


Figure 3.15 Purification of 1625

a. A linear diagram illustrating the *Bam*HI fragment spaning US5 in wild type 17^{+} (and 1622) and recombinant virus, 1625, and the size of *Bam*HI fragments following digestion. The 1.2 kbp gD/ORF P fragment was inserted in US5.

b. A Southern blot of HSV DNA prepared from recombinant virus, 1625, and its parent, 1622. This Southern blot shows HSV DNA digested with *Bam*HI, probed with [^{32}P]dCTP labelled pAT5.1/ORF P and exposed to an autoradiograph for 48h at -70^{0}C. Lane 1: semipurified recombinant virus, 1625; lane 2: purified 1625; lane 3: 1625 stock; lane 4: 1622; lane 5: 17^{+}; lane 6: shows a 1 kbp molecular weight marker. Hybridizing bands are identified (►).

Fig 3. 16 HSV-1 (17+) ICP34.5/ORF P DNA sequence

The upper line is ICP34.5 and the lower one is the ORF P. On ICP34.5 map, Dra I site at 380 bp is the TATA box of the promoter, NcoI site at 512 bp is start codon (ATG) and TAA at 1257 bp is stop codon. On ORF P map, the promoter is possibly around 1300 bp but it has not been confirmed experimentally. The ORF P ATG is on the bottom strand at 1186 bp and the stop codon is at 480 bp. For expression of ICP34.5, the NcoI/Alu I and for ORF P a PCR fragment spanning the ATG to the stop codon were cloned.

1600 bp.

```
AGCCCGGGCCCCCCGCGGGCGCGCGCGCGCGCAAAAAAGGCGGGCGGCGGTCCGGGCGGC
         10        20        30        40        50        60
----:----|----:----|----:----|----:----|----:----|----:----|
TCGGGCCCGGGGGGCGCCCGCGCGCGCGCGCGTTTTTTCCGCCCGCCGCCAGGCCCGCCG


GTGCGCGCGCGCGGCGGGCGTGGGGGGCGGGGCCGCGGGAGCGGGGGGAGGAGCGGGGGG
         70        80        90        100       110       120
----:----|----:----|----:----|----:----|----:----|----:----|
CACGCGCGCGCGCCGCCCGCACCCCCCGCCCCGGCGCCCTCGCCCCCCTCCTCGCCCCCC


AGGAGCGGGGGGAGGAGCGGGGGGAGGAGCGGGGGGAGGAGCGGGGGGAGGAGCGGGGGG
         130       140       150       160       170       180
----:----|----:----|----:----|----:----|----:----|----:----|
TCCTCGCCCCCCTCCTCGCCCCCCTCCTCGCCCCCCTCCTCGCCCCCCTCCTCGCCCCCC


AGGAGCGGGGGGAGGAGCGGGGGGAGGAGCGGGGGGAGGAGCGGGGGGAGGAGCGGGGGG
         190       200       210       220       230       240
----:----|----:----|----:----|----:----|----:----|----:----|
TCCTCGCCCCCCTCCTCGCCCCCCTCCTCGCCCCCCTCCTCGCCCCCCTCCTCGCCCCCC


AGGAGCGGGGGGAGGAGCGGGGGGAGGAGCGGGGGGAGGAGCGGGGGGAGGAGCGGGGGG
         250       260       270       280       290       300
----:----|----:----|----:----|----:----|----:----|----:----|
TCCTCGCCCCCCTCCTCGCCCCCCTCCTCGCCCCCCTCCTCGCCCCCCTCCTCGCCCCCC


AGGAGCGGGGGGAGGAGCGGCCAGACGCCGAAAACGGGCCCCCCCCAAAACACACCCCCC
         310       320       330       340       350       360
----:----|----:----|----:----|----:----|----:----|----:----|
TCCTCGCCCCCCTCCTCGCCGGTCTGCGGCTTTTGCCCGGGGGGGGGTTTTGTGTGGGGGG


                   DraI
                   \
GGGGGTCGCGCGCGGCCCTTTAAAGCGGTGGCGGCGGGCAGCCCGGGCCCCCCGCGGCCG
         370       380       390       400       410       420
----:----|----:----|----:----|----:----|----:----|----:----|
CCCCCAGCGCGCGCCGGGAAATTTCGCCACCGCCGCCCGTCGGGCCCGGGGGGCGCCGGC
                   /
                   DraI


AGACTAGCGAGTTAGACAGGCAAGCACTACTCGCCTCTGCACGCACATGCTTGCCTGTCA
         430       440       450       460       470       480
----:----|----:----|----:----|----:----|----:----|----:----|
TCTGATCGCTCAATCTGTCCGTTCGTGATGAGCGGAGACGTGCGTGTACGAACGGACAGT


                             NcoI
                             \
AACTCTACCACCCCGGCACGCTCTCTGTCTCCATGGCCCGCCGCCGCCGCCATCGCGGCC
         490       500       510       520       530       540
----:----|----:----|----:----|----:----|----:----|----:----|
TTGAGATGGTGGGGCCGTGCGAGAGACAGAGGTACCGGGCGGCGGCGGCGGTAGCGCCGG
                             /
                            NcoI


CCCGCCGCCCCCGGCCGCCCGGGCCCACGGGCGCCGTCCCAACCGCACAGTCCCAGGTAA
         550       560       570       580       590       600
----:----|----:----|----:----|----:----|----:----|----:----|
GGGCGGCGGGGGCCGGCGGGCCCGGGTGCCCGCGGCAGGGTTGGCGTGTCAGGGTCCATT
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CCTCCACGCCCAACTCGGAACCCGCGGTCAGGAGCGCGCCCGCGGCCGCCCCGCCGCCGC
          610       620       630       640       650       660
----:----|----:----|----:----|----:----|----:----|----:----|
GGAGGTGCGGGTTGAGCCTTGGGCGCCAGTCCTCGCGCGGGCGCCGGCGGGGCGGCGGCG


CCCCCGCCGGTGGGCCCCCGCCTTCTTGTTCGCTGCTGCTGCGCCAGTGGCTCCACGTTC
          670       680       690       700       710       720
----:----|----:----|----:----|----:----|----:----|----:----|
GGGGGCGGCCACCCGGGGGCGGAAGAACAAGCGACGACGACGCGGTCACCGAGGTGCAAG


CCGAGTCCGCGTCCGACGACGACGATGACGACGACTGGCCGGACAGCCCCCCGCCCGAGC
          730       740       750       760       770       780
----:----|----:----|----:----|----:----|----:----|----:----|
GGCTCAGGCGCAGGCTGCTGCTGCTACTGCTGCTGACCGGCCTGTCGGGGGGCGGGCTCG


CGGCGCCAGAGGCCCGGCCCACCGCCGCCGCCCCCCGGCCCCGGCCCCCACCGCCCGGCG
          790       800       810       820       830       840
----:----|----:----|----:----|----:----|----:----|----:----|
GCCGCGGTCTCCGGGCCGGGTGGCGGCGGCGGGGGGCCGGGGCCGGGGGTGGCGGGCCGC


TGGGCCCGGGGGGCGGGGCTGACCCCTCCCACCCCCCCTCGCGCCCCTTCCGCCTTCCGC
          850       860       870       880       890       900
----:----|----:----|----:----|----:----|----:----|----:----|
ACCCGGGCCCCCCGCCCCGACTGGGGAGGGTGGGGGGGAGCGCGGGGAAGGCGGAAGGCG


CGCGCCTCGCCCTCCGCCTGCGCGTCACCGCGGAGCACCTGGCGCGCCTGCGCCTGCGAC
          910       920       930       940       950       960
----:----|----:----|----:----|----:----|----:----|----:----|
GCGCGGAGCGGGAGGCGGACGCGCAGTGGCGCCTCGTGGACCGCGCGGACGCGGACGCTG


GCGCGGGCGGGGAGGGGGCGCCGGAGCCCCCCGCGACCCCCGCGACCCCCGCGACCCCCG
          970       980       990       1000      1010      1020
----:----|----:----|----:----|----:----|----:----|----:----|
CGCGCCCGCCCCTCCCCCGCGGCCTCGGGGGGCGCTGGGGGCGCTGGGGGCGCTGGGGGC


CGACCCCCGCGACCCCCGCGCGGGTGCGCTTCTCGCCCCACGTCCGGGTGCGCCACCTGG
          1030      1040      1050      1060      1070      1080
----:----|----:----|----:----|----:----|----:----|----:----|
GCTGGGGGCGCTGGGGGCGCGCCCACGCGAAGAGCGGGGTGCAGGCCCACGCGGTGGACC


TGGTCTGGGCCTCGGCCGCCCGCCTGGCGCGCCGCGGCTCGTGGGCCCGCGAGCGGGCCG
          1090      1100      1110      1120      1130      1140
----:----|----:----|----:----|----:----|----:----|----:----|
ACCAGACCCGGAGCCGGCGGGCGGACCGCGCGGCGCCGAGCACCCGGGCGCTCGCCCGGC


ACCGGGCTCGGTTCCGGCGCCGGGTGGCGGAGGCCGAGGCGGTCATCGGGCCGTGCCTGG
          1150      1160      1170      1180      1190      1200
----:----|----:----|----:----|----:----|----:----|----:----|
TGGCCCGAGCCAAGGCCGCGGCCCACCGCCTCCGGCTCCGCCAGTAGCCCGGCACGGACC


GGCCCGAGGCCCGTGCCCGGGCCCTGGCCCGCGGAGCCGGCCCGGCGAACTCGGTCTAAC
          1210      1220      1230      1240      1250      1260
----:----|----:----|----:----|----:----|----:----|----:----|
```

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CCGGGCTCCGGGCACGGGCCCGGGACCGGGCGCCTCGGCCGGGCCGCTTGAGCCAGATTG


                                    AluI      AluI
                                    \         \
GTTACACCCGAGGCGGCCTGGGTCTTCCGCGGAGCTCCCGGGAGCTCCGCACCAAGCCGC
         1270      1280      1290      1300      1310      1320
----:----|----:----|----:----|----:----|----:----|----:----|
CAATGTGGGCTCCGCCGGACCCAGAAGGCGCCTCGAGGGCCCTCGAGGCGTGGTTCGGCG
                                    /         /
                                    AluI      AluI


TCTCCGGAGAGACGATGGCAGGAGCCGCGCATATATACGCTTGGAGCCAGCCCGCCCTCA
         1330      1340      1350      1360      1370      1380
----:----|----:----|----:----|----:----|----:----|----:----|
AGAGGCCTCTCTGCTACCGTCCTCGGCGCGTATATATGCGAACCTCGGTCGGGCGGGAGT


CAGGGCGGGCCGCCTCGGGGGCGGGACTGGCCAATCGGCGGCCGCCAGCGCGGCGGGGCC
         1390      1400      1410      1420      1430      1440
----:----|----:----|----:----|----:----|----:----|----:----|
GTCCCGCCCGGCGGAGCCCCCGCCCTGACCGGTTAGCCGCCGGCGGTCGCGCCGCCCCGG


CGGCCAACCAGCGTCCGCCGAGTCTTCGGGGCCCGGCCCATTGGGCGGGAGTTACCGCCC
         1450      1460      1470      1480      1490      1500
----:----|----:----|----:----|----:----|----:----|----:----|
GCCGGTTGGTCGCAGGCGGCTCAGAAGCCCCGGGCCGGGTAACCCGCCCTCAATGGCGGG


AATGGGCCGGGCCGCCCACTTCCCGGTATGGTAATTAAAAACTTGCAAGAGGCCTTGTTC
         1510      1520      1530      1540      1550      1560
----:----|----:----|----:----|----:----|----:----|----:----|
TTACCCGGCCCGGCGGGTGAAGGGCCATACCATTAATTTTTGAACGTTCTCCGGAACAAG


CGCTTCCCGGTATGGTAATTAGAAACTCATTAATGGGCGG
         1570      1580      1590      1600      1610      1620
----:----|----:----|----:----|----:----|----:----|----:----|
GCGAAGGGCCATACCATTAATCTTTGAGTAATTACCCGCC
```

```
# Enzymes that cut  Frequency       Isoschizomers
     AluI            2
     DraI            1
     NcoI            1


# Enzymes < MINCUTS Frequency       Isoschizomers


# Enzymes > MAXCUTS Frequency       Isoschizomers


# Enzymes that do not cut


# Number of enzymes not matching SITELEN, BLUNT, STICKY, COMMERCIAL criteria
0
```

4.0 *In vitro* characterization of HSV-1 recombinant viruses

4.1 Introduction:

To investigate the role of ICP34.5 and ORF P in the HSV-1 lifecycle, a number of HSV-1 recombinant viruses were constructed. These are described in detail in the previous chapter (section 3). All recombinants were made in the background of the HSV-1 ICP34.5/ORF O/P null mutant, 1716. 1622 expresses ICP34.5 in the UL43/43.5 locus in the UL43.5 orientation. 1624 expresses ORF P in the UL43/43.5 locus in the UL43 orientation. 1624.5 expresses ORF P in the UL43/43.5 locus in the UL43.5 orientation. 1625, based on 1622, expresses ICP34.5 in the UL43/43.5 locus and also gD/ORF P in the US5 locus in the same orientation as US5. In addition, all recombinants express *LacZ* in the UL43/43.5 locus in the opposite orientation to that of the inserted HSV gene. In this chapter (i) expression of the inserted genes in these recombinants is examined; and (ii) the recombinants are characterised *in vitro* compared to both wild type, 17^+, and their parental virus, 1716.

4.2 Analysis of ICP34.5 expression

Sequence analysis of ICP34.5 from several HSV-1 strains has identified variation in the number of copies of the DNA sequence encoding a PAT repeat within the protein. HSV-1 strain F has 10, strain 17^+ 5, and strain KOS 3 copies of this repeat. Migration of ICP34.5 by SDS-PAGE consequently varies between strains. Initially, an antiserum raised against a synthetic peptide, PAT_{10}, was used in Western blots (McKay *et al.*, 1993). This antiserum strongly recognises ICP34.5 in strain F with 10 copies but only weakly recognises ICP34.5 in strain 17^+ probably because it has only 5 PAT repeats. Subsequently antisera against a GST/ICP34.5 fusion protein were raised and demonstrated equal expression of ICP34.5 in both strain F and 17^+ (Brown *et al.*, 1997).

Using one of these antisera, 137, ICP34.5 expression has been analysed in the recombinants. Using this antibody, Holman (2000) had previously reported that 1622 overexpresses ICP34.5 compared to strain F and 17^+.

4.3 Expression of ICP34.5 in recombinant viruses

Using BHK infected cell extracts, expression of ICP34.5 was analysed in 1622, 1624, 17^+ and 1716 by Western blotting with antiserum, 137 (Fig.4.1). As expected ICP34.5 was not detected from 1716 and 1624 or mock infected extracts (Lanes 3, 4 and 5, respectively), but was from 17^+ and 1622 (lanes 1 and 2, respectively). 1622 clearly expresses more ICP34.5 than 17^+. By Western blotting of BHK cell extracts with antiserum 137, the level of expression of ICP34.5 from 1622 and 1625 was compared to 17^+ (Fig.4.2). Two-fold serial dilutions were made of 1622 and 1625 infected BHK cells 16 h pi to ascertain a semiquantitative level of ICP34.5 expression. The level of ICP34.5 expression in 1622 (lanes 3-6) is the same as 1625 (lanes 7-10). These two recombinants express nearly 8 times more ICP34.5 than 17^+ (lane 1). Apparently, expression of ORF P in 1625 does not effect expression of ICP34.5 compared to its parent virus, 1622. As expected, ICP34.5 is not expressed by 1624 (lane 11), 1624.5 (lane 12) or 1716 (lanes 2 and 13).

4.4 Analysis of ORF P expression

ORF P protein expressed by HSV-1(F) is predicted to contain 248 amino acids. It contains a short amino terminal domain followed by an amino acid trimer of Ala-Gly-Val repeated 10 times and a long carboxy terminal domain (Lagunoff and Roizman, 1994). Similarly to ICP34.5, there is variation in the number of trimers and in length of the protein between strains. The protein in HSV-1 (17^+) contains 233 amino acids with

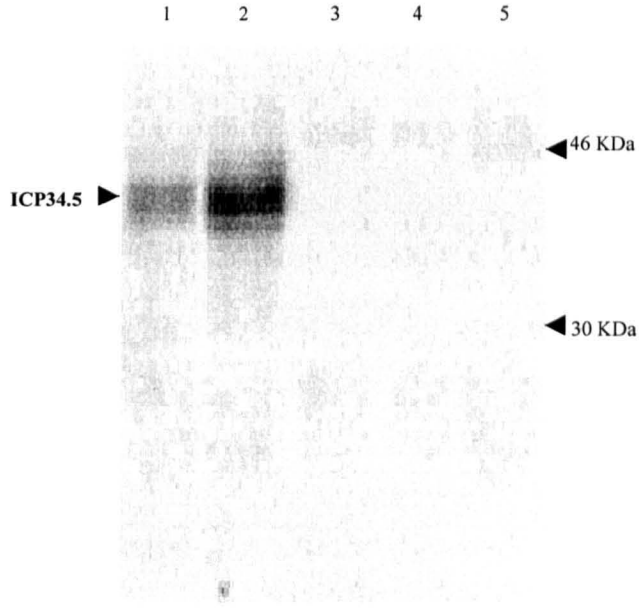


Figure 4.1 Western blotting of ICP34.5 expression from recombinant viruses

BHK cells were infected with viruses at a m.o.i of 20 pfu/cell and harvested at 24 h pi. Analysis of ICP34.5 expression was carried out by 12.5% SDS-PAGE and Western blotting with antiserum 137 (1/500 dilution) and protein A-HRP, reacted with ECL and exposed to autoradiography. Lane1: 17^{+}; lane 2: 1622; lane 3: 1716; lane 4: 1624; lane 5: MI. Molecular weight markers are indicated on the right and ICP34.5 is marked on the left (►).

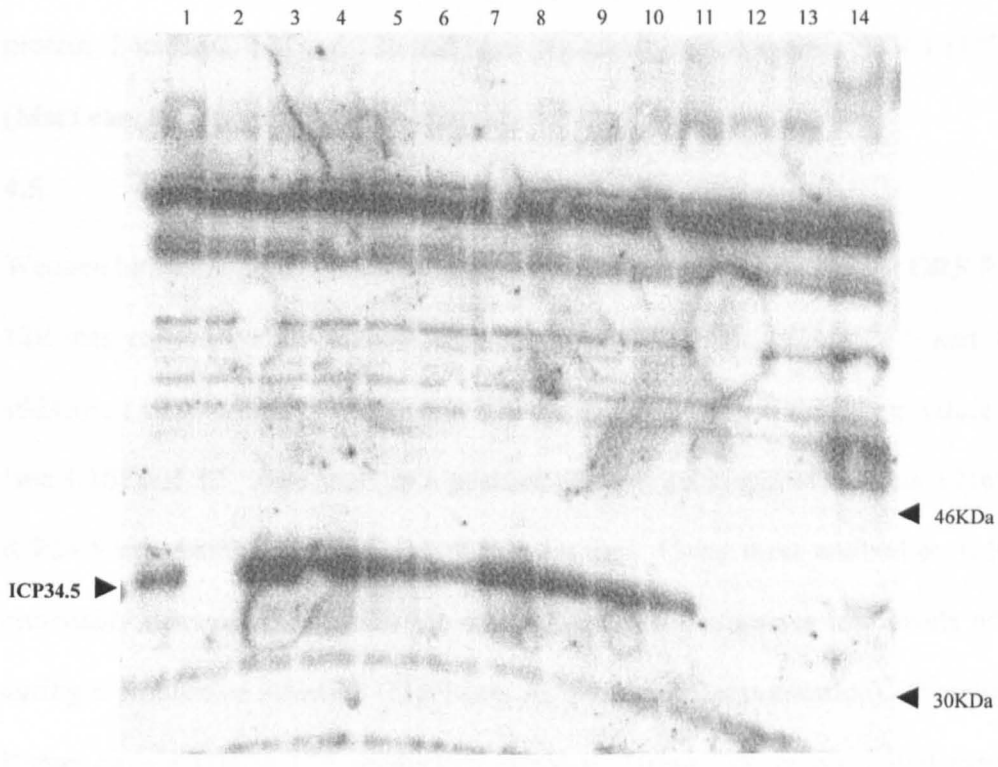


Figure 4.2 Semiquantitative expression of ICP34.5 by 1622 and 1625

BHK cells were infected with viruses at a m.o.i of 20 pfu/cell, extracts prepared 24 h pi and serial 2-fold dilutions made of both 1622 and 1625 infected BHK cells. Analysis of ICP34.5 expression was carried out on a 12.5% SDS-PAGE which was Western blotted with 137 antiserum (1/500 dilution) and protein A-HRP, reacted with ECL and exposed to autoradiography. Lane1: 17^{+}; lane 2:1716; lane 3:1622 (N); lane 4:1622 (1:2 dilution); lane 5:1622 (1:4 dilution); lane 6: 1622 (1:8 dilution); lane 7: 1625 (N); lane 8:1625 (1:2 dilution); lane 9: 1625 (1:4 dilution); lane 10: 1625 (1:8 dilution); lane 11: 1624; lane 12:1624.5; lane 13: 1716; lane 14: MI. Molecular weight markers are indicated on the right and ICP34.5 is marked on the left (►).

an amino acid trimer of Ala-Gly-Val repeated 5 times. Using a GST-ORF P fusion protein, 2 antisera, 128 and 129 had been previously raised against HSV-1 (17^+) ORF P (MacLean, A., personal communication).

4.5 Expression of ORF P in recombinant viruses

Western blotting of BHK infected cell extracts with two antisera against ORF P 128 and 129 was carried out to analyse expression of ORF P in 1624/1624.5 and 1625. In addition, a virus with a *ts* mutation in ICP4 at the NPT (*ts*K) which overproduces ORF P (see 1.16) and 17^+ were used as a positive control. As negative controls 1716 and the ICP34.5 expressing recombinant (1622) were used. Using these antibodies, it had been previously demonstrated in our lab that HSV-1 (17^+) expresses low levels of ORF P during a productive infection (MacLean, A., personal communication). Therefore, ORF P may have a role in 17+ productive infection. Using 128, in our initial experiments (normal western blotting method) (Fig.4.3a) we could only detect ORF P from the overproducing *ts*K (lane 3) but from neither 17^+ (lanel), 1716 (lane 2), 1622 (lane 4) nor any ORF P containing recombinant viruses 1624, 1624.5 and 1625 (lanes 5-7, respectively). Using 129, the same results were obtained (Fig.4.4a). ORF P was only detected from the overproducing *ts*K (lane 1) but from neither 17^+ (lane 2), 1716 (lane 3), 1624.5 (lane 4) nor 1625 (lane 5). This was at least partly because of the high background in the gel. To try to minimise the background, 1M NaCl was added to the antisera (Fig.4.3b). This figure shows Western blotting of BHK cell extracts with antiserum 128 containing 1M NaCl. Expression of ORF P was detected from both *ts*K and 17^+ (lanes 1 and 2) but not from recombinants 1622, 1624, 1624.5 and 1625 (lanes 4-7, respectively) or from 1716 (lane 2). The same results were obtained using antiserum 129 containing 1M NaCl (Fig 4.4b). In *ts*K an ORF P band of 28 KDa is seen (Fig.4.3b and 4.4b lane 1) whereas the ORF P band in 17^+ (Fig.4.3b and 4.4b lane 2) is 32 KDa

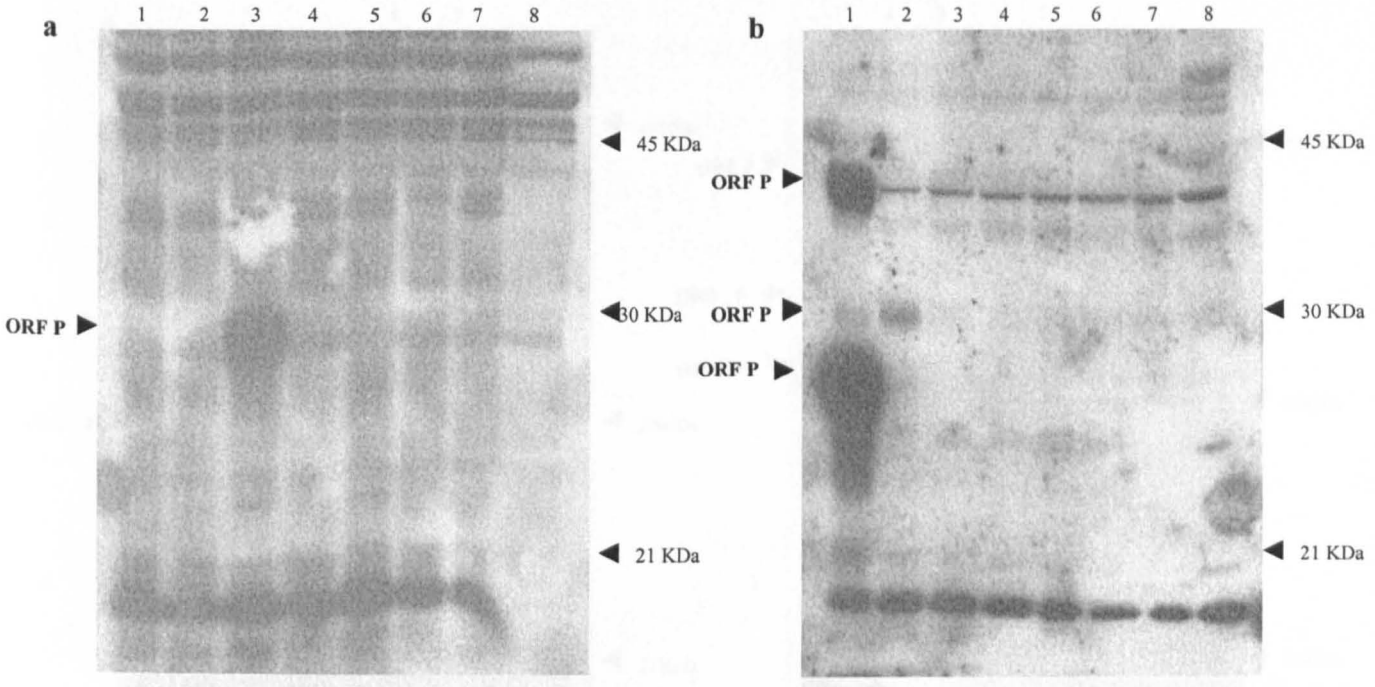


Figure 4.3 Western blotting of ORF P expression using antiserum 128

BHK cells were infected with viruses at a m.o.i of 20 pfu/cell and harvested at 24 h pi. Analysis of ORF P expression was carried out by 12.5% SDS-PAGE and Western blotting with 128 antiserum and protein A-HRP, reacted with ECL and exposed to autoradiography. a) initial (normal western blotting method) and b) final experiments (using 1 molar NaCl containing antisera) . Molecular weight markers are indicated on the right and ORF P related bands are marked on the left (▶).

a) Lane 1: 17^{+}; Lane 2: 1716; lane 3: *ts*K; lane 4: 1622; lane 5: 1624; lane 6: 1624.5; lane 7: 1625; lane 8: MI.

b) Lane1: *ts*K; lane 2: 17^{+}; lane 3: 1716; lane 4: 1622; lane 5: 1624; lane 6: 1624.5; lane 7: 1625; lane 8: MI.

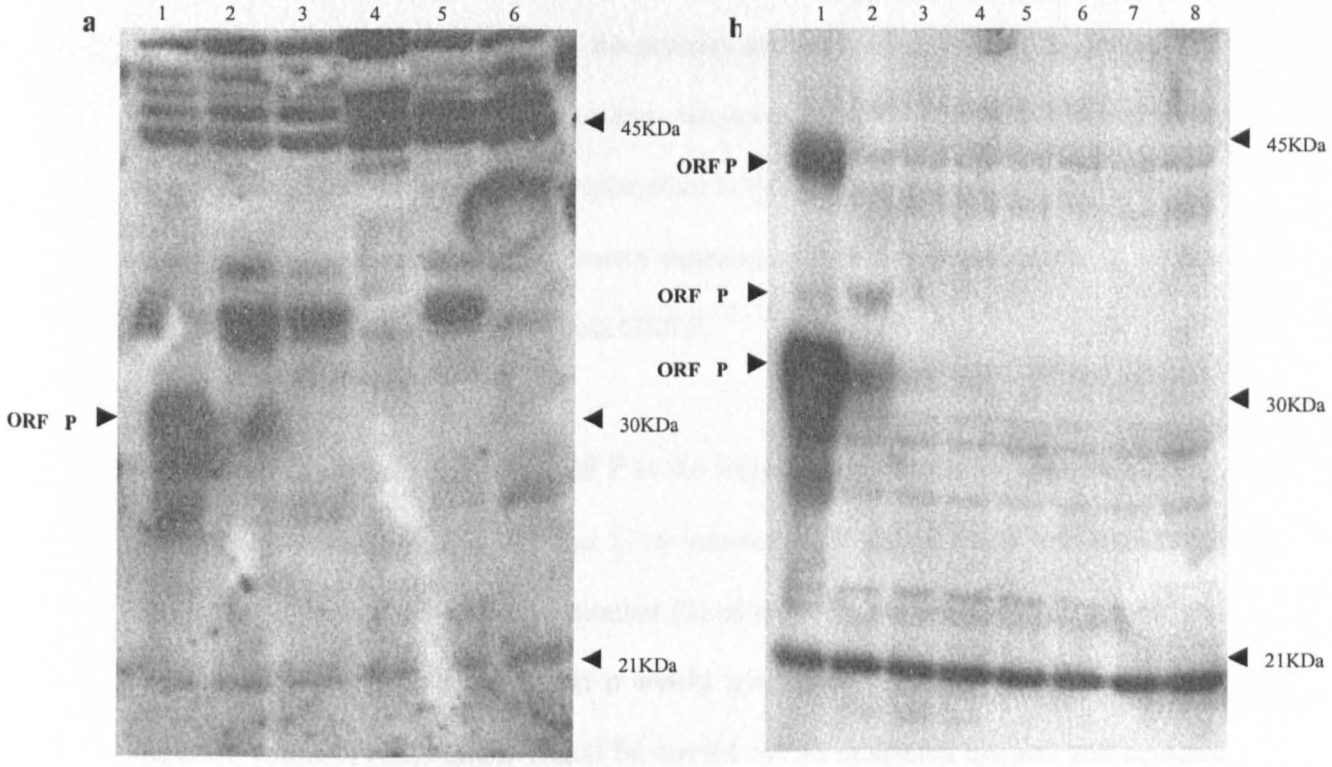


Figure 4.4 Western blotting of ORF P expression using antiserum 129

BHK cells were infected with viruses at a m.o.i of 20 pfu/cell and harvested at 24 h pi. Analysis of ORF P expression was carried out by 12.5% SDS-PAGE and Western blotting with 129 antiserum and protein A-HRP, reacted with ECL and exposed to autoradiography. a) initial (normal western blotting method) and b) final experiments (using 1 molar NaCl containing antisera) Molecular weight markers are indicated on the right and ORF P is marked on the left (►).

a) Lane1: *ts*K; lane 2: 17^{+}; lane 3: 1716; lane 4: 1624.5; lane 5: 1625; lane 6: MI.

b) Lane1: *ts*K; lane 2: 17^{+}; lane 3: 1716; lane 4: 1622; lane 5: 1624; lane 6: 1624.5; lane 7: 1625; lane 8: MI.

indicating possible processing during a productive infection. In *ts*K a high molecular weight band of 45 KDa is detected possibly due to the overproduction of ORF P leading to aggregate formation. By varying conditions such as use of different dilutions of antisera and using 0.5-1 M NaCl in the primary antibody, we attempted to demonstrate expression of ORF P in the recombinants. However, no ORF P protein detected in the recombinants. The most probable explanation is that the antisera are not strong enough to detect low amounts of ORF P protein expression from the recombinants. Also it is possible that the viruses do not express ORF P.

As neither 128 nor 129 detected ORF P in the recombinant viruses by Western blotting, immunoprecipitation of *ts*K, 17^+ and 1716 infected BHK cell extracts was carried out (Fig.4.5). As there is only a small number (2) of methionines in ORF P (Lagunoff and Roizman, 1994) it was decided that it would label poorly with [^{35}S] methionine and therefore immunoprecipitations would be carried out on unlabeled extracts and detected by Western blotting. Immunoprecipitations were carried out at both 4^0C overnight (lanes 1-6) and at 37^0C for 2 h (lanes 7-12) with both 128 (lanes 1-3 and 7-9) and 129 (lanes 4-6 and 10-12) and Western blotted with 128. Again although the two antibodies detected ORF P in *ts*K (lanes 1, 4, 7 and 10), indicating immunoprecipitation of ORF P, they were not able to detectably precipitate the low amounts of ORF P in 17^+ (lanes 1, 4, 7 and 10) and 1716 (lanes 3, 6, 9 and 12). An immunoprecipitation carried out against ICP34.5 with 137 and Western blotted with 128, as a negative control, failed to detect ORF P (lanes 13-15). As ORF P could not be detected from 17^+ extracts, we did not carry out an immunoprecipitation with the recombinants 1624, 1624.5 and 1625. As expected the 50 KDa band of the precipitated IgG heavy chain was detected by the protein-A-HRP in the western blots.

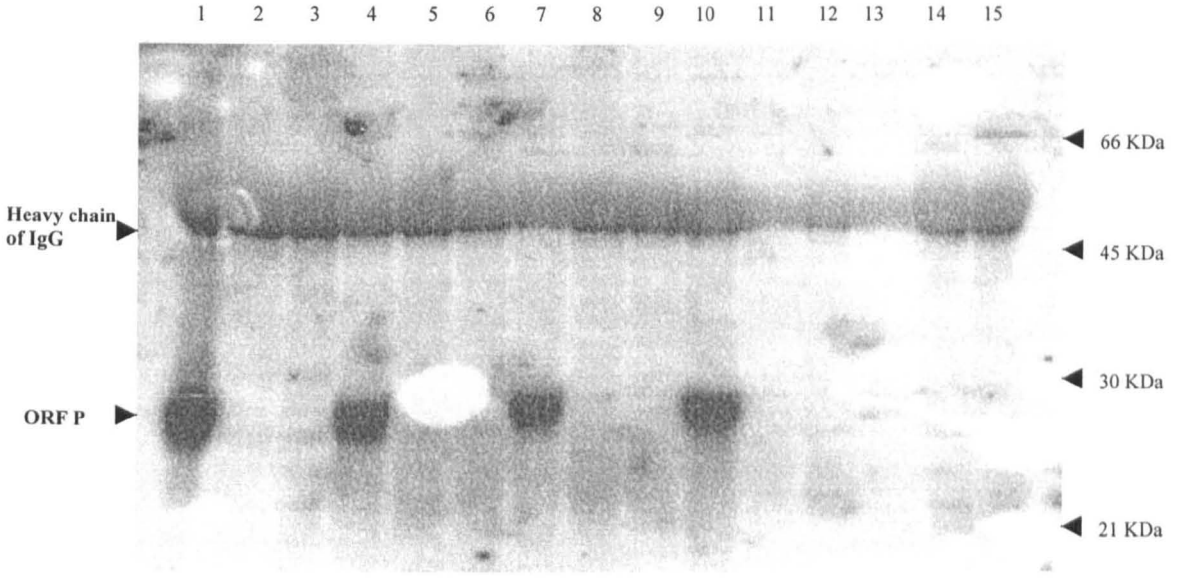


Figure 4.5 Western blotting of immunoprecipitated ORF P

BHK cells were infected at a m.o.i of 20 pfu/cell, harvested at 16h pi and immunoprecipitated with ORF P antiserum 128 (lanes 1-3; 7-9) and ORF P antiserum 129 (lanes 4-6; 10-12) or an anti-ICP34.5 serum 137 (lanes 13-15). Immunoprecipitations were carried out at either 4^0C (lanes 1-6) or 37^0C (lanes 7-15). Samples were electrophoresed on a 12.5% SDS-PAGE and Western blotted with 128 and protein-A-HRP. Lanes1, 4, 7, 10 and 13: *ts*K; lanes 2, 5, 8, 11 and 14: 17^+; lane 3, 6, 9, 12 and 15: 1716. Molecular weight markers are indicated on the right, ORF P and the heavy chain of IgG are indicated on the left (►).

4.6 Analysis of ORF P RNA synthesis by recombinant viruses

As expression of ORF P protein was not detected from the recombinants, ORF P RNA synthesis was analysed. Fig.4.6 shows Northern blotting of RNA extracts from the recombinants using GST-ORF P as probes. As ORF P is inserted in the UL43/43.5 loci, GST-ORF P will hybridize with and detect UL43/43.5 spanning transcripts. In addition, it will also hybridize with and detect the ORF P (and ICP34.5) transcripts in 1624, 1624.5 and 1625.

Using ORF P as a probe (Fig.4.6), a number of HSV-1 transcripts were detected. Based on a RNA size marker, the sizes of these transcripts are 1: 7.0 kbp; 2: 4.0 kbp; 3: 2.7 kbp; 4: 2 kbp; 5: 1 kbp (Fig.4.6). These transcripts spanning UL41-44 and based on transcript map for this region, these transcripts are probably 1: uncharacterised; 2: UL43/44; 3: UL44; 4: UL41; 5: UL43. The uncharacterised long transcript probably arises from the non specific transcription detected from the HSV genome late in infection (Jacquemont and Roizman, 1975a, b).

In addition to the HSV transcripts, a band with the size expected for ORF P RNA of about 700 bp was detected in 1624, 1624.5, and 1625 (lanes 5-7, respectively). As ORF P is used as the probe and there is sequence identity between ORF P and ICP34.5, the similarly sized ICP34.5 transcript was detected in 1622 (lane 4). As expected ORF P did not hybridize to the UL41-44 HSV transcripts in *ts*K, 17^{+} and 1716 (lanes 1-3, respectively). Surprisingly no native ORF P RNA was detected in tsK and 17+ (lanes 1 and 2). As expected, no hybridization was detected to mock infected RNA (lane 8). The ORF P probe blot has failed to detect ORF P even in positive control so no conclusion can be drawn.

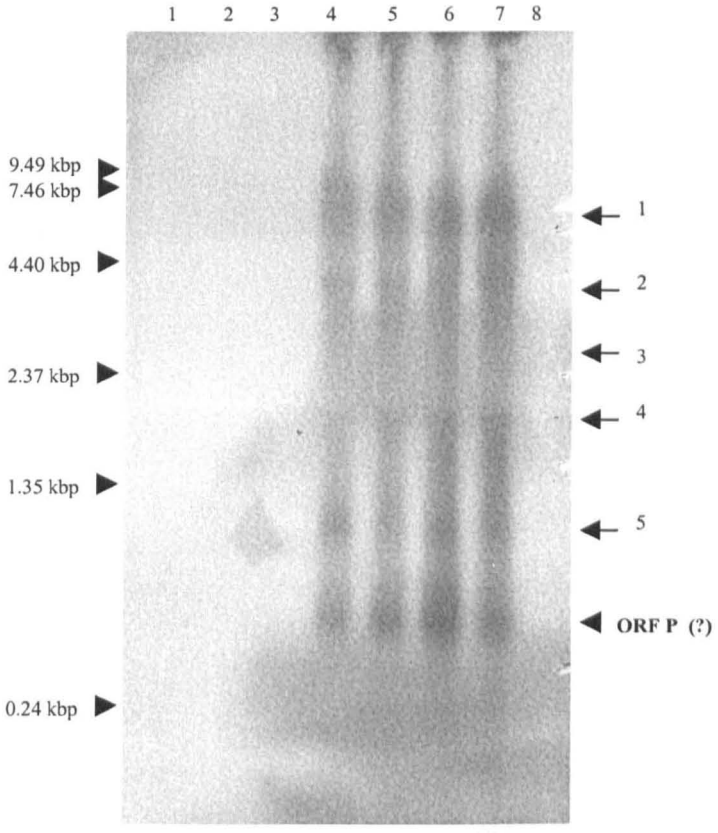


Figure 4.6 ORF P RNA synthesis from recombinant viruses

Northern blot of HSV-1 RNA probed with [^{32}P]dCTP labelled GST-ORF P and exposed to an autoradiograph for 72h at -70^{0}C . Lanel: *ts*K; lane 2: 17^{+}; lane 3: 1716; lane 4: 1622; lane 5: 1624; lane 6: 1624.5; lane 7: 1625; lane 8: MI. RNA ladder with the sizes marked is shown ( ). Hybridizing bands ( ) and a band with the size expected for ORF P are indicated ( ).

To confirm that RNA was loaded in all tracks, a northern blot was carried out with a cellular gene, γ1 actin. The membrane used for the ORF P probe was stripped and reprobed with γ1 actin (Fig.4.7). All tracks contained RNA with a excess amount in tsK infected (lane 1).

4.7 (was removed)

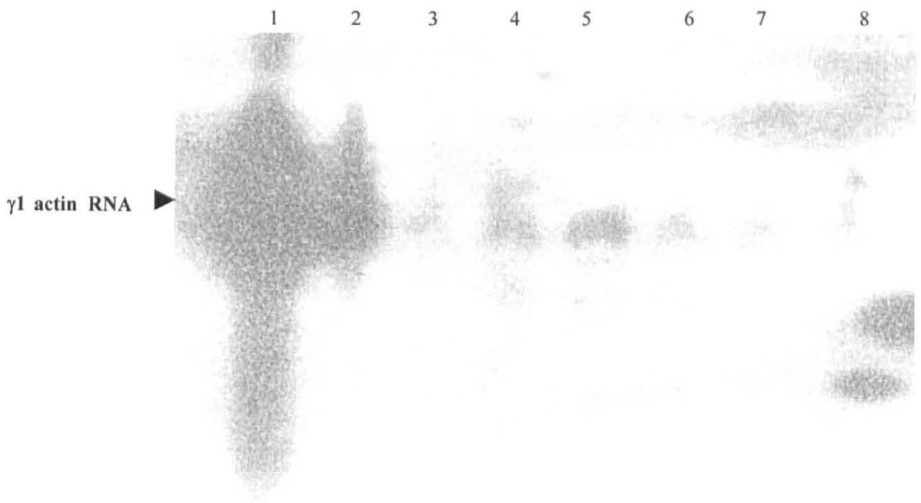


Figure 4.7 Northern blotting with γ1 actin

Northern blot of HSV-1 RNA probed with γ1 actin [^{32}P]dCTP labelled and exposed to an autoradiograph for 72h at -70^{0}C . Lane1: *ts*K; lane 2: 17^{+}; lane 3: 1716; lane 4: 1622; lane 5: 1624; lane 6: 1624.5; lane 7: 1625; lane 8: MI. γ1 actin band is indicated (►).

Fig 4.8 was removed (deleted).

4.8 *In vitro* replication kinetics of recombinant viruses

Having analysed expression of ICP34.5 and ORF P from the recombinant viruses, we then proceeded to analyse their phenotypes *in vitro*. It has been previously shown that ICP34.5 is essential for HSV-1 replication in some but not all cell lines (Brown *et al.*, 1994a; Chou *et al.*, 1992). Thus the replication behaviour of recombinant viruses was compared to 17^+ (wild type) and 1716 (ICP34.5 –ve, ORF O/P -ve) in a variety of cell types. These recombinants were 1622 (ICP34.5+ve), 1624 (ORF P +ve), 1624.5 (ORF P +ve), and 1625 (ICP34.5 +ve, ORF P +ve). The cell lines used were BHK21/C13 cells which are fully permissive for ICP34.5 negative viruses; 3T6 cells which are non permissive for ICP34.5 negative viruses; and SK-N-SH cells whose permissivity had not previously been fully analysed but which exhibit protein synthesis shutoff following infection with ICP34.5 negative viruses (Chou *et al.*, 1992). The multicycle growth kinetics of the recombinants was analysed in all 3 cell types.

4.8.1 Multicycle replication kinetics in BHK cells

In BHK cells, as illustrated in Fig.4.9 (a and b) all viruses with the exception of 1624 exhibited a similar growth pattern, reaching a maximum titre of 10^8-10^9 pfu/ml by 48-72 h pi. 1624 consistently grew 5 to 10 fold less well than the rest. In (c) only the growth of 1622 and 1625 has been compared to 17^+ and 1716 and again exhibited similar growth.

4.8.2 Multicycle replication kinetics in 3T6 cells

In stationary state 3T6 cells, Fig.4.10 (a, b and c) 17^+ grew well reaching a maximum titre of 10^6-10^7 pfu/ml by 48-72 h pi whereas 1716, expressing neither ICP34.5 nor ORF O/P failed to grow. The recombinants 1624 and 1624.5 which express only ORF P, similarly to 1716, failed to grow whereas 1622 and 1625 expressing ICP34.5 grew.

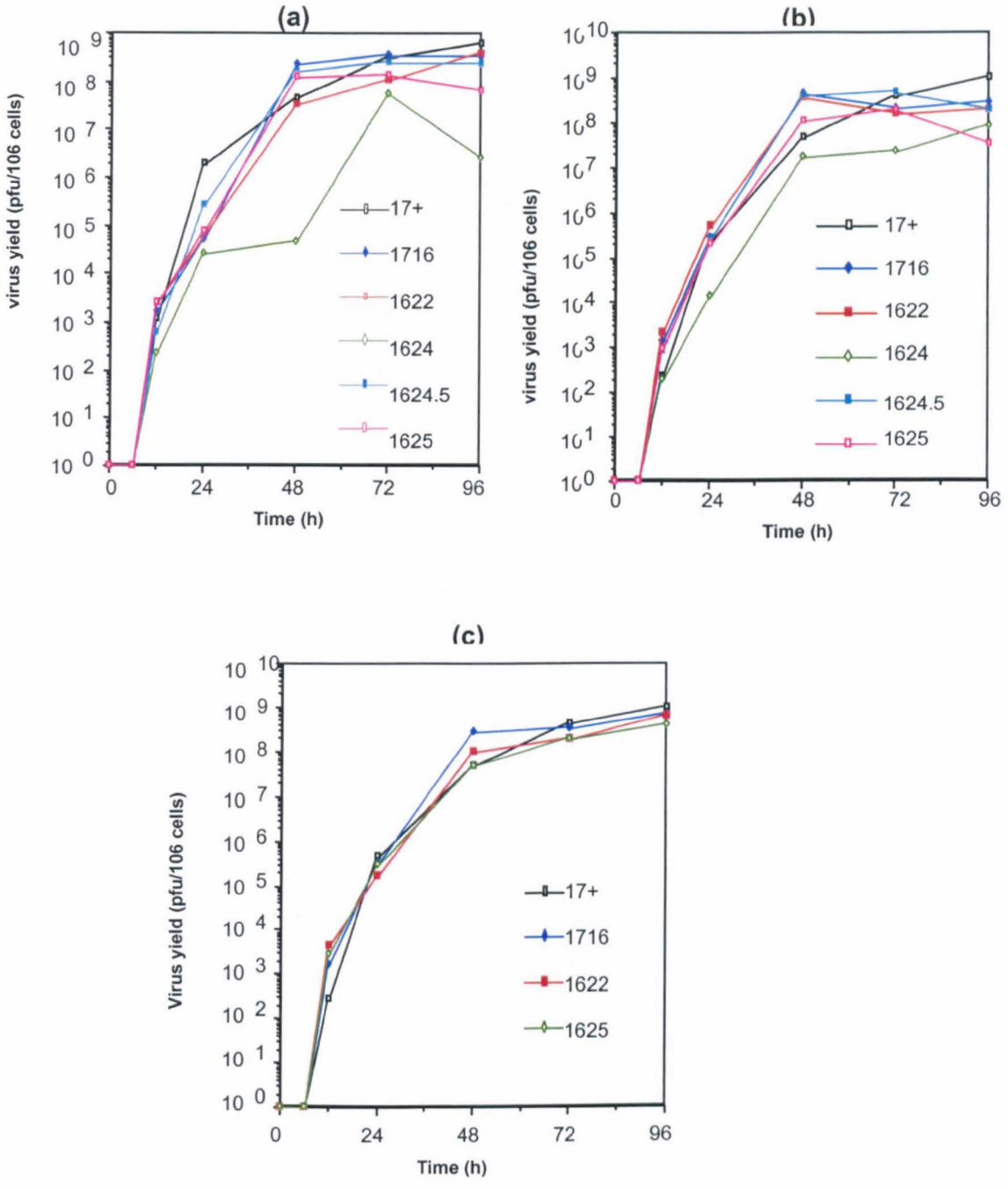


Figure 4.9 Multicycle replication kinetics in BHK cells

BHK cells were infected at a m.o.i of 0.001 pfu/cell. At O, 6, 12, 24, 48, 72, 96 and 120 h pi, infected cells were harvested. Infected cells were scraped into the medium, sonicated and titrated onto a BHK monolayer. Three separate experiments (a, b, and c) are shown.

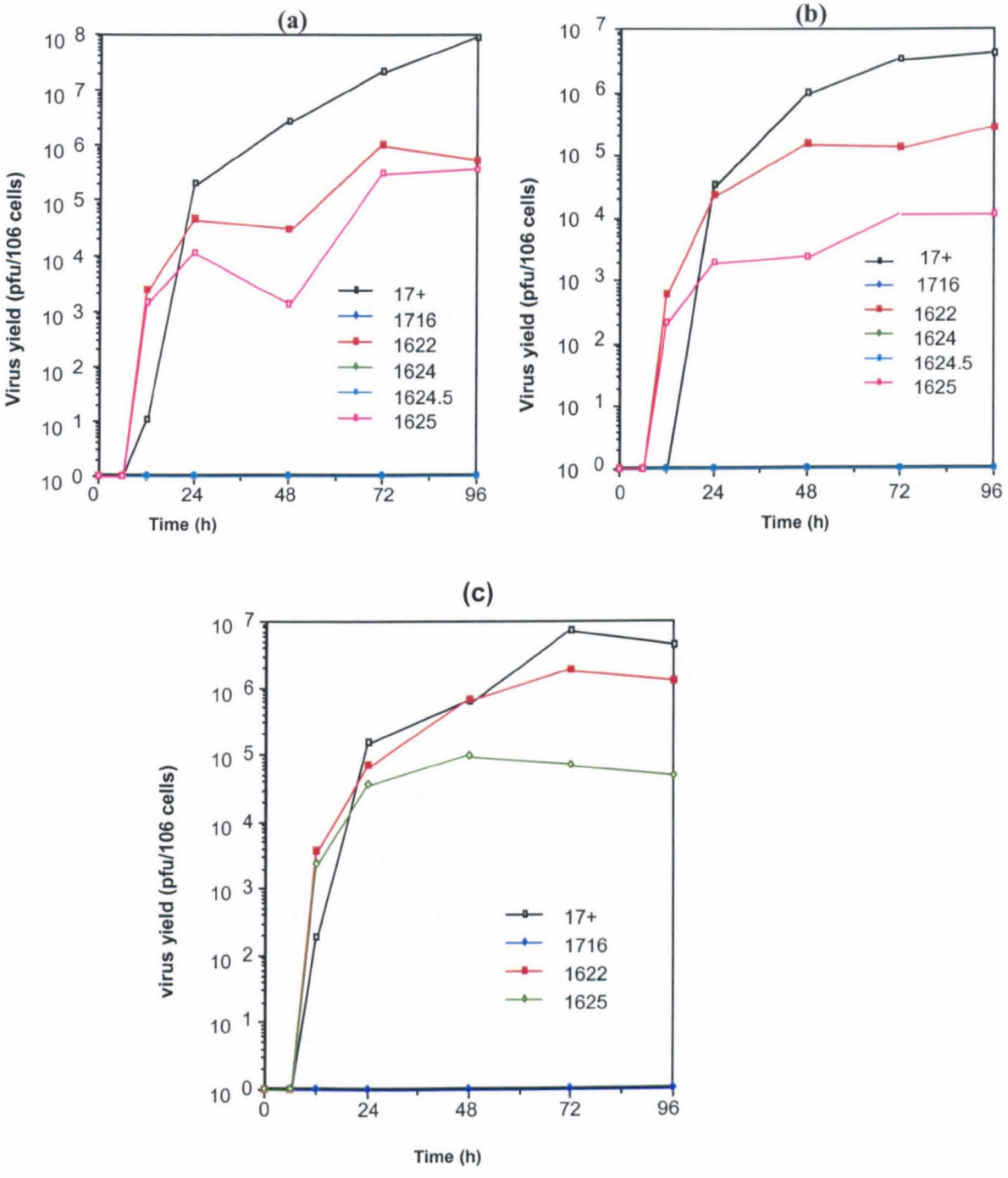


Figure 4.10 Multicycle replication kinetics in 3T6 cells

3T6 cells were infected at a m.o.i of 0.001 pfu/cell. At O, 6, 12, 24, 48, 72, 96 and 120 h pi infected cells were harvested. Infected cells were scraped into the medium, sonicated and titrated onto a BHK monolayer. Three separate experiments (a, b, and c) are shown.

Although both 1622 and 1625 grew they plataued at a maximum titre of 10^1 to 10^2 fold lower than 17^+ (a, b and c). In some experiments both 1622 and 1625 grew similarly (Fig.4.10a) whereas in others 1625 grew 10 fold less well than 1622. Most consistently, 1625 grew to a maximum titre 10 fold lower than 1622 (Fig.4.10b and c)

4.8.3 Multicycle replication kinetics in SK-N-SH cells

In SK-N-SH cells, Fig.4.11 (a, b and c), 17^+ grew well reaching a maximum titre of 10^7-10^8 pfu/ml by 48-72 h pi. 1716 and 1624.5 although exhibiting some growth reached a maximum titre 10^4 lower than 17^+. In agreement with its impaired growth in BHK cells, 1624 grew consistently 10 fold poorer. Both 1622 and 1625 grew similarly to 17^+ (Fig.4.11a). In some experiments, 1622 and 1625 grew similarly (Fig.4.11a) whilst in others 1625 grew about 5 fold poorer than 1622 (Fig.4.11b and c).

4.9 Analysis of 1625 profile at the end of the growth experiments

As discussed in 3.5.3, we could only obtain 1625 with 90% purity. As 1625 was slightly impaired compared to its parent 1622, we were concerned that the growth of 1625 was partly due to amplification of the parental 1622 contamination. To check this a DNA profile analysis was carried out on the final timepoint from each of the 3 growth experiments. The DNA profile of 1625 from the final timepoint in each cell type was compared to that of the stock. Fig.4.12 represents the DNA profile from the 1st experiment.

The DNA profile of 1625 at the end of the growth experiment from each cell type (lanes 3, 4 and 5) was identical to that of the stock (lane 2) with 90% 1625 and 10% 1622. Thus there was no amplification of the 1622 contamination and hence the growth of

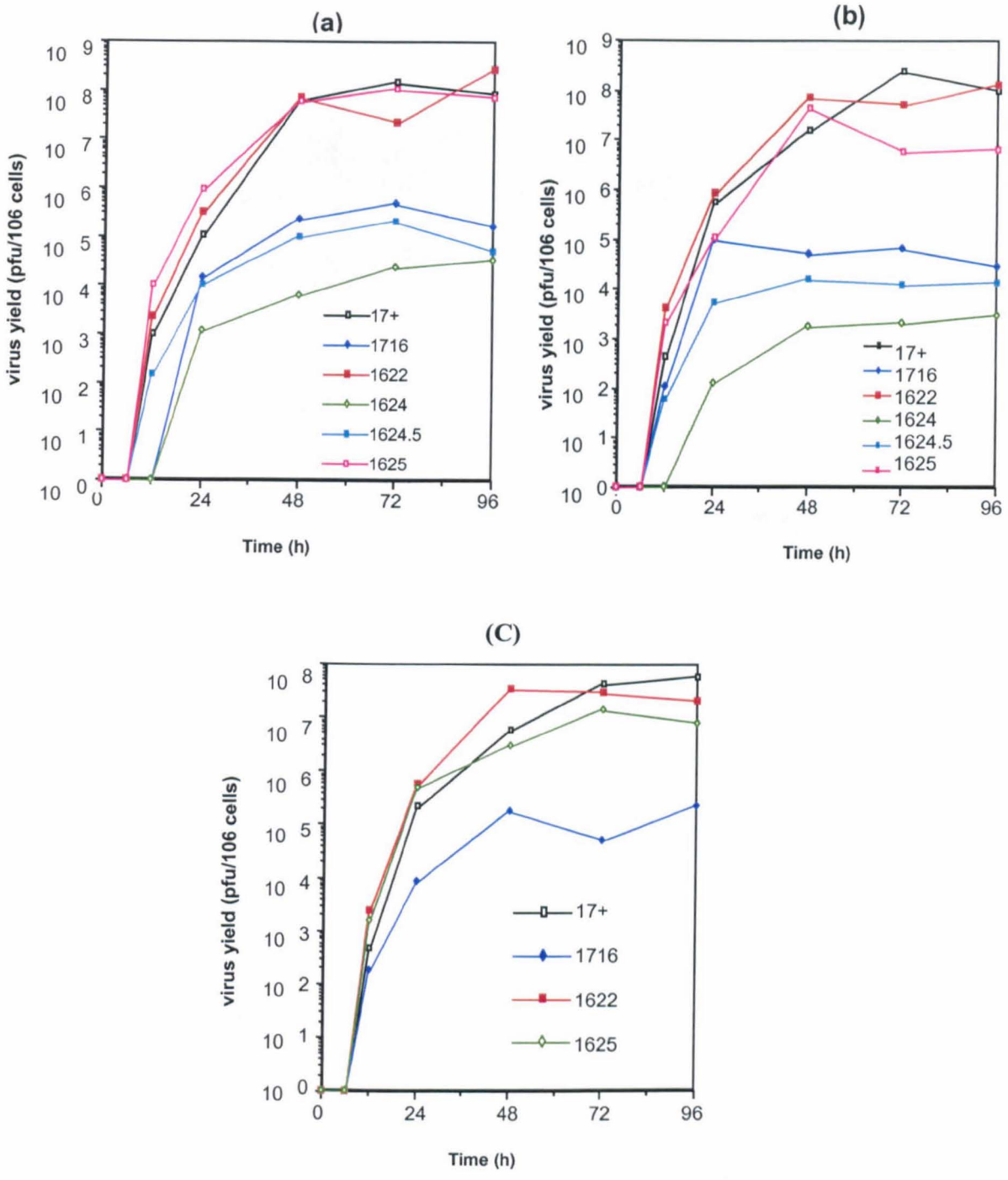


Figure 4.11 Multicycle replication kinetics in SK-N-SH cells

SK-N-SH cells were infected at a m.o.i of 0.001 pfu/cell. At O, 6, 12, 24, 48,72, 96 and 120 h pi infected cells were harvested. Infected cells were scraped into the medium, sonicated and titrated onto a BHK monolayer. Three separate experiments (a, b, and c) are shown.

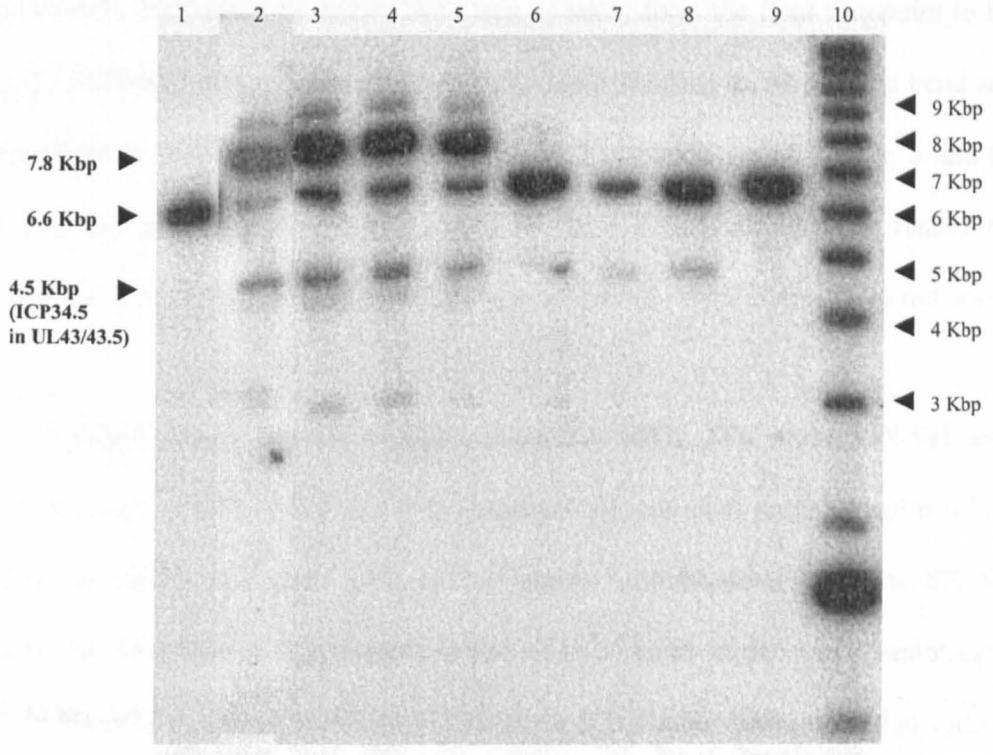


Figure 4.12 DNA profile of 1625 at the end of growth curve 1 (G1)

Southern blot of HSV-1 DNA digested with BamHI and probed with [^{32}P]dCTP labelled pAT5.1/ORF P and exposed to an autoradiograph for 24h at -70^{0}C. Lane1: 17^{+}/BHK; lane 2: 1625 (stock); lane 3: 1625/BHK (96 h); lane 4: 1625/SK (96 h); lane 5: 1625/3T6 (120 h); lane 6: 1622/BHK; lane 7: 1624/BHK; lane 8: 1624.5/BHK; lane 9: 1716/BHK; lane 10: 1 kbp DNA ladder with the sizes marked and hybridising bands are indicated (◀).

1625 was not due to preferential growth of 1622. As expected 17^{+} (lane1), 1716 (lane 9), 1622 (lane 6), 1624 (lane 7) and 1624.5 (lane 8) taken from the final timepoint in BHK cells still exhibited one hundred percent purity. Hybridization to the 4.5 kbp band is due to hybridization of ORF P to either ICP34.5 (1622 and 1625, lanes 2, 3, 4, 5 and 6) or ORF P (1624 and 1624.5, lanes 7 and 8) in the UL43 locus. Similar results were obtained when analysing the DNA profile from all growth experiments (data not shown).

4.10 Analysis of host protein synthesis shutoff in BHK, 3T6, and SK-N-SH cells

One of the roles of HSV-1 ICP34.5 is to maintain host and viral protein synthesis late in infection in certain cell types such as the human neuroblastoma cell line SK-N-SH (1.15.3). To determine if this phenotype had been restored in the recombinant viruses 1622, 1624, 1624.5, and 1625, SK-N-SH, BHK, and 3T6 cells were infected at a m.o.i of 10 pfu/cell, labelled with [^{35}S] methionine late in infection, harvested and analysed by SDS-PAGE.

In Fig.4.13a the 2 varients, 1622 (lane 3) and 1625 (lane 5), expressing ICP34.5 gave a similar protein profile to 17+ (lane 1) whereas the recombinants expressing only ORF P, 1624 (lane 4) and 1624.5 (lane 6), gave a protein profile similar to 1716 (lane 2) and protein synthesis was not restored. Thus the growth of 1622 and 1625 in SK-N-SH cells is most likely due to restored protein synthesis and the lack of growth of 1624/1624.5 is probably due to the failure to restore protein synthesis. In BHK cells (Fig.4.13b), as expected, all viruses showed a similar protein synthesis profile with no shutoff of protein synthesis. In 3T6 cells (Fig.4.13c), all viruses again also showed a similar protein synthesis profile with no shutoff of protein synthesis. In these cells the lack of inhibition

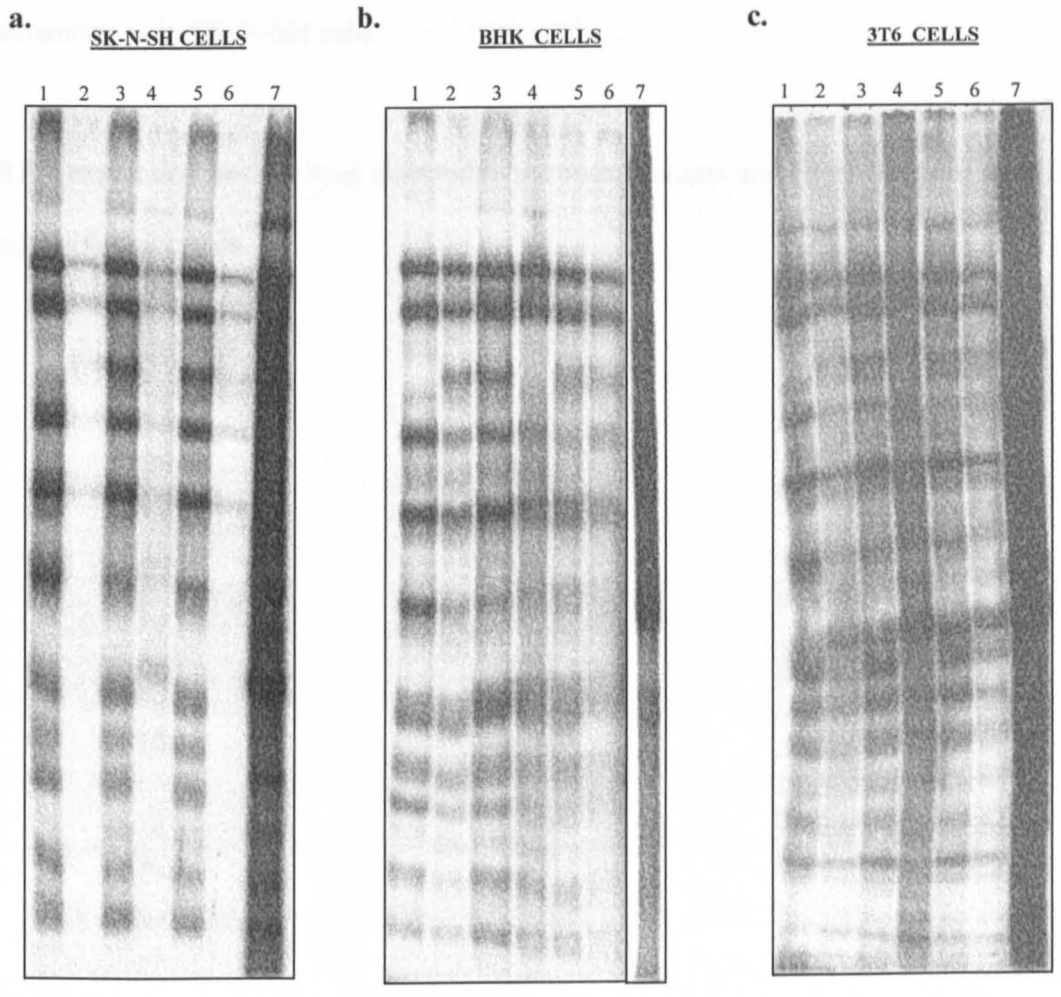


Figure 4.13 Host cell protein synthesis shutoff in SK-N-SH, 3T6 and BHK cells

SK-N-SH (a), BHK (b), and 3T6 (c) cells were infected with HSV-1 variants at a m.o.i of 10 pfu/cell and pulse labelled with [^{35}S] methionine from 14-16 h pi. The infected cells were harvested and analysed by 10% SDS-PAGE and exposure to an autoradiograph o/n at –70^{0}C. Lane 1: 17^{+}; lane 2: 1716; lane 3: 1622; lane 4: 1624; lane 5: 1625; lane 6: 1624.5; lane 7: MI.

of protein synthesis by 1716 showed that the growth defect was not due to the same mechanism as in SK-N-SH cells.

ORF P expression has not been detected in our recombinants and all conclusions must be made with that caveat.

5.0 Optimization of GST-P pull down experiments

5.1 Optimization of GST-ORF P fusion protein expression

5.1.1 Introduction

A pGEX based system can be used for inducible protein expression in *E.coli*. In this system target genes are cloned as a glutathione-S-transferase (GST) fusion under the control of the T7 promoter, which is not recognised by *E.coli* RNA polymerase and therefore virtually no expression occurs until a source of T7 RNA polymerase is provided. This decreases the posibility of plasmid instablility due to the production of proteins potentially toxic to the host cell. Once cloned, plasmids are transferred into an expression host containing a chromosomal copy of the T7 RNA polymerase gene under the control of the *Lac I* promoter and expression is induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) which induces expression from the *Lac* promoter. Alternatively, T7 RNA polymerase can be provided by infection of the original cloning host with CE6 Lambda phage which expresses T7 RNA polymerase (Smith and Johnson, 1988).

Routinely, the aminoterminus of a protein is fused inframe to the carboxyterminus of GST. During fusion protein expression, several factors can be varied to maximise the amount, length, and solubility of the protein. Increasing the IPTG concentration may increase the amount of product. Decreasing the temperature at which fusion proteins are expressed will decrease total yield but will also decrease the action of proteolytic enzymes which may degrade fusion proteins, leading to increased amounts of full length protein. It can also increase the solubility of the fusion protein by altering its folding. Increasing the time of induction will increase fusion protein expression but will also increase the length of time proteases have to work in and therefore may lead to a

decrease in the amount of full length protein. The expression of GST-ORF P where ORF P was cloned as a 3' GST fusion protein in a bacterial system had not been previously optimized.

Using a number of *E.coli* strains at different temperatures, expression of the amount and length of GST-ORF P was optimised.

5.1.2 Optimization of GST-ORF P expression in different *E-coli* strains BL21, C41, and Novablue.

To choose the best expression system for GST-ORF P, *E.coli* strains BL21, C41, and Novablue were used.

(i) BL21

BL21 is the most widely used host backgroud and has the advantage of being deficient in both lon and ompT proteases. BL21 contains a plasmid containing the T7 polymerase gene under the *Lac I* promoter (Fig.5.1a) which is induced by IPTG to express T7 RNA polymerase. This enzyme attaches to both the GST and GST-ORF P promoter and hence leads to expression. In this system, bacteria are grown at 37°C to midlog phase and then induced by IPTG for two hours.

(ii) C41

The principle of GST fusion protein expression in C41 is the same as that in BL21. However, in C41 the lac repressor binds the *Lac I* promoter tighter and therefore there is less uninduced expression of the T7 polymerase and hence fusion protein (Fig.5.1a).

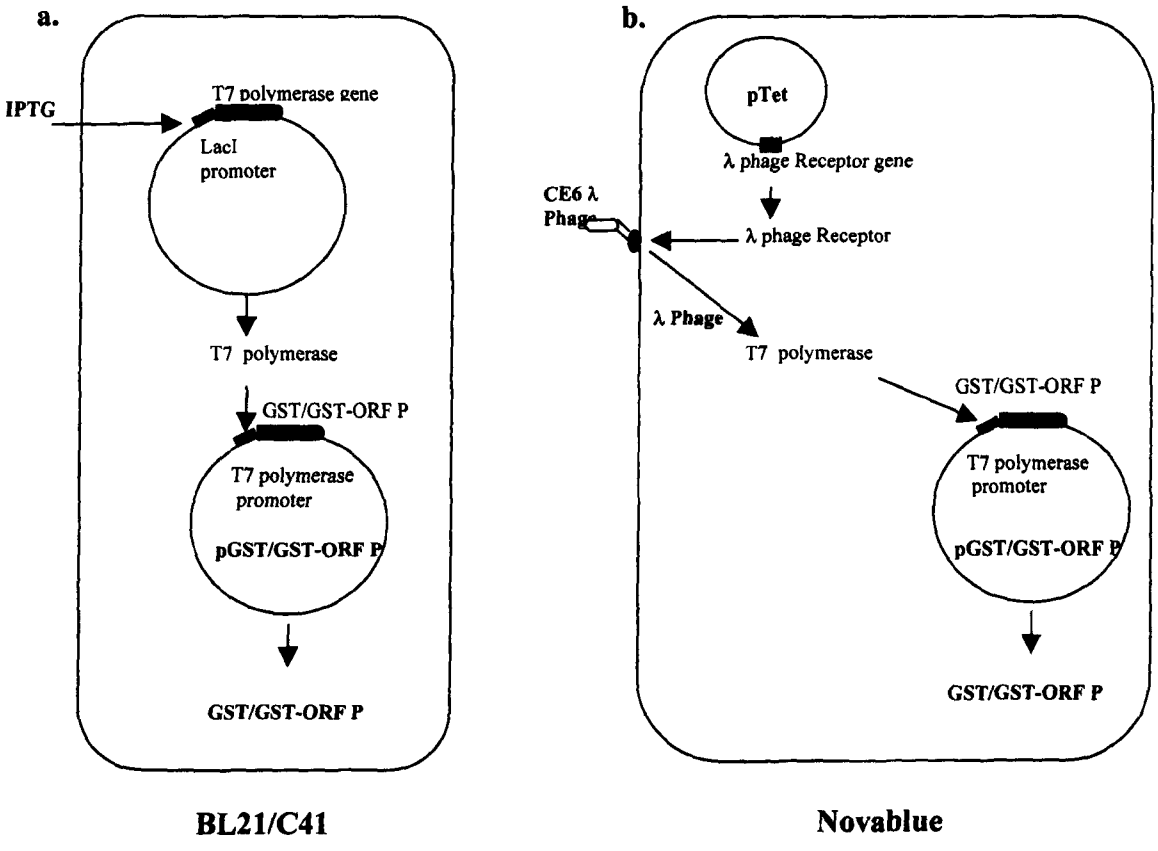


Figure 5.1 Mechanism of GST/GST-ORFP expression in BL21, C41, and Novablue

a. BL21/C41. IPTG induces the T7 polymerase promoter to express T7 polymerase and this enzyme attaches to the GST and GST-ORF P promoter to synthesise both GST and GST-ORF P.

b. Novablue. λ CE6 phage expresses T7 polymerase in phage infected *E.coli* and this enzyme attaches to both the GST and GST-ORF P promoter to synthesise both GST and GST-ORF P. pTet produces the λ phage receptor on the surface of *E.coli* to allow phage entry.

(iii) Novablue

Novablue is an *E.coli* K-12 strain ideally suited as an initial cloning host due to its high transformation efficiency which results in high yields of excellent quality plasmid DNA. This strain does not contain the T7 RNA polymerase gene containing plasmid and superinfection with CE6 λ phage containing the T7 RNA polymerase gene is used to induce expression of fusion protein (Studier and Moffat, 1986). Novablue cells contain a plasmid, pTet, which encodes the phage λ receptor gene and produces the phage receptor on the bacterial surface. This system is used when a target gene is so toxic that a plasmid cannot be maintained in a DE3 lysogenic host such as BL21 where there will always be minimal recombinant protein expression. In this system, bacteria are grown at 37°C to midlog phase and then superinfected with λ phage for two hours (Fig.5.1b).

Expression of both GST and GST-ORF P was detected in these 3 strains by Western blotting with an anti-GST serum (Fig.5.2a) and Commassie blue staining (Fig.5.2b). Expression of the 28 KDa GST is best in BL21 (lane 1), slightly lower in C41 (lane 3), and significantly lower in Novablue (lane 5). Expression of truncated GST-ORF P in BL21 which was detected as a 40 KDa doublet is shown in lane 2. No expression of GST-ORF P is detected in either C41 (lane 4) or Novablue (lane 6). Thus expression of GST-ORF P was best in BL21.

5.1.3 Optimization of GST-ORF P and GST-ICP27 expression in BL21 using different temperatures

The optimum temperature for expression of GST-ORF P in BL21 was investigated. As we were going to use GST-ICP27 in subsequent experiments (see 5.2.2), the optimum temperature for the expression of GST-ICP27 in BL21 was also investigated. Expression

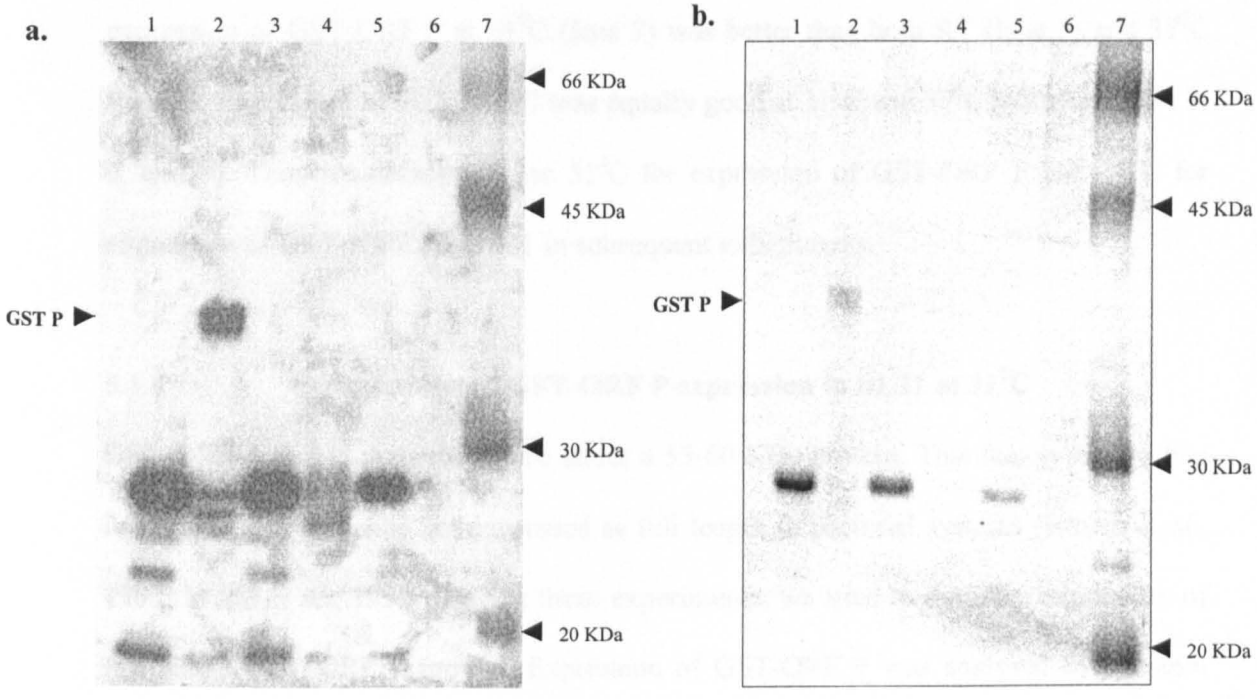


Figure 5.2 Expression of GST and GST-ORF P in *E.coli* strains BL21, C41, and Novablue

GST pulldown extracts were run on a 12.5% SDS-PAGE and analysed by **a)** Western blotting using an anti-GST serum and **b)** Commassie blue staining. Lanes 1 and 2: BL21; lanes 3 and 4: C41; lanes 5 and 6: Novablue. Lanes 1, 3, and 5: GST; lanes: 2, 4, and 6 GST-ORF P; lane 7: molecular weight markers with sizes are shown. GST-ORF P related bands are indicated (►).

at different temperatures was analysed by Western blotting with an anti-GST serum (Fig.5.3a) and Commassie blue staining (Fig.5.3b).

Expression of GST at all three temperatures was the same (lanes 1, 4, and 7). However, expression of GST-ORF P at 31^0C (lane 2) was better than both RT (lane 5) and 37^0C (lane 8). Expression of GST-ICP27 was equally good at 31^0C and 37^0C (compare lanes 3, 6, and 9). Thus we decided to use 31^0C for expression of GST-ORF P and 37^0C for expression of GST-ICP27 in BL21 in subsequent experiments.

5.1.4 Optimization of GST-ORF P expression in BL21 at 31^0C

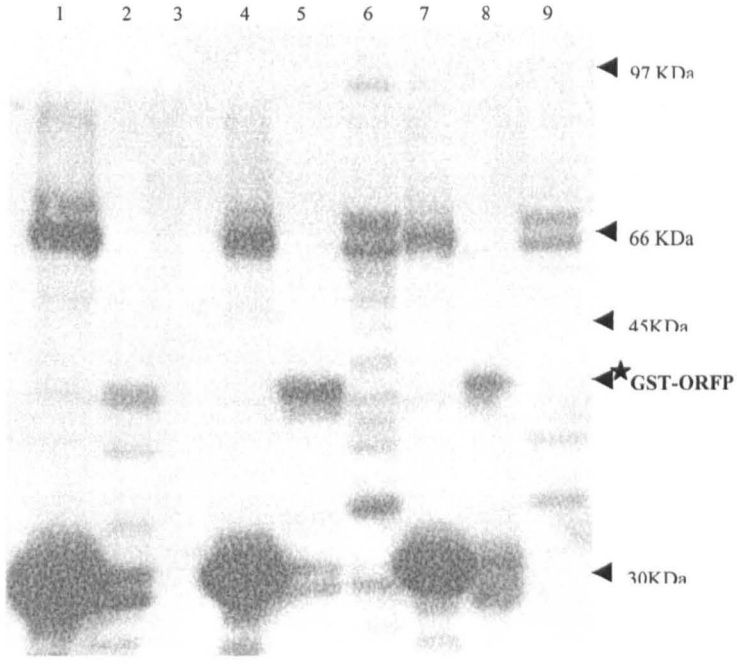
GST-ORF P should theoretically be about a 55-60 KDa protein. This fusion protein like many HSV-1 proteins is not expressed as full length in bacterial systems (Brown *et al.*, 1997; Wadd *et al.*, 1999). During these experiments, we tried to optimise expression of full length GST-ORF P protein. Expression of GST-ORF P was analysed by Western blotting with an anti GST serum (Fig.5.4a) and Commassie blue staining (Fig.5.4b). Expression of GST was similar in both the initial (using normal western blotting and Commassie blue staining) and final experiments (repetition of experiments and keeping everything on ice) (lanes 1 and 3). In the initial experiments, a 40 KDa GST-ORF P doublet was detected in both Western blotting and Commassie staining (lane 2). In the final experiments the 40 KDa doublet and a higher molecular weight band of 45 KDa was detected (lane 4).

Expression of GST-ORF P from the final experiment was also analysed on a 5-12.5% gradient SDS-PAGE by Commassie staining (Fig.5.5). Again the 28 KDa GST was detected (lane 1). In this gel as well as detecting the 40 KDa doublet and the 45 KDa band, two higher molecular weight bands of 50 and 60 KDa were detected (lane 2). These may correspond to full length GST-ORF P.

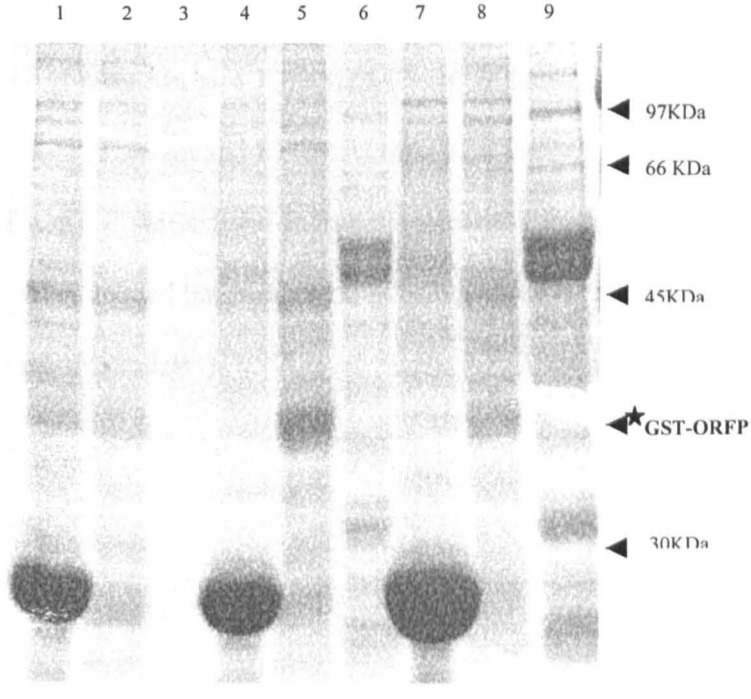
Figure 5.3 Expression of GST, GST-ORF P, and GST-ICP27 at RT, 31°C and 37°C

GST pulldown extracts were run on a 12.5% SDS-PAGE and analysed by **a)** Western blotting using an anti-GST serum and **b)** Commassie blue staining. Lanes 1, 2, and 3: room temperature; lanes 4, 5, and 6: 31°C; lanes 7, 8, and 9: 37°C; lanes 1, 4, and 7: GST; lanes 2, 5, and 8: GST-ORF P; lanes 3, 6, and 9: GST-ICP27. GST-ORF P related bands are indicated (★). Molecular weight markers with sizes are shown (►).

a.


1 2 3 4 5 6 7 8 9
97 KDa
66 KDa
45KDa
GST-ORFP
30KDa

b.


1 2 3 4 5 6 7 8 9
97KDa
66 KDa
45KDa
GST-ORFP
30KDa

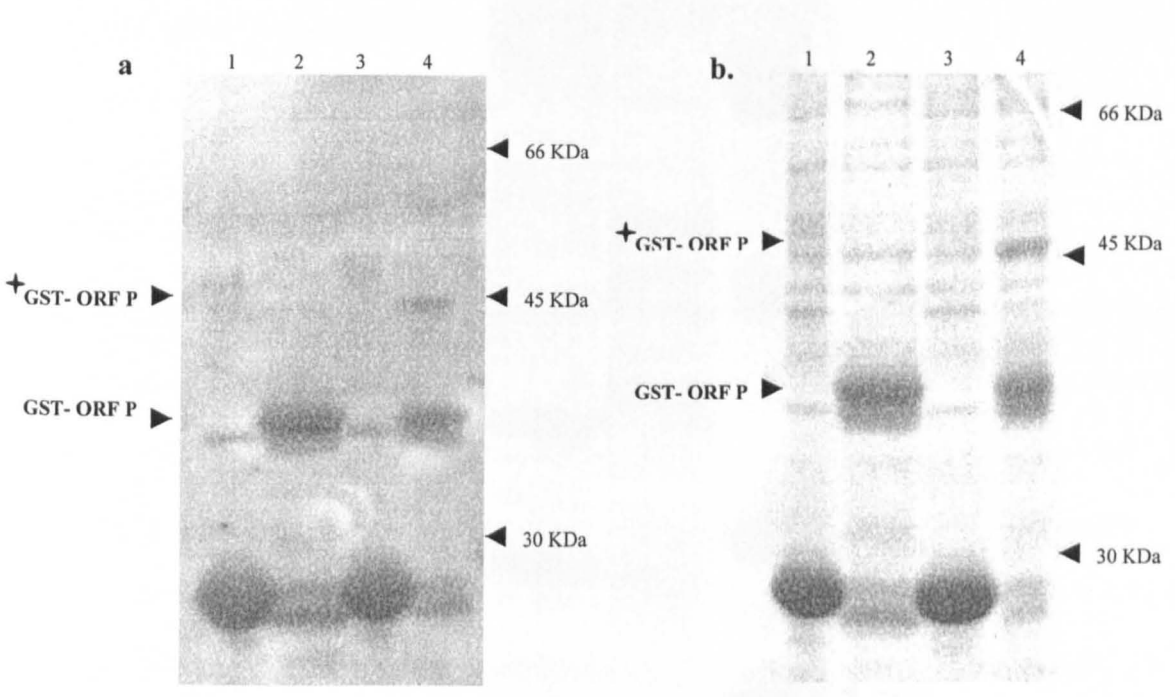


Figure 5.4 Expression of GST-ORF P in both initial and final experiments

GST pulldown extracts were run on a 10% SDS-PAGE and analysed by **a)** Western blotting using an anti-GST serum and **b**) Commassie blue staining. Lanes 1 and 3: GST; lanes 2 and 4: GST–ORF P; lanes 1 and 2: initial expression; lanes: 3 and 4: final expression. GST-ORF P related bands 40 KDa (►) and 45 KDa (✦►)are indicated. Molecular weights are marked (►).

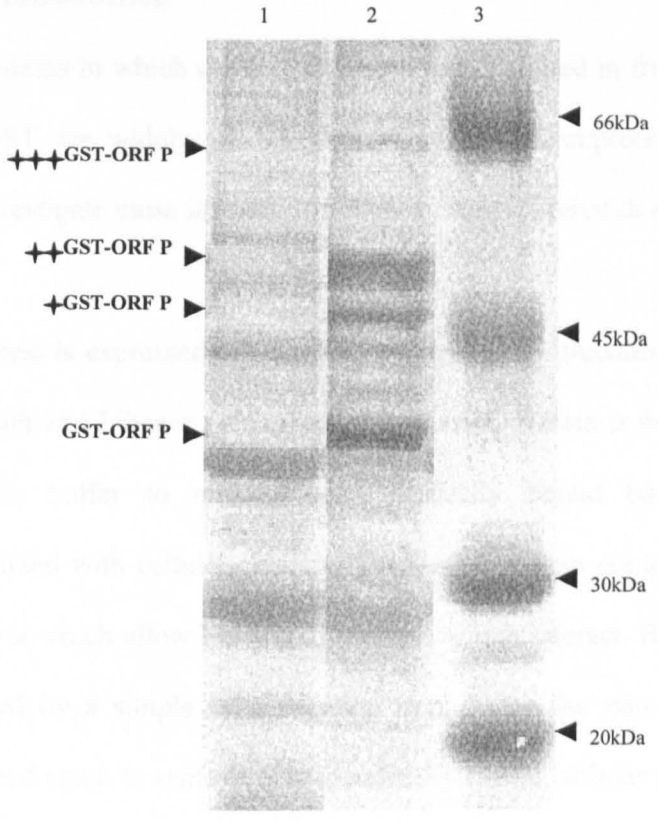


Figure 5.5 Expression of GST-ORF P in the final experiment

GST pulldown extracts were run on a 5-12.5 % gradient SDS-PAGE and analysed by Commassie blue staining. Lane 1: GST; lane 2: GST-ORF P; lane 3: molecular weight markers with sizes shown. GST-ORF P related bands of 40 KDa (►), 45 KDa (+), 50 KDa (++) and 60 KDa (+++) are indicated. Molecular weight markers are indicated on the right hand side (►).

5.2 GST pulldown assays

5.2.1 Introduction

GST fusion proteins in which the protein of interest is cloned in frame with the carboxy terminus of GST are widely used to identify new protein:protein interactions or to confirm and investigate those identified in other systems (Meredith *et al.*, 1994).

The fusion protein is expressed and purified by binding to glutathione which is attached to agarose (Smith and Johnson, 1988). After the fusion protein is bound to the matrix, it is washed with buffer to remove non-specifically bound bacterial proteins and subsequently mixed with cellular extracts. The fusion protein containing beads serve as an affinity matrix which allow binding of proteins which interact. Bound proteins can be partially purified by a simple centrifugation step, hence the name "pulldown" assay. Beads are washed again to remove non-specifically bound cellular proteins and proteins analysed by SDS-PAGE. An overview of this method can be seen in (Fig.5.6).

GST pulldown assays have been successfully used by many workers. For example, a similar approach to the one used here was used to identify an interaction between the immediate early HSV-1 protein ICPO and a 135 KDa cellular protein (Meredith *et al.*, 1994). As a part of this project, this method was used to attempt to identify both cellular and viral proteins which interact with GST-ORF P.

5.2.2 Control of pulldown assay by use of GST-ICP27.

Using the standard conditions for a GST pulldown assay, we were not able to recognize any ORF P interacting proteins (data not shown). This method had previously identified a

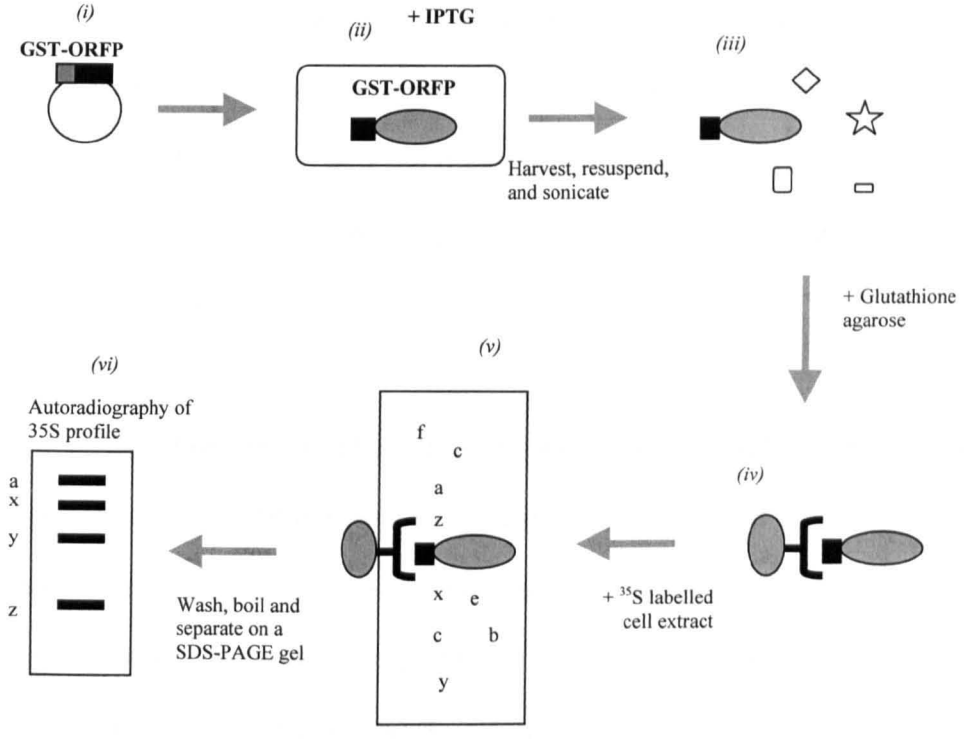


Figure 5.6 GST pulldown assay

(i) GST-ORF P is transformed into *E.coli.* (ii) GST-ORF P expression is induced by the addition of IPTG. (iii) bacteria are harvested, resuspended in a small volume and split open by sonication. (iv) GST -ORF P is purified from bacterial proteins by addition of glutathione agarose. (v) A pulldown assay was performed by the addition of [^{35}S] methionine labelled cellular extracts to purified GST-ORF P bound onto beads. (vi) The complex is washed to remove nonspecific binding, boiled to denature the proteins and break protein:protein interactions, separated by SDS-PAGE and autoradiographed.

number of proteins interacting with GST-ICP27 (Bryant, 2000). Thus to investigate that I was getting the method to work, the GST-ICP27 protein was used as a positive control and its interaction with 17^+ infected cellular proteins was investigated. A pulldown of 17^+ infected cell proteins was performed with GST, GST-ICP27 and GST-ORF P (Fig.5.7). GST-ICP27 (lane 1) interacted with a number of proteins compared to GST (lane 2) e.g previously identified bands of 66 KDa (hnRNPK) and 44 KDa (TK) (Bryant, 2000). Thus the pulldown procedure was working correctly. Again, GST-ORF P (lane 3) did not show any specific interaction with viral or cellular proteins.

5.2.3 Optimization of the pulldown assay by varying NaCl concentration

As with co-immunopercipitations, salt and detergent concentrations in the buffers along with target and interacting protein concentrations affect which proteins interact with the bound fusion protein. The previous experiment (Fig.5.7) showed that although the GST pulldown method was working no ORF P interacting proteins could be identified. Thus we attempted to optimise the detection of interacting bands by GST-ORF P by changing the salt concentration of the washing buffer. The standard concentration used was 0.5 mM NaCl and by increasing the salt concentration we would increase the stringency of the washing buffer and hopefully remove non specific binding bands leaving specific interacting bands visible. Obviously we would reach a salt concentration where both nonspecific and specific interacting bands were removed. Fig.5.8 shows an experiment when a pulldown was carried out and samples were washed in either 0.5, 5, 50 or 500 mM NaCl containing wash buffer, respectively.

As before, washing with 0.5 mM NaCl containing wash buffer resulted in no specific bands being detected. (see Fig 5.7 and data not shown). Using 5 mM NaCl containing

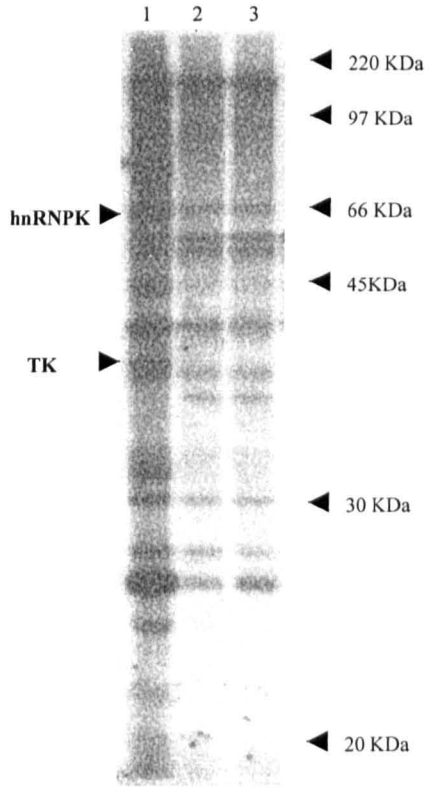


Figure 5.7 GST-ICP27 pulldown

A GST pulldown was performed on a [^{35}S] methionine labelled 17^{+} infected cell extract, analysed by 12.5% SDS-PAGE and autoradiography. Lane 1:GST-ICP27; lane 2: GST and lane 3: GST-ORF P. Two predominant bands which interact with ICP27, hnRNPK and TK are indicated (►). Molecular weights are marked (►).

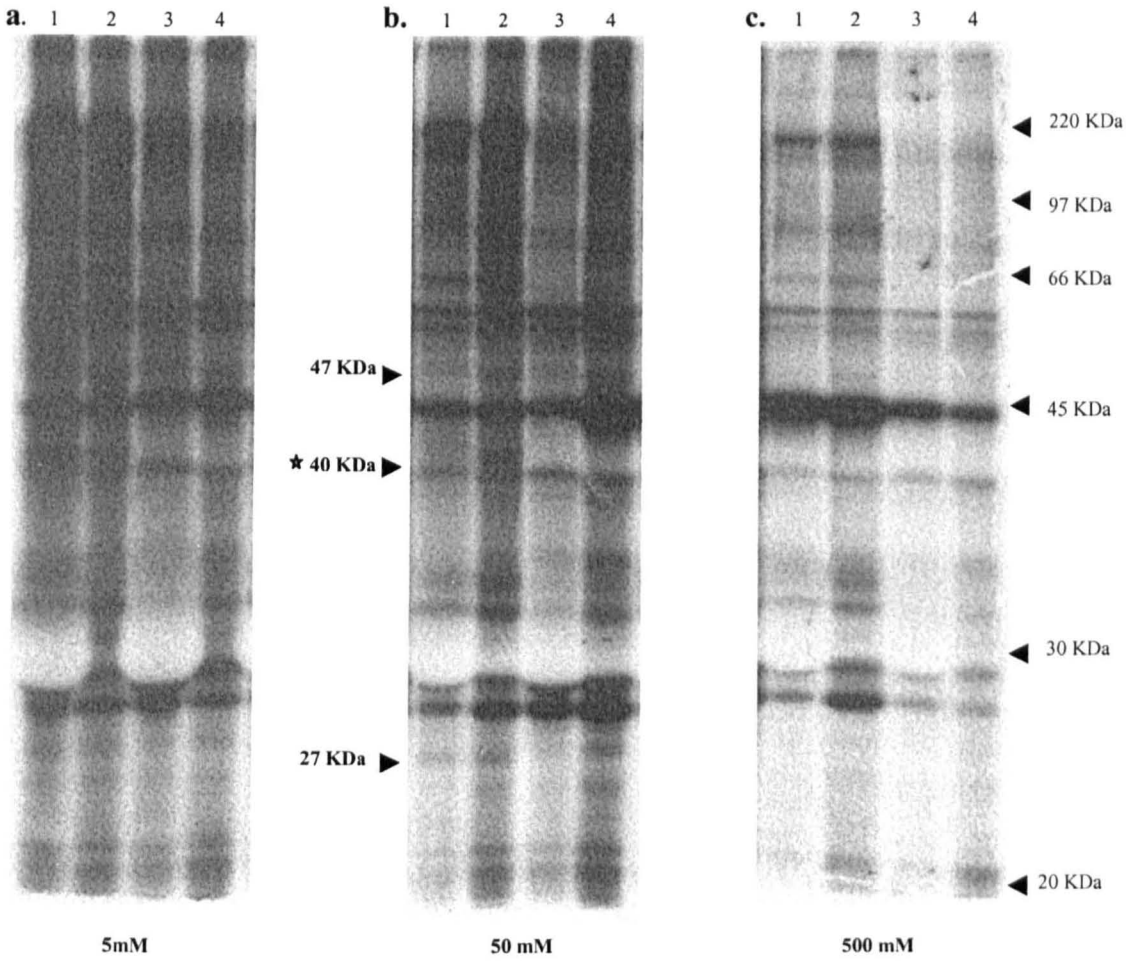


Figure 5.8 Optimization of GST pulldown experiment by the use of different concentrations of NaCl in the washing buffer

A GST pulldown was performed on [^{35}S] methionine labelled 17^+ infected (lanes 1 and 2) and MI cell extracts (lanes 3 and 4), and analysed by SDS-PAGE and autoradiography. Pulldowns were washed with a) 5 mM, b) 50 mM and c) 500 mM NaCl. Lanes 1 and 2: 17^+ infected extract; lanes 3 and 4: MI infected extracts; lanes 1 and 3: GST; lanes 2 and 4: GST-ORF P. GST-ORF P interacting cellular (▶) and viral (★ ▶) bands with their molecular weights are indicated. Molecular weights are marked (▶).

wash buffer (Fig.5.8a), no specific bands were still detected. However, increasing the NaCl to 50 mM (Fig.5.8b) resulted in a number of specific bands being detected. These bands include at least two viral and possibly one cellular protein with sizes of 27, 40 (viral) and 47 KDa (cellular) (lanes 2 and 4). However, the photograph did not reproduce this well. Increasing the NaCl to 500 mM, resulted in these bands being lost (Fig.5.8c).

6.0 Identification of proteins which interact with ORF P

6.1 Introduction

Using a GST pulldown assay, it was shown in the previous chapter that ORF P interacts with a number of cellular proteins and also with one viral protein. The aim of the work in this section was to identify with which cellular and viral gene products ORF P interacts. There is some previous evidence indicating that ORF P interacts with a number of splicing factors and may play a role in splicing (Bruni and Roizman, 1996). For this reason we screened our GST pulldowns with a range of antibodies against proteins with some role in splicing and posttranscriptional processing and which were of an approximate molecular weight to those identified in the previous chapter (Chapter 5). Western blotting of cellular extracts was carried out with 14 available antibodies. Of these we could only get positive results with 10 against cellular extracts. These 10 working antibodies were used to analyse GST-ORF P pulldowns by Western blotting to detect an interaction of ORF P with these proteins. The antibodies used and their characteristics are shown in table 6.1.

6.2 Interaction of ORF P with ICP27

It had previously been shown that ICP27 interacts with cellular posttranscriptional processing factors (Bryant *et al.*, 2000). Thus it seemed reasonable to investigate whether ORF P interacts with ICP27. A GST-ORF P pulldown was carried out and Western blotted with an anti-ICP27 antibody. Both GST and GST-ORF P interact with ICP27 and its related bands (Fig.6.1). To try to reduce this non specific binding, the amount of beads was reduced from 50 (lanes 2 and 3) to 12.5 ul (lanes 4 and 5) and 50 mM NaCl containing washing

Primary antibody	Type	Function	References
α CPSF (25 KDa)	Mouse	3' cleavage and polyadenylation of mRNA	(Maneley, 1995)
hnRNP K	Rabbit	Interacts with a number of cellular proteins involved in transcription, translation and signal transduction	(Bustello et al., 1995)
CK II β sununit	Mouse	phosphorylates and modulates the function of a number of viral proteins	(Sanz-Ezquerro et al., 1998)
Thymidine Kinase	Mouse	Deoxypyrimidine kinase (phosphorylation of thimidine)	(Evans et al., 1998
P32	Mouse	Involved in splicing	(Krainer et al., 1990)
SC35 [1]	Mouse	Splicing factor	(Sandri-Goldin et al., 1995)
hnRNP J	Mouse	Multifunctional protein involved in posttranscriptiona regulation	(Bustello et al., 1995)
α ASF	Mouse	Recognition and cleavage of 5' splice sites	(Krainer et al., 1991)
ICP27	Rabbit	Multifunctional protein involved in posttranscriptiona regulation	(Bryant et al., 2000
α CFI (25 KDa)	Rabbit	3' cleavage and polyadenylation of mRNA	(Takagaki et al., 1989)
α CFI (68 KDa)	Rabbit	3' cleavage and polyadenylation of mRNA	(Takagaki et al., 1989)
α CPSF (30 KDa)	Rabbit	3' cleavage and polyadenylation of mRNA	(Maneley, 1995)

Table 6.1: Cellular and viral proteins whose *in vitro* interaction with ORF P was investigated. Identified proteins interacting with ORF P indicated [1].

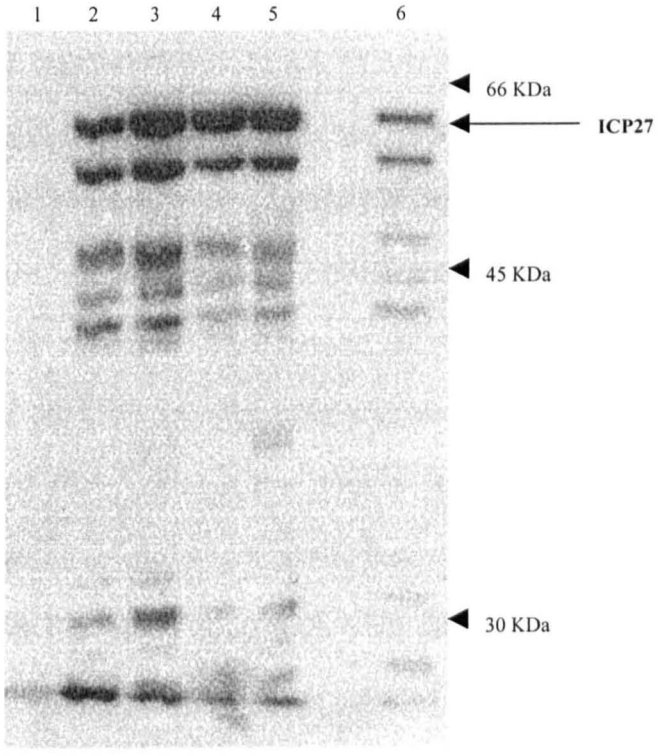


Figure 6.1 Western blotting of a GST-ORFP pulldown with an anti ICP27 antibody

A GST pulldown was run on a 12.5% SDS-PAGE and analysed by Western blotting using an anti-ICP27 antibody. Lane1: MI extract; lanes 2-6: 17^+ infected cell extracts; lane 2: 50 ul GST beads; lane 3: 50 ul GST-ORF P beads; lane 4: 12.5 ul GST beads; lane 5: 12.5 ul GST-ORF P beads and lane 6: whole 17^+ infected cell extract. Full length ICP27 is indicated (→). Molecular weights are marked (►).

buffer was used. Decreasing the amounts of beads had no effect on the nonspecific interaction of ICP27 with GST. As the GST-ICP27 fusion protein expressed better than GST-ORF P, the reverse experiment was carried out where GST-ICP27 was used to try to pulldown ORF P from *ts*K infected cell extracts and Western blotted with an anti-ORF P serum (Fig.6.2). In initial Western blotting, serum 128 cross reacted with the GST-ICP27 related bands as it had been raised against a GST fusion protein (data not shown). Therefore, we had to preadsorb 128 against GST to remove the GST specific bands. When this preadsorbed serum was used, no specific band was detected in lane 3, indicating no interaction of ORF P with ICP27. Also in lane 2 no specific band was detected indicating that no ORF P self interaction ocured. A nonspecific band of 30 KDa (presumably GST) was detected in lanes 1-3 and two bands of 50 KDa were detected in lane 3 presumably the predominant GST-ICP27 bands. These bands are detected due to incomplete adsorption of anti-GST antibodies and thus cross reaction with the major GST containing bands. The antiserum still recognized ORF P in the *ts*K extract (lane 4) indicating that the anti-ORF P antibodies were still present.

6.3 Identification of proteins which do not interact with ORF P

Antibodies against 10 cellular and one viral protein were used in Western blotting of GST-ORF P pulldowns to look for specific interactions (Table 6.1). Of these, only one recognised an identified protein interacting with ORF P. Examples of Western blotting results from two of the non interacting proteins are shown in Fig.6.3 and Fig.6.4. Fig.6.3 shows Western blotting of pulldown extracts with an antibody against p32. In lane 5 p32 can be detected in the mock infected whole cell extract, whereas no interaction can be seen in 17^{+} infected

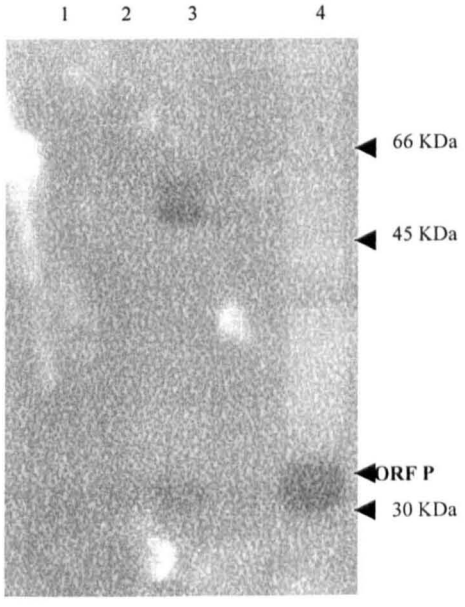


Figure 6.2 Western blotting of a GST-ICP27 pulldown with preadsorbed anti-ORF P serum 128

GST pulldowns of *ts*K infected cell extracts were run on a 12.5% SDS-PAGE and analysed by Western blotting using anti-ORF P serum 128. Lane 1: GST pulldown; lane 2: GST-ORF P pulldown; lane 3:GST-ICP27 pulldown; lane 4: *ts*K infected cell extract. The ORF P band is indicated (►). Molecular weights are marked (►).

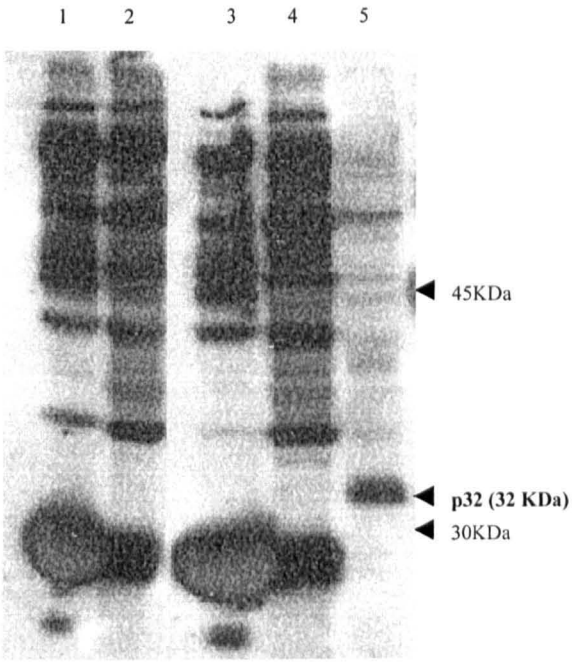


Figure 6.3 Western blotting of a GST-ORF P pulldown with p32 antibody

GST pulldowns were run on a 12.5% SDS-PAGE and analysed by Western blotting using an anti-p32 antibody. Lanes 1 and 2: 17^{+} infected cell extracts; lanes 3-5: MI extracts; lanes 1 and 3: GST pulldown; lanes 2 and 4: GST-ORF P pulldown; lane 5: whole mock infected cell extract. The p32 band is indicated (▶). Molecular weights are marked (▶).

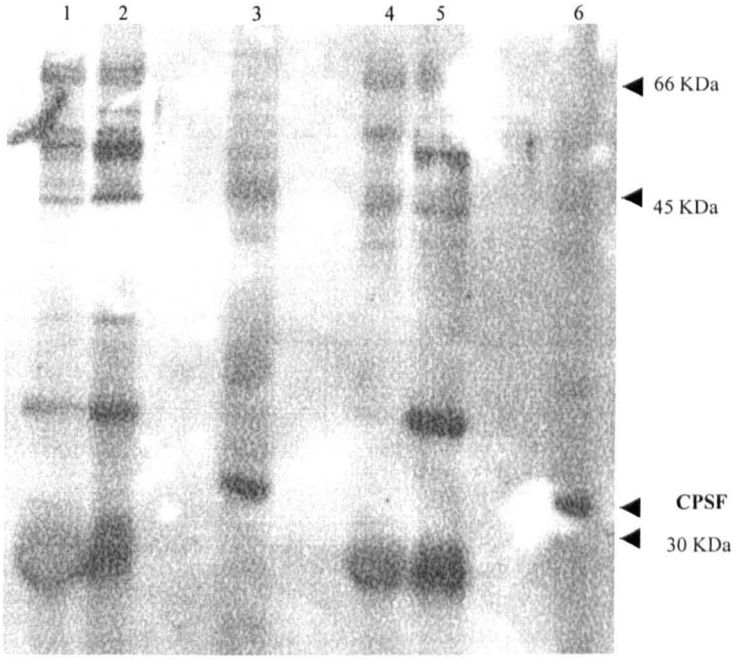


Figure 6.4 Western blotting of a GST-ORF P pulldown with CPSF antibody

GST pulldowns were run on a 12.5% SDS-PAGE and analysed by Western blotting using an anti-CPSF antibody. Lanes 1–3: 17^+ infected cell extracts; lanes 4-6: MI extract; lanes 1 and 4: GST pulldown; lanes 2 and 5: GST-ORF P pulldown; lane 3: whole 17^+ infected cell extracts and lane 6: whole MI cell extract. The CPSF band is indicated (◄). Molecular weights are marked (◄).

(lane 1, 2) or in mock infected pulldowns (lanes 3 and 4) with either GST or GST-ORF P, respectively. Fig.6.4 shows Western blotting of pulldown extracts with an antibody against CPSF. CPSF can be seen in both 17^+ infected (lane 3) and MI whole cell extracts (lane 6), whereas no interaction can be seen in either GST or GST-ORF P pulldowns from 17^+ infected (lanes 1 and 2) or mock infected extracts (lanes 4 and 5).

6.4 Identification of proteins which interact with ORF P

Using Western blotting of pulldown extracts with an antibody against CKII (β), an unexpected result was obtained. No specific interaction with CKII (β) was detected. CKII (β) is detected in both 17^+ infected whole extract (Fig.6.5, lane 5) and mock infected whole cell extract (lane 8), whereas no specific interaction can be seen in either 17^+ infected (lane 4) or mock infected extracts (lane 7) with GST or GST-ORF P. However, a 45 KDa band is specifically detected in lanes 4 and 7, indicating an unidentified cellular protein, which was also detected by the CKII (β) antibody, interacted with ORF P. As the band detected was slightly lower than that detected in both 17^+ (lane 5) and mock infected cell extracts (lane 8), GST (lane 1) and GST-ORF P (lane 2) extracts were used in western blotting to rule out cross reaction of the antibody with the GST-ORF P fusion protein. No 45 KDa protein was detected in either of these extracts.

To try to identify the 40 KDa viral band interacting with GST-ORF P, Western blotting of pulldown extracts was carried out with an antibody against the viral thymidine kinase (TK), which is about 40 KDa (Fig.6.6). As expected, TK is detected in 17^+ infected whole cell extract (lane 5) but not in mock whole cell extract (lane 6). No specific interaction can be

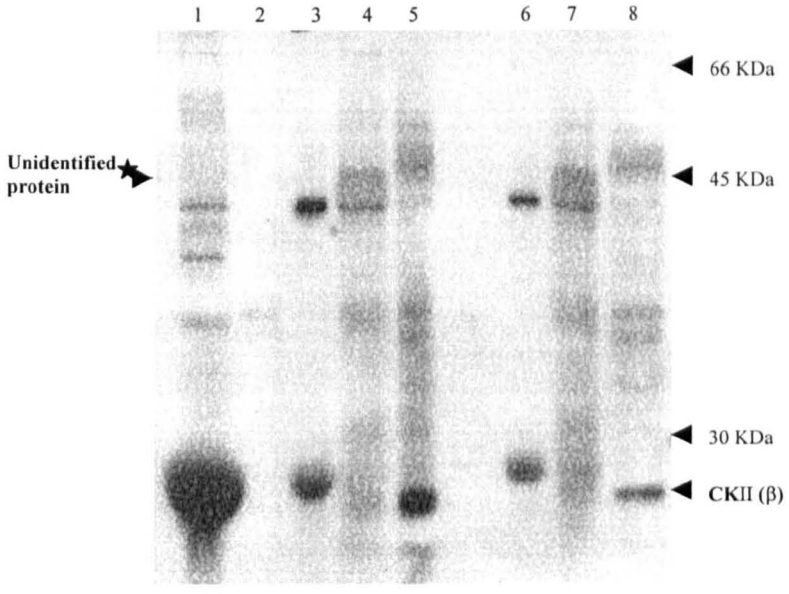


Figure 6.5 Western blotting of a GST-ORF P pulldown with CKII (β) antibody

GST pulldown extracts were run on a 12.5% SDS-PAGE and analysed by Western blotting using an anti-CKII (β) antibody. Lane 1: GST; lane 2: GST-ORF P; lane 3-5: 17^+ infected cell extracts; lanes 6-8: MI extract; lanes 3 and 6: GST pulldown; lanes 4 and 7: GST-ORF P pulldown; lane 5: whole 17^+ infected cell extracts and lane 8: whole MI cell extract. CKII (β) (►) and an unidentified protein (★►) are indicated. Molecular weights are marked (►).

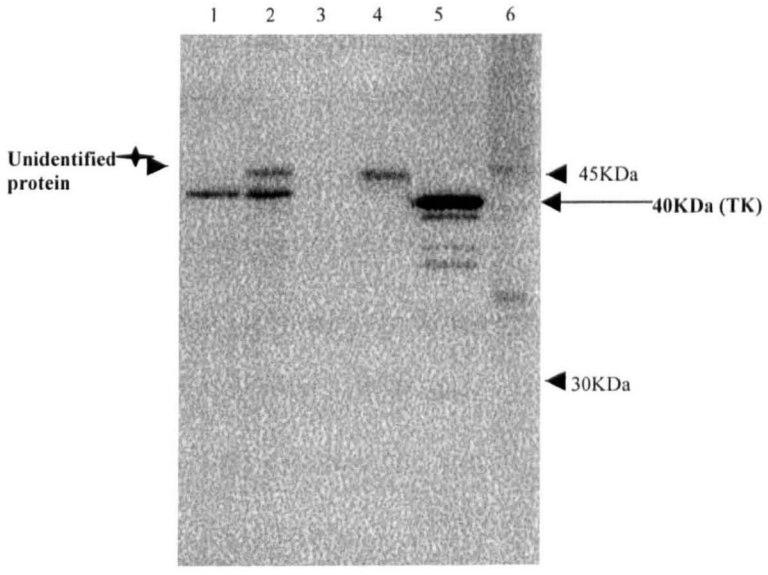


Figure 6.6 Western blotting of GST-ORFP pull down with TK antibody

GST pulldown extracts were run on a 12.5% SDS-PAGE and analysed by Western blotting using an anti-TK antibody. Lane1, 2 and 5: 17^{+} infected cell extracts; lanes 3, 4, and 6: MI extracts; lanes 1 and 3: GST pulldown; lanes 2 and 4: GST-ORF P pulldown; lane 5: whole 17^{+} infected cell extracts and lane 6: whole MI cell extract. The TK band (⟵) and the unidentified protein (✦) are indicated. Molecular weights are marked (◀).

seen in 17^+ infected extract with either GST (lane 1) or GST-ORF P (lane 2), as both GST and GST-ORFP interacted with TK. Again the pulldown was carried out with decreasing amounts of GST and GST-ORF P and the interaction with TK was lost equally by GST and GST-ORF P indicating no specific interaction was occuring. However, a 45 KDa band was specifically detected in both infected (lane 2) and mock infected (lane 4) GST-ORF P pulldowns and also in mock infected whole cell extract (lane 6), indicating that an unidentified 45 KDa cellular protein which cross reacted with the TK antibody was interacting with ORF P. On a long exposure, the 45 KDa band could also be identified in 17^+ whole cell infected extracts (data not shown).

The only identified cellular protein interacting with ORF P detected in these experiments was SC35. Fig.6.7 shows Western blotting of GST and GST-ORF P pulldown extracts with an antibody against SC35. As expected the specific 65 KDa SC35 band was detected in both mock infected (lane 8) and 17^+ (lane 5) cell extracts. The 65 KDa SC35 band was detected specifically in the GST-ORF P pulldowns (lanes 2 and 5) but not in GST pulldowns (lanes 3 and 6).

6.5 Nuclear and cytoplasmic fractionation of SC35/ORF P

In section 6.4 we demonstrated that ORF P interacted *in vitro* with SC35. If this is representative of an *in vivo* interaction it would be expected that both proteins will be located in the same cellular compartment. It has been previously demonstrated that SC35 localises mainly to the nucleus (Boe *et al.*, 1998) and thus we wished to confirm this and determine if ORF P was also located in the nucleus. To investigate the intracellular location of ORF P

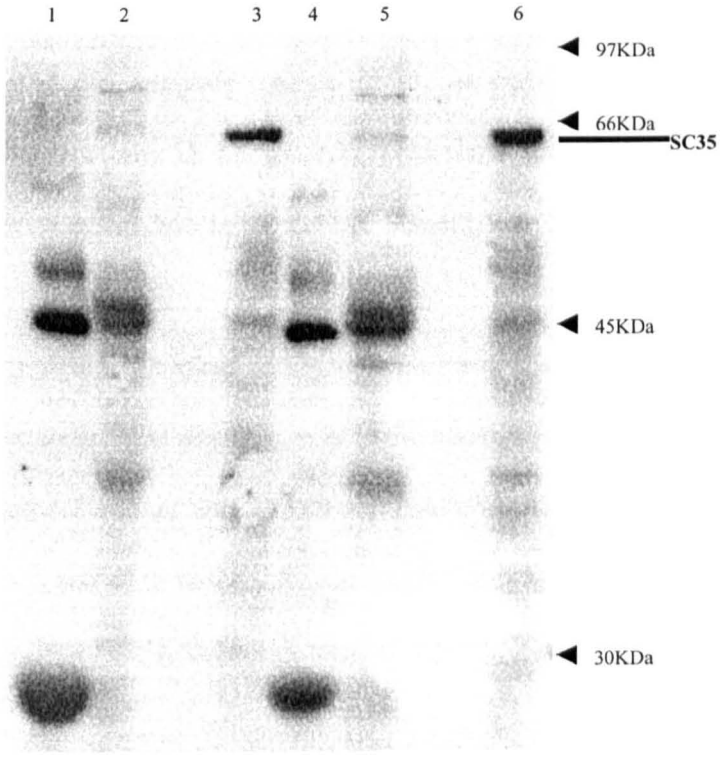


Figure 6.7 Western blotting of GST-ORF P pulldown with SC35 antibody

GST pulldown extracts were run on a 12.5% SDS-PAGE and analysed by Western blotting using an anti-SC35 antibody. Lanes 1 –3: 17^+ infected cell extracts; lanes 4-6: MI extracts; lanes 1 and 4: GST pulldown; lanes 2 and 5: GST-ORF P pulldown; lane 3: whole 17^+ infected cell extract and lane 5: whole MI cell extract. The SC35 band is indicated (⟵). Molecular weights are marked (►).

and SC35, BHK cells were infected with *ts*K, 17^+ and 1716 or mock infected, their nuclear and cytoplasmic proteins separately extracted and Western blotted with both an antibody against SC35 and antiserum 128 against ORF P. Fig.6.8 shows a Western blot of nuclear and cytoplasmic extracts with the antibody against SC35. As previously published, SC35 is located in the nucleus of *ts*K (lane 1), 17^+ (lane 3), 1716 (lane 5) and mock infected (lane 7) BHK cells with no protein being detected in the cytoplasm (lanes 2, 4, 6 and 8).

Fig.6.9 shows a Western blot of nuclear and cytoplasmic extracts with antiserum 128. ORF P is located in approximately equal amounts in both the nucleus and cytoplasm of BHK cells infected with *ts*K (lanes 1 and 2) and 17^+ (lanes 3 and 4). As expected no ORF P was detected in 1716 (lanes 5 and 6) or mock infected (lanes 7 and 8) extracts. In lane 9 a whole cell extract from *ts*K infected BHK cells was used as a positive control to show the 30 kDa ORF P protein.

6.6 Coimmunoprecipitation of ORF P and SC35

In section 6.4 we demonstrated that ORF P interacted *in vitro* with SC35 and in section 6.5 we demonstrated that both SC35 and ORF P are located in the nucleus. To investigate if ORF P interacts with SC35 *in vivo*, immunoprecipitation of BHK cell extracts infected with *ts*K, 17^+ and 1716 and mock infected was carried out separately using both an antibody against SC35 and the anti-ORF P serum 128 and coimmunoprecipitation of the other protein analysed by Western blotting.

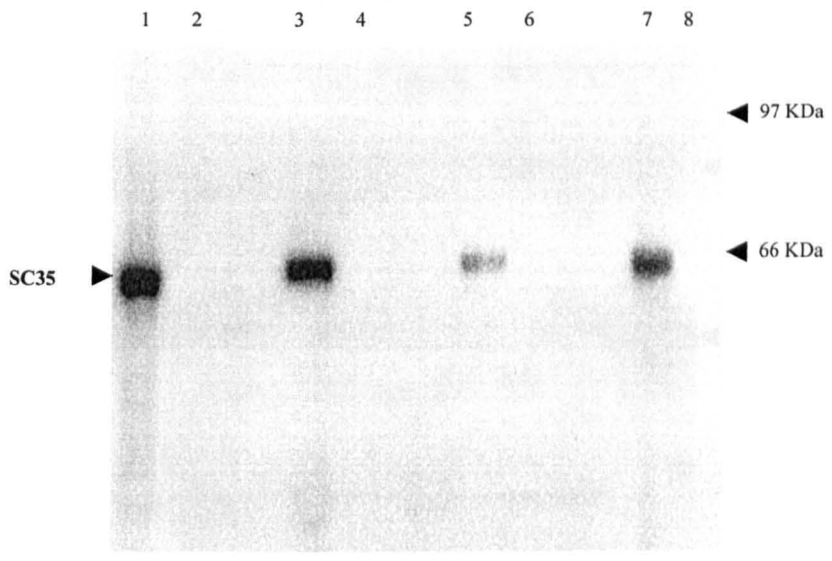


Figure 6.8 Western blotting of BHK nuclear and cytoplasmic extracts with SC35 antibody

BHK cells were infected with HSV-1 at a m.o.i of 20 pfu/cell and harvested at 24 h pi. Analysis of SC35 distribution in both BHK cell nuclear and cytoplasmic extracts was carried out by 7.5% SDS-PAGE and Western blotting with SC35 antibody and anti IgG-HRP, reacted with ECL and exposed to autoradiography. Lanes 1, 3, 5 and 7: nuclear extracts; lanes 2, 4, 6 and 8: cytoplasmic extracts. Lanes 1 and 2: *ts*K; lanes 3 and 4: 17^{+}; lanes 5 and 6: 1716; lanes 7 and 8: MI. Molecular weight markers are indicated on the right and SC35 related bands are marked on the left (►).

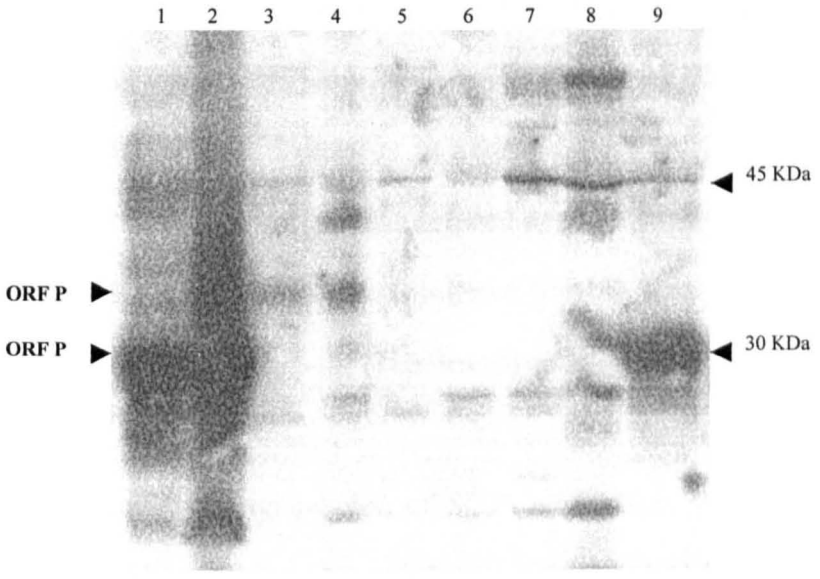


Figure 6.9 Western blotting of BHK nuclear and cytoplasmic extracts with anti-ORF P serum 128

BHK cells were infected with HSV-1 at a m.o.i. of 20 pfu/cell and harvested at 24 h pi. Analysis of ORF P distribution in both BHK cell nuclear and cytoplasmic extracts was carried out by 12.5% SDS-PAGE and Western blotting with antiserum 128 and protein-A-HRP, reacted with ECL and exposed to autoradiography. Lanes 1, 3, 5 and 7: nuclear extracts; lanes 2, 4, 6 and 8: cytoplasmic extracts. Lanes 1 and 2: *ts*K; lanes 3 and 4: 17^{+}; lanes 5 and 6: 1716; lanes 7 and 8: MI; lane 9: *ts*K infected whole cell extract. Molecular weight markers are indicated on the right and ORF P related bands are marked on the left (►).

Initially, samples were immunoprecipitated with the ORF P antiserum and Western blotted with 128 and the SC35 antibody. Fig.6.10a shows a Western blot carried out with 128 to confirm ORF P immunoprecipitation. Lane 5 shows that ORF P was precipitated from *ts*K infected BHK cell extracts. However, no immunoprecipitated ORF P was detected from 17^+ infected BHK cell extracts (lane 6) or as expected from 1716 (lane 7) or mock infected extracts (lane 8). ORF P expression was detected from whole *ts*K infected BHK cell extracts (lane 1) but not from BHK cell extracts infected with 17^+ (lane 2) or as expected from the negative controls infected with both 1716 (lane 3) and mock infected (lane 4).

To investigate coimmunoprecipitation of SC35 by ORF P, Western blotting of ORF P immunoprecipitated samples was carried out with the SC35 antibody (Fig 6.11a). As expected SC35 was detected in *ts*K (lane 1), 17^+ (lane 2), 1716 (lane 3) and mock infected (lane 4) whole BHK cell extracts. This figure shows that SC35 was coimmunoprecipitated by ORF P from both the *ts*K and 17^+ infected BHK cell extracts, where ORF P was present (lanes 5 and 6, respectively), but also from both 1716 and mock infected BHK cell extracts, where ORF P was not present (lanes 7 and 8, respectively). Thus there was nonspecific coimmunoprecipitation.

To try to remove these non specific bands and determine if there was also specific coimmunoprecipitation of SC35 by ORF P, washing of the immunoprecipitation was carried out with Zweig's buffer containing 1M NaCl. However, no difference was seen (data not shown). To further increase the stringency to attempt to remove non specific binding, Zweig's buffer containing 0.1% (w/v) SDS was used to prepare infected BHK cell extracts

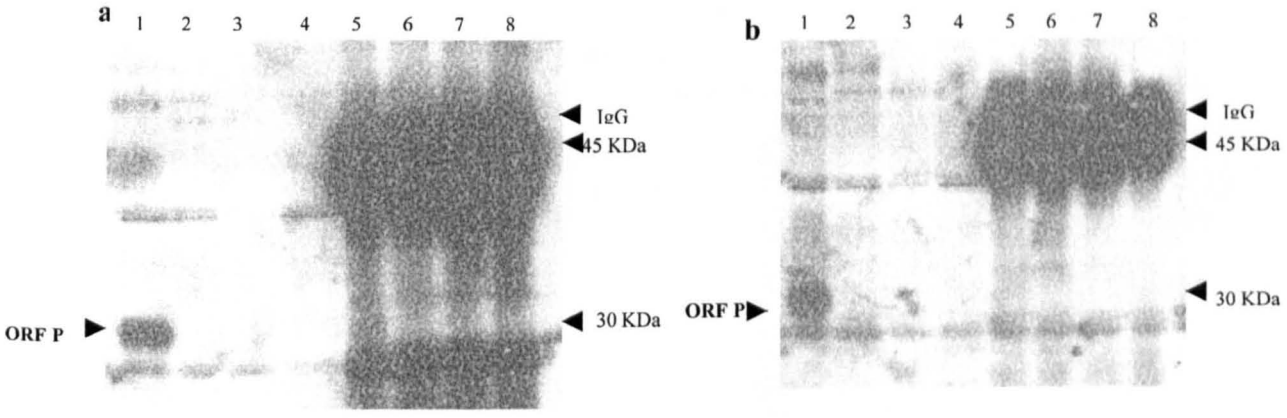


Figure 6.10 Western blotting of immunoprecipitated ORF P protein with anti-ORF P serum 128

BHK cells were infected with HSV-1 at a m.o.i. of 20 pfu/cell, harvested at 24 h pi and immunoprecipitated with ORF P antiserum 128 in a) the absence of SDS and b) the presence of 0.1% (w/v) SDS. Samples were analysed by 12.5% SDS-PAGE and Western blotting with antiserum 128 and protein-A-HRP. Lanes 1-4: whole cell extract; lanes 5-8: immunoprecipitated samples. Lanes 1 and 5: *ts*K; lanes 2 and 6: 17^{+}; lanes 3 and 7:1716; lanes 4 and 8: MI. Molecular weight markers and the heavy chain of IgG are indicated on the right and ORF P on the left (►).

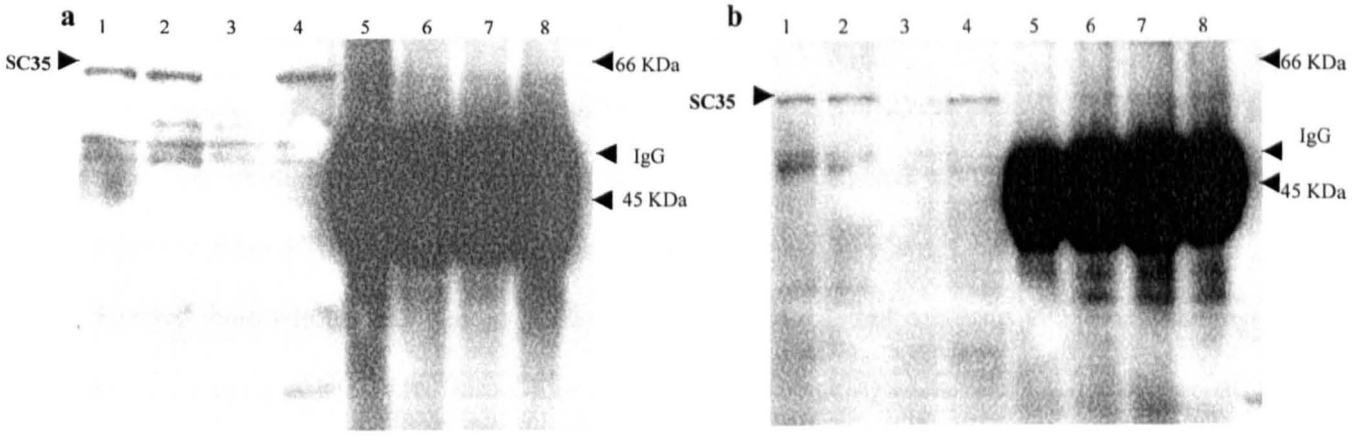


Figure 6.11 Western blotting of anti-ORF P immunoprecipitation with SC35 antibody

BHK cells were infected with HSV-1 at a m.o.i. of 20 pfu/cell, harvested at 24 h pi and immunoprecipitated with ORF P antiserum 128 in a) the absence of SDS and b) the presence of 0.1% (w/v) SDS. Samples were analysed by 12.5% SDS-PAGE and Western blotting with SC35 antibody and anti IgG-HRP. Lanes 1-4: whole cell extract; lanes 5-8: immunoprecipitated samples. Lanes 1 and 5: *ts*K; lanes 2 and 6: 17^{+}; lanes 3 and 7:1716; lanes 4 and 8: MI. Molecular weight markers and the heavy chain of IgG are indicated on the right (►). SC35 is indicated on the left (►).

and carry out the immunoprecipitation with 128 followed by Western blotting with both 128 and SC35 antibody.

Western blotting of SDS containing ORF P immunoprecipitated samples with 128 is shown in Fig.6.10b. The band previously seen in Fig.6.10a lane 5 indicating that ORF P protein had been immunoprecipitated by 128 from *ts*K infected BHK cells was absent. As before, no band was immunoprecipitated from BHK cell extracts infected with 17^+ (lane 6), or as expected from 1716 (lane 7) or mock infected (lane 8). As before, ORF P expression was detected from whole *ts*K infected BHK cell extracts (lane 1) but not from 17^+ (lane 2) or as expected from either 1716 (lane 3) or mock infected (lane 4) cells. Thus the presence of 0.1% (w/v) SDS prevented the immunoprecipitation of ORF P by 128 and we will not expect SC35 to coimmuoprecipitate.

However, we decided to carry out Western blotting of SDS containing ORF P immunoprecipitated samples with the SC35 antibody (Fig.6.11b). Again SC35 was detected in *ts*K, 17^+, 1716 and mock infected immunoprecipitations (lanes 5, 6, 7 and 8, respectively), indicating that SC35 is being nonspecifically immunoprecipitated. Whole BHK cell extracts infected with *ts*K (lane 1), 17^+ (lane 2), 1716 (lane 3) and mock (lane 4) were used as positive controls for the Western blot.

We then proceeded to carry out the reverse experiment by immmunoprecipitating SC35 and looking for coimmunoprecipitation of ORF P. Immunoprecipitation of SC35 was carried out both at 4^0C overnight and at 37^0C for 2 h and the samples Western blotted with the SC35 antibody. Fig.6.12 shows the result of the immunoprecipitation carried out at 4^0C. Whole

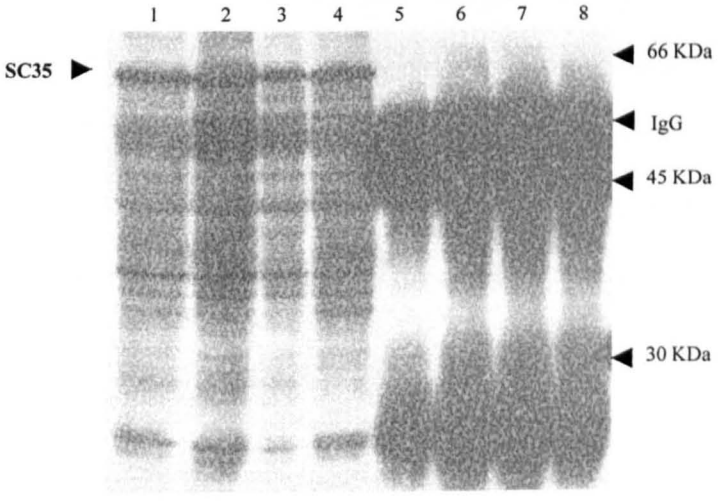


Figure 6.12 Western blotting of immunoprecipitated SC35 with SC35 antibody

BHK cells were infected with HSV-1 at a m.o.i of 20 pfu/cell, harvested at 24 h pi and immunoprecipitated with SC35 antibody. Samples were electrophoresed on a 12.5% SDS-PAGE and Western blotted with SC35 antibody and anti-IgG-HRP. Lanes 1-4: whole cell extracts; lanes 5-8: immunoprecipitated samples. Lanes 1 and 5: *ts*K; lanes 2 and 6: 17^{+}; lanes 3 and 7:1716; lanes 4 and 8: mock infected. Molecular weight markers and the heavy chain of IgG are indicated on the right and SC35 on the left (►).

*ts*K (lane 1), 17^+ (lane 2), 1716 (lane 3) and mock (lane 4) infected BHK cell extracts were used as positive controls and the 65 KDa SC35 protein was detected in all extracts. However, no SC35 protein was detected in the *ts*K, 17^+, 1716 or mock infected immunoprecipitations (lanes 5, 6, 7 and 8, respectively) indicating no immunoprecipitation of SC35 by SC35 antibody had occurred. An identical result was obtained with the immunoprecipitation carried out at 37^0C (data not shown). As we were unable to precipitate SC35, it was not possible to determine if SC35 coimmunoprecipitated ORF P.

7.0 Discussion

The work carried out in this project comprises two main sections: i) characterisation of the role of ICP34.5 and ORF P in HSV-1 replication; and ii) identification of cellular and viral proteins which interact with ORF P. These two areas will be discussed separately.

7.1 Characterisation of the role of ICP34.5 and ORF P in HSV-1 replication

A complication in the analysis of the role of ICP34.5 in the virus lifecycle is the presence of overlapping antisense genes, ORF O/P, which are also deleted in ICP34.5 negative mutants. In 1991, MacLean, A. *et al.* isolated 1716, a HSV-1 17^+ ICP34.5/ORF O/P deletion mutant. This mutant has been demonstrated to have specific characteristics both *in vitro* and *in vivo*. Following intracranial inoculation, 1716 is unable to replicate in the CNS of mice and is totally avirulent with a LD_{50} of 10^6 pfu/mouse higher than wild type or rescued virus (MacLean, A. *et al.*, 1991). Following peripheral inoculation via the eye, 1716 does not appear to replicate in the cornea or trigeminal ganglia (TG) and although establishing latency in TG does so at a significantly lower frequency than wild type virus. Following footpad or vaginal inoculation, virus may undergo limited replication at the initial site of inoculation but no replication takes place in the dorsal rout ganglia (DRG) and latency is established although not as inefficiently as in the TG and can be reactivated *in vitro* following explantation (Roberston *et al.*,1992; Spivack *et al.*, 1995).

In tissue culture, 1716 replicates normally in many non neuronal cell types such as Vero and BHK 21/C13 cells, but is restricted in 3T6 cells (Brown *et al.*, 1994a). In general, in nonpermissive cells the growth defect of ICP34.5 null mutants is greater when they are in the stationary phase (Brown *et al.*, 1994a). In certain cell types such as SK-N-SH cells

infected with ICP34.5 null mutants, PKR is activated, eIF-2α is phosphorylated and protein synthesis is shutoff (Chou and Roizman, 1992; Chou *et al.*, 1995).

Both the *in vitro* and *in vivo* phenotypes of 1716 were demonstrated to be due to the absence of the deleted sequences by the wild type growth characteristics of two independently constructed rescuants of 1716 (MacLean, A. *et al.*, 1991; McKie *et al.*, 1994; Spivack *et al.*, 1995).

To attribute characteristics which were originally attributed solely to ICP34.5 to each of the two genes, a number of HSV-1 recombinant viruses that express ICP34.5 and ORF O/P independently were constructed, purified and characterised.

The basic design of all recombinant viruses was that an expression cassette was inserted into the 1716 UL43/UL43.5 locus. In this cassette the gene of interest was inserted under a HSV-1 gD promoter with a HSV-2 IE4/5 polyadenylation signal. In the opposite orientation, the *lac*Z gene was inserted under a SV40 promoter with its own polyadenylation signal. Construction of these recombinants is described in Section 3. The delayed early HSV-1 gD promoter (Everett, 1984) was used to express both ICP34.5 and ORF P as it was believed that it would express both genes strongly, and ICP34.5 with similar kinetics to those of the wild type. As UL43/43.5 are non essential genes and it had previously been shown that deletion and insertional inactivation of these genes has no effect on virus replication either *in vitro* or *in vivo* (MacLean, C. *et al.*, 1991), this locus was selected as the site of insertion. Disruption of these two genes should have no effect on the phenotype of the recombinants. ICP34.5 and *lac*Z were inserted at the 5' end of UL43 and thus this insertion should inactivate UL43. However,

these two genes were inserted at the 3' end of UL43.5 and thus this insertion might not inactivate UL43.5. By this method, 1622 expressing ICP34.5 and 1624, 1624.5 and 1625 ,in which ORF P sequence were inserted, constructed.

To construct 1625, gD/ORF P was inserted in the middle of the non essential gene US5 which encodes glycoprotein J (McGeoch *et al.*, 1985). As it had been demonstrated that insertion of *LacZ* in this position inactivated US5 (Balan *et al.*, 1994), insertion of ORF P should also inactivate this gene. Although US5 is also not essential for HSV-1 replication *in vitro* or *in vivo* (Longnecker and Roizman, 1987; Balan *et al.*, 1994), we were not able to completely purify 1625 from its parent, 1622 with a level of about 10% contamination. It seems unlikely that 1625 is non viable and requires 1622 as a helper virus. The contamination is more likely to be due to instability of 1625 DNA with deletion of the inserted sequences. This deletion is unlikely to be precisely limited to the inserted sequence and could either leave some of the inserted sequences or remove some of the adjacent viral DNA with the deletion being within 100 bp of the size of the insert as no size alteration was detected by Southern blotting. To address this possibility, PCR could be carried out across the insertion site followed by sequence analysis. Also, the instability may be due to multiple insertion of gD promoter.

The recombinants need to properly characterised to conclude if they actually contain/express ORF P by using specific ORF P fragment with no extra sequences.

Western blotting analysis of 1622 and 1625 infected BHK cells demonstrated at least an eight-fold increase in ICP34.5 levels compared with wild type 17^{+}. Both 1622 and 1625 express ICP34.5 under the gD promoter and in the UL43.5 orientation. 1625 also contain

gD/ORF P inserted in US5. The most probable explanation is that the gD promoter is stronger than the ICP34.5 native promoter. Alternatively, as there is a ATG in the 5' untranslated leader of the natural ICP34.5 gene and this ATG is absent in the 5' untranslated leader of gD/ICP34.5, this ATG might be having a negative effect on ICP34.5 translation in wild type virus. The higher level of ICP34.5 expression might also be due to an orientation effect in the UL43/43.5 locus (see below). Analysis of ICP34.5 RNA levels might allow determination of the reason for this overexpression.

Expression of L/STs and their protein products, ORF P and ORF O, has not been detected during productive infection by HSV-1 strain F (Lagunoff *et al.*, 1996). However, it had been previously demonstrated in our laboratory (McKie and MacLean, personal communication) that in BHK21/C13 cells RL1 and ORF P transcripts and proteins were simultaneously expressed during a productive infection by HSV-1 (17^+) in the presence of functional ICP4. My work also confirmed that HSV-1 (17^+) produces ORF P during a productive infection. Thus, ORF P may have a role in 17^+ productive infection. It has been suggested that ORF O specifically binds to and inhibits *in vitro* binding of ICP4 to its recognition sites in DNA (Randall *et al.*, 1997). Thus, the higher production of ORF P in 17^+ than strain F may be due to higher production of ORF O in 17^+ than in strain (F). Alternatively, ORF O of 17^+ might bind to ICP4 stronger than that of strain F and thus allow more expression of L/STs and hence ORF O/P in strain 17^+. Evidence to support the different function between ORF O protein in strains 17^+, F and KOS is their different length. ORF O consists of 245 amino acids in 17^+ whilst in strains F and KOS consists of 105 amino acids (Randall *et al.*, 1997; Yea and Schaffer, 1998).

In the recombinants 1624, 1624.5 and 1625 no ORF P protein was detected. This would suggest that these recombinants express low levels of ORF P. In 1624 ORF P sequence inserted in the UL43 orientation, in 1624.5 in the UL43.5 orientation and in 1625 in US5. The antibodies used, 128 and 129, were rabbit polyclonal sera raised against a GST-ORF P fusion protein. Use of GST fusion proteins tends to generate rabbit polyclonal sera with high background as evidenced by the antisera against ICP34.5 and ICP27 used in this thesis. Also, as evidenced from our results the GST fusion protein produces a truncated protein. Therefore, this antiserum will be against a limited number of epitopes from the aminoterminus of the protein. These antisera exhibited problems of detection with low amount of ORF P protein from the recombinants and to a lesser extent from 17^+ but not from the overproducing *ts* mutant in ICP4 (*ts*K) at the nonpermissive temperature (NPT). If monoclonal antibodies were raised against ORF P these might produce less background and thus the Western blots could be exposed longer to detect low levels of ORF P.

When gD/ORF P was originally cloned, it was thought that ORF O was translated from a different initiating methionine codon upstream from that of ORF P (Lagunoff *et al.*, 1996; Yeh and Schaffer, 1993) and thus only ORF P would be expressed. Subsequently, it was shown that both ORF O and P share the same initiating methionine codon and ORF O then frameshifts to the ORF O frame after amino acid 35 of ORF P (Randall *et al.*, 1997). If the normal (L/ST) RNA 5' untranslated leader is required for the correct RNA structure to allow this frameshifting from the ORF P to ORF O reading frame, then the recombinants will not express ORF O. However, if the sequence determining frameshifting is in the 5' part of the ORF they will express both ORFs O and P. Without

experimental evidence of ORF O expression by antibody detection, it is not possible to conclusively say whether or not ORF O is expressed from our recombinants.

As neither antisera 128 nor 129 detected ORF P in the recombinant viruses by Western blotting, immunoprecipitation of *ts*K, 17^+ and 1716 infected BHK cell extracts was carried out. As there is only a small number (2) of methionines in ORF P it was decided that it would label poorly with [^{35}S] methionine and therefore immunoprecipitation was carried out on unlabelled extracts and detected by Western blotting. Again although both antibodies immunoprecipitated ORF P in *ts*K at the NPT, they were not able to detectably precipitate the lower amounts of ORF P expressed in 17^+. As ORF P could not be detected from 17^+ extracts, we did not carry out an immunoprecipitation with the recombinants 1624, 1624.5 and 1625.

As we were not able to detect ORF P protein from our recombinant viruses, we proceeded to look for the RNA. As ORF P is inserted in the UL43/43.5 loci, the GST-ORF P probe will detect the HSV UL43/43.5 spanning transcripts. The size of transcripts were 7.0 kbp; 4.0 kbp; 2.7 kbp; 2 kbp; 1 kbp and are probably respectively uncharacterised; UL43/44; UL44; UL41; and UL43. The uncharacterised long transcript probably arises from the non specific transcription detected from the HSV genome late in infection (Jacquemont and Roizman, 1975a, b). In addition, the GST-ORF P probe also detects a band with the size expected for ORF P RNA of about 700 bp in 1624, 1624.5, and 1625. As ORF P is used as the probe and there is sequence identity between ORF P and ICP34.5, a 700 bp ICP34.5 transcript was detected in 1622. As expected ORF P did not hybridise to the UL41-44 HSV transcripts in *ts*K, 17^+ and 1716. Surprisingly no native ORF P RNA was detected in *ts*K or 17^+ and thus the ORF P probe blot has failed

to detect ORF P even in positive control so no conclusion can be drawn. Although we did confirm the presence of RNA using a γ1 actin probe.

No Southern blotting definitely showing ORF P had been performed. Neither ORF P protein nor RNA was detected from the recombinants. Using a specific ORF P fragment with no extrasequence as a probe both Southern and northern blotting should be carried out to detect ORF P gene and ORF P RNA synthesis, respectively in the recombinants. Alternatively, PCR could be carried out across the insertion site followed by sequence analysis.

As previously stated, in tissue culture ICP34.5 null mutants replicate normally in many non neuronal cell types such as Vero and BHK21/C13 cells, but are restricted in SK-N-SH neuroblastoma cells, human foreskin fibroblasts (HFF), murine 10T1/2 cells, stationary phase primary mouse embryo cells, and 3T6 cells (Chou *et al.*, 1990, 1994; Chou and Roizman, 1992; Bolovan *et al.*, 1994; Brown *et al.*, 1994a). In some cell types such as SK-N-SH cells infected with ICP34.5 null mutants, PKR is activated, eIF-2α is phosphorylated and protein synthesis is shutoff (Chou *et al.*, 1995).

It had been previously shown that neither ICP34.5 nor ORFO/P are essential for replication of HSV-1 (17^+) in BHK cells (MacLean, A. *et al.*, 1991). Therefore as expected, in these cells all our recombinant viruses with the exception of 1624 exhibited a similar growth pattern to wild type virus, confirming that neither ICP34.5 nor ORF O/P are essential for replication of HSV-1 (17^+) in BHK cells. 1624 seems to be impaired in growth in BHK cells compared with the other viruses, indicating a probable secondary mutation elsewhere in the genome.

In stationary state 3T6 cells, 17^+ grew well reaching a maximum titre of 10^6-10^7 pfu/10^6 cells whereas 1716, expressing neither ICP34.5 nor ORF O/P failed to grow. The recombinants 1624 and 1624.5 which express only ORF O/P also failed to grow whereas 1622 and 1625 expressing ICP34.5 grew. However, 1622 and 1625 plataued at a maximum titre of 10^1 to 10^2 fold lower than 17^+. Previous work (Adams, 1999) had demonstrated that an insertion into UL43/43.5 did not affect growth in 3T6 cells. There are at least two possible explanations for the poorer growth of the recombinants compared to 17^+. Either the higher levels of expression of ICP34.5 in 1622 than in 17^+ or different kinetics of ICP34.5 expression in 1622 compared with 17^+ might be deleterious to growth. In some experiments both 1622 and 1625 grew similarly whereas in others 1625 grew 5-10 fold less well than 1622. Most consistently, 1625 grew to a maximum titre 5-10 fold lower than 1622. Thus, in 3T6 cells, expression of ICP34.5 partially restored 1716 growth to 17^+ levels. Expression of ORF O/P either by itself or in conjunction with ICP34.5 has no positive effect on 1716 growth.

In SK-N-SH cells, 17^+ grew well reaching a maximum titre of 10^7-10^8 pfu/10^6 cells. The growth of 1716 in SK-N-SH cells had not been previously characterised. As expected based on the host protein synthesis shutoff phenotype, SK-N-SH cells are not fully permissive for 1716. However, our results showed that this cell line is semi permissive for 1716, exhibiting limited growth with a maximum titre 10^4 fold lower than 17^+. There are at least two possibilities to explain this semi rather than non permissive phenotype. As SK-N-SH cells are a heterogenous cell line and a small percentage of cells have dedifferentiated and reverted to fibroblasts, these dedifferentiated cells might fully support 1716 replication whilst the differentiated cells are nonpermissive. Alternatively, host protein synthesis shutoff might not completely inhibit growth of 1716 in infected

SK-N-SH cells allowing limited growth of 1716 in these cells. 1624.5 grew similarly to 1716 and thus expression of ORF O/P has no positive effect on 1716 growth. In agreement with its impaired growth in BHK cells, 1624 grew consistently 10 fold poorer than 1716. Both 1622 and 1625 grew similarly to 17^+. Although in some experiments 1625 grew about 5 fold poorer than 1622. In SK-N-SH cells, expression of ICP34.5 restores 1716 growth to nearly 17^+ levels.

One role of ICP34.5 is to maintain host and viral protein synthesis late in infection in some cell lines such as SK-N-SH (Chou *et al.*, 1995). In HSV-1 infected cells, transcription of the complementary DNA strands results in the formation of double stranded RNA, induction of IFN and activation of the PKR pathway and eIF-2α phosphorylation which results in premature shutoff of host protein synthesis. However, the HSV encoded ICP34.5 blocks this shutoff of host protein synthesis (Chou and Roizman, 1994) by forming a cytoplasmic complex containing protein phosphatase 1α (PP1α) to redirect PP1α to dephosphorylate eIF-2α (He *et al.*, 1997a, 1998) and thus prevent premature shutoff of host protein synthesis induced after the onset of viral DNA synthesis (Chou *et al.*, 1995). In cells infected with ICP34.5 null mutants, PKR is activated, eIF-2α is phosphorylated and protein synthesis is shutoff (Chou *et al.*, 1995). Restoration of ICP34.5 function in both 1622 and 1625 infection was confirmed *in vitro* by the maintenance of host cell and viral protein synthesis in SK-N-SH cells. Thus, expression of this gene in 1716 prevents host cell protein synthesis shutoff. However, expression of ORF O/P fails to prevent host cell protein synthesis shutoff in 1716.

Brown *et al.* (1994a) demonstrated that the growth of ICP34.5 null mutants was impaired in mouse embryo fibroblasts (3T6) with the impairment amplified in the stationary state.

In 3T6 cells, expression of ICP34.5 restored growth of 1716 to nearly 17^+ growth levels. In 3T6 cells, the mechanism of lack of growth seems to be different from that in SK-N-SH cells in that I have demonstrated that lack of ICP34.5 has no effect on host protein synthesis in these cells. Brown *et al.* (1994b) demonstrated that the impairment in growth in 3T6 cells was due to a defect in envelopement and egress of virus particles with an accumulation of unenvelopped capsids in the nucleus of infected 3T6 cells with little or no effect on the behaviour of the virus in BHK cells.

ICP34.5 seems to confer two functions: growth and maintenance of protein synthesis. The relationship between these is not clear. In SK-N-SH cells, these are linked but in 3T6 cells they are not. The relationship of host shutoff to the *in vivo* phenotype may be complicated (1.15.3).

ORF P insertion into the viruses has not been conclusively proven and there is no evidence of expression at all. Therefore the conclusions made with regard to growth curves etc. may be incorrect.

7.2 Analysis of ORF P interacting proteins

As mentioned in the previous section of this chapter, ORFO/P overlap and are antisense to ICP34.5. As determined by characterisation of mutants in these genes, these two genes seem to have little if any role in the HSV lifecycle (this thesis; section 1.16). An alternative way to analyse their function is to determine those cellular and viral proteins which interact with ORF P and O. This was carried out for ORF P. There are a number of methods such as GST pulldown, the yeast two-hybrid system and coimmunoprecipitation which can be used to determine protein-protein interactions both *in vitro* and *in vivo*.

Using GST-ICP27 and GST-ICP34.5, it was previously shown in our laboratory that GST pulldown is useful for determining protein-protein interactions *in vitro* (Brown *et al.*, 1997; Bryant *et al.*, 2000) and thus this method was used in this work to determine the interaction of ORF P with cellular and viral proteins. The principle of the GST pulldown assay is shown in Fig.6.6.

Expression of the GST-ORF P fusion protein where ORF P was cloned as a 3' GST fusion protein in a bacterial system had not been previously optimised. Using a number of *E.coli* strains and different temperatures of induction, I attempted to optimize expression of both the amount and length of GST-ORF P.

Firstly, expression of both GST and GST-ORF P was investigated in 3 different *E.coli* strains BL21, C41 and Novablue. BL 21 is the most widely used host backgroud and has the advantage of being deficient in both lon and ompT proteases. BL21 contains a plasmid containing the T7 polymerase gene under the *Lac I* promoter which is induced by IPTG to express T7 RNA polymerase. The principle of GST fusion protein expression in C41 is the same as that in BL21. However, in C41 the lac repressor binds the *LacI* promoter for the T7 gene tighter and therefore there is less uninduced expression of T7 polymerase and hence the fusion protein. This promotes plasmid stability if the fusion protein is toxic. Novablue does not contain the T7 RNA polymerase gene containing plasmid and superinfection with CE6 λ phage containing the T7 RNA polymerase gene is required to induce expression of the fusion protein (Studier and Moffat, 1986). This system is used when a target gene is so toxic that a plasmid cannot be maintained in a DE3 lysogenic host such as BL21 where there will always be minimal recombinant protein expression.

Expression of GST was similar in all 3 strains although slightly higher in BL21. Expression of GST-ORF P which was initially detected as a truncated 40 KDa doublet was only detected in BL21. No expression of GST-ORF P was detected in either C41 or Novablue. Thus BL21 was the strain used for future analysis.

The optimum temperature for expression of GST-ORF P in BL21 was investigated. Expression of GST at all three temperatures was similar. However, expression of GST-ORF P at 31^0C was better than at either RT or 37^0C. Thus we decided to use 31^0C for expression of GST-ORF P in BL21 in subsequent experiments.

The main problem faced in carrying out the pulldown experiments was the lack of expression of full length GST-ORF P fusion protein. This could either be a problem of protease degradation or lack of expression of full length protein. This fusion protein like many HSV-1 proteins does not express as full length in bacterial systems (Brown *et al.*, 1997; Wadd *et al.*, 1999). As most HSV genes, unlike bacterial genes, consist of a high G + C content, bacterial cells will not favour expression of these proteins. By repeated experiments, we tried to optimise expression and harvesting to obtain full length GST-ORF P. In the initial experiments, only a 40 KDa GST-ORF P doublet was detected. In the final experiments the 40 KDa doublet, a higher molecular weight band of 45 KDa and two higher molecular weight bands of 50 and 60 KDa were detected. ORF P in 17^+ contains 233 amino acids (Lagunoff and Roizman, 1994). As the molecular weight of each amino acid is approximately 110 Dalton ORF P is about 28 KDa. As GST is about 28 KDa, GST-ORF P should theoretically be about a 55-60 KDa protein. Thus the higher molecular weight products may correspond to full length GST-ORF P. Our results show at the end, some nearly full length GST-ORF P was expressed and this preparation was

used for our pulldown assays. Purification of some full length GST-ORF P might be due to a number of factors, such as taking greater care during the harvesting procedure, obtaining more experience and better skills enabling the experiment to be carried out quicker and keeping everything on ice. All these would give proteases less opportunity to work.

In our initial experiments, using the standard conditions for a GST pulldown assay, we were not able to recognize any ORF P interacting proteins. This method had previously identified a number of cellular proteins interacting with GST-ICP27 (Bryant, 1999) and GST-ICP34.5 (Brown *et al.*, 1997). As a positive control, a GST-ICP27 pulldown was carried out with HSV-1 17+ infected cellular proteins. GST-ICP27 interacted with a number of proteins, for example hnRNP and HSV-1 TK, previously shown to interact with ICP27 (Bryant, 1999). This result showed that the pulldown procedure was working correctly. As with coimmunopercipitations, salt and detergent concentrations in the buffers along with target and interacting protein concentrations affect which proteins are pulled down. As we had shown that, although the GST pulldown method was working, no ORF P interacting proteins could be identified, we attempted to optimise the detection of proteins interacting with GST-ORF P by changing the salt concentration of the washing buffer. The standard buffer used contained 0.5 mM NaCl and by increasing the salt concentration we would increase the stringency of the washing buffer and hopefully remove non specific binding proteins leaving specific interacting proteins visible. Obviously we would reach a salt concentration where both non specific and specific interacting proteins were removed. NaCl concentrations of 0.5, 5, 50 and 500 mM NaCl were used.

Washing with 0.5 and 5mM NaCl containing wash buffer resulted in no specifically bound proteins being detected. However, increasing the NaCl to 50 mM resulted in

binding by a number of specific proteins being detected. These proteins include at least two viral proteins with sizes of 27 and 40 KDa and one cellular protein of 47 KDa. Although the photograph did not reproduce this well. Increasing the NaCl to 500 mM, resulted in these protein interactions being lost.

As ORF P was cloned as a 3' GST fusion protein and different lengths of GST-ORF P fusion protein were expressed, all the fusion proteins will include the aminoterminus but be truncated at the carboxyterminus which will thus be underrepresentated. Thus the protein identified might interact with the aminoterminus of ORF P.

We then proceeded to try to identify these interacting proteins. There is some previous evidence indicating that ORF P interacts with a number of splicing factors and may play a role in splicing (Bruni and Roizman, 1996). Thus we chose to look at proteins involved in posttranscriptional regulation. It had previously been shown that ICP27 interacts with a number of proteins involved in posttranscriptional gene regulation (Bryant *et al.*, 2000). Thus it seemed reasonable to investigate whether ORF P interacts with ICP27. A GST-ORF P pulldown was carried out and Western blotted with an anti-ICP27 antibody. Both GST and GST-ORF P interact with ICP27 and its related bands. Despite attempts to reduce non specific binding by decreasing the amount of beads, we were unable to do so without losing binding to both GST and GST- ICP27. Thus we could not screen for an interaction. Non-specific binding could be either because GST binds non-specifically to ICP27, or that ICP27 is trapped between the GST beads. Reducing the amount of beads trends to rule out the possibility of capture of the protein by GST beads and the second idea that ICP27 binds non-specifically to GST is more acceptable.

As the GST-ICP27 fusion protein is expressed better than GST-ORF P, the reverse experiment was carried out where GST-ICP27 was used to try to pulldown ORF P from *ts*K infected cell extracts and Western blotted with an anti ORF P serum. In initial Western blotting, serum 128 cross reacted with the GST-ICP27 related bands as it had been raised against a GST fusion protein. Therefore, we had to preadsorb 128 against GST to remove the GST specific bands. When this preadsorbed serum was used, no specific bands were detected binding to GST-ICP27 or GST-ORF P. This indicated that no interaction of ORF P with ICP27 or self interaction was occurring.

We next screened our GST-ORF P pulldowns with a range of antibodies against proteins with a role in posttranscriptional regulation and which were of an approximate molecular weight to those previously identified. Western blotting of GST-ORF P pulldowns was carried out with 10 available cellular antibodies (table 6.1). Of these, only one recognised an identified protein interacting with ORF P. In addition, two unidentified proteins were demonstrated to interact specifically with ORF P.

Using an antibody against CKII (β), an unexpected result was obtained. No specific interaction of GST-ORF P with CKII (β) was detected. However, a 45 KDa band was detected both in cell extracts and from the GST pulldown, indicating an unidentified cellular protein was interacting with ORF P.

To try to identify the 40 KDa viral band interacting with GST-ORF P, Western blotting of pulldown extracts was carried out with an antibody against HSV-1 TK, which is about 40 KDa. Both GST and GST-ORFP interacted with TK. Again the pulldown was carried out with decreasing amounts of GST and GST-ORF P and the interaction with TK was

lost equally by GST and GST-ORF P indicating no specicfic interaction was occuring. However, a 45 KDa cellular band was specifically detected, indicating that an unidentified 45 KDa cellular protein which cross reacted with the TK antibody was interacting with ORF P.

The only characterized cellular protein interacting with ORF P detected in these experiments was SC35. SC35 is an essential component of small non nuclear ribonucleoprotein particles (snRNPs) and is a splicing factor (Sandri-Goldin *et al.*,1995; Zahler *et al.*, 1992).

Having demonstrated that ORF P interacted *in vitro* with SC35, we wished to determine if this was representative of an *in vivo* interaction. For this to occur, it would be expected that both proteins would be located in the same cellular compartment. It has been previously demonstrated that SC35 localises mainly to the nucleus (Boe *et al.*, 1998) and thus we wished to confirm this and determine if ORF P was also located in the nucleus. To investigate the intracellular location of ORF P and SC35, BHK cells were infected with *ts*K and 17^{+}, their nuclear and cytoplasmic proteins separately extracted and Western blotted with antisera against SC35 and ORF P.

As previously published, SC35 is located in the cell nucleus with no protein being detected in the cytoplasm. ORF P is located in approximately equal amounts in both the nucleus and cytoplasm of infected BHK cells. This agrees with the finding of Lagunoff *et al.* (1995) who detected ORF P in the nucleus of cells infected with a mutant which overproduced ORF P by using immunofluorescence and Western blotting of fractionated cell extracts.

Having demonstrated that ORF P interacted *in vitro* with SC35 and that both SC35 and ORF P are located in the nucleus, we investigated if ORF P interacts with SC35 *in vivo* by carrying out an immunoprecipitation of BHK cell extracts infected with *ts*K and 17^+. Immunoprecipitation was carried out separately with antibodies against both SC35 and ORF P and coimmunoprecipitation of the other protein analysed by Western blotting.

Initially, samples were immunoprecipitated with the ORF P antiserum and Western blotted with anti-ORF P which confirmed succesful immunoprecipitation of ORF P from *ts*K but not from 17^+ infected BHK cell extracts. To investigate coimmunoprecipitation of SC35 by ORF P, Western blotting of ORF P immunoprecipitated samples was carried out with the SC35 antibody. SC35 was coimmunoprecipitated by the ORF P antisera non-specifically as it was detected when both ORF P was present (*ts*K/17^+) and absent (1716/mock infected). To try to remove these non specific bands and determine if there was also specific coimmunoprecipitation of SC35 by ORF P, the immunoprecipitated samples were washed with high salt. However, no difference was seen. To further increase the stringency to attempt to remove non specific bands, SDS was added to the buffers. However, the presence of SDS prevented the immunoprecipitation of ORF P by the anti-ORF P serum and we could not look for coimmunoprecipitation of SC35.

We then proceeded to carry out the reverse experiment by immmunoprecipitating SC35 and looking for coimmunoprecipitation of ORF P. However, we were unable to immunoprecipitate SC35 under a number of conditions. As we were unable to precipitate SC35, it was not possible to determine if SC35 coimmunoprecipitated ORF P.

Antisera 128 and 129 do not work in immunofluorescence due to their high background (MacLean, A, personal communication) and thus we did not try to investigate colocalization of ORF P and SC35 by immunofluorescecne.

Overall, these results show that ORF P interacts with SC35 *in vitro* and also both are in the nucleus of infected cells. However, due to technical difficulties we were unable to determine if an *in vivo* interaction was occurring. This data is in agreement with previously published work. Previous workers using immunofluorescense had demonstrated that ORF P colocalises with SM antigens and with SC35 in the nuclei of infected cells, interacts with SM components in a GST pulldown assay and interacts with p32 by in a yeast-two-hybrid system (Brunei and Roizman, 1996).

Conclusively, the work in this project in addition to confirmation (previously shown) that 17+ expresses ORF P in productive infection showed that i); if the recombinants express ORF P, this protein seems to have no role in the HSV-1 17+ replication in vitro; ii) SK-N-SH cells are semi permissive (rather than non permissive) for 1716; iii) over expression of ICP34.5 protein might have deleterious effect on growth of 17+; iv) expression of ORF P in a GST fusion protein is optimized and an ORF P protein ranging of 40-60 KDa was detected; v) ORF P interacts with at least two viral and one cellular protein.

7.3 Methods for analysis of protein-protein interactions

To investigate if any cellular or viral proteins interact with ORF P *in vitro*, a GST pulldown assay was carried out. In this method, the fusion protein is expressed and purified by binding to glutathione which is attached to agarose (Smith and Johnson,

1988). After the fusion protein is bound to the matrix, it is washed with buffer to remove nonspecifically bound bacterial proteins and subsequently mixed with cellular extracts. The fusion protein containing beads serve as an affinity matrix to allow binding of proteins which interact with the protein fused to GST. Beads are washed again to remove nonspecifically bound cellular proteins and proteins analysed by SDS-PAGE. Non specific proteins are eliminated as they also bind to a GST control. Bound proteins can be partially purified by a simple centrifugation step, hence the name "pulldown" assay. An overview of this method can be seen in Fig.5.6. A disadvantage of this method is that interacting proteins are not identified directly and the GST pulldowns need to be screened by Western blotting to identify the protein or proteins eluted from the gel and sequenced. Using this method, a number of ORF P interacting proteins were detected. However we were able to identify only one of these proteins by Western blotting.

An alternative method to identify interacting proteins is the yeast two-hybrid system. This method exploits the ability of a pair of interacting proteins to bring the activation domain (AD) of a transcription factor in close proximity with the DNA-binding domain (BD) of the same transcription factor which can bind to a cis-acting regulatory element and thus stimulate the expression of adjacent reporter genes, *LacZ* and HIS3 (reviewed in Mendelsohn and Brent, 1999). In this system two different cloning vectors are used to generate fusions of the AD and the genes encoding the target proteins to construct a library and the BD and the gene encoding the protein used to screen the library. The hybrid proteins are coexpressed in a strain of yeast that lacks the HIS3, TRP1, LEU2 and *LacZ* genes. Since the cloning vector that encodes the activation domain/target protein also carries the TRP1 gene and the vector that encodes the DNA-binding domain/bait protein encodes the LEU2 gene, yeast colonies containing two plasmids encoding

interacting proteins can be selected for by culture on a medium lacking tryptophan, leucine and histidine. An assay for *β-galactosidase* activity confirms that colonies contain two interacting proteins.

The two-hybrid system is a very sensitive method for detecting protein-protein interactions and can detect binding that is often beyond the limits of other detection methods such as immunoprecipitation. As the two-hybrid system is performed *in vivo*, conditions are similar to those in which protein interactions normally occur. A major advantage is that as positive plasmids are identified, a portion of the gene encoding the interacting protein is immediately available for sequencing and the identity of interacting proteins can be determined very quickly. Purified target protein or antibody against the protein is not required for this system. However, the high sensitivity of the two-hybrid system can create a large number of false positives, especially if the bait protein encodes a transcriptional activator. Also, some hybrid proteins may not be stably expressed in yeast cells (Estojak *et al.*, 1995).

Coimmunoprecipitation has been used to investigate protein:protein interactions *in vivo* and for example the interaction of the HSV-1 glycoproteins gH and gL was discovered by this method (Hutchinson *et al.*, 1992). Due to their high specificity, antibodies may be used to isolate specifically interacting proteins from a complex such as cell lysates. Identification of the immunoprecipitated protein and proteins which coimmunoprecipitate with it can be achieved by separation of the complex on a SDS-PAGE gel, followed by detection of radiolabelled proteins by autoradiography or non-labelled proteins by Western blotting. However, there are a number of limitations. An antibody may mask an interaction site or a protein:protein interaction may mask the

antibody binding site. Thus polyclonal antisera are better than monoclonal antibodies, although in general the latter give a cleaner result with low background. Salt and detergent concentrations in the buffer used to immunoprecipitate and wash the antibody:antigen complex will effect the ability of the proteins to maintain their interaction and this may affect the stringency of the immunoprecipitation.

Another method used to identify colocalization of two proteins in the same cellular compartment is confocal microscopy. Here antibodies labeled with different fluors for example, fluorescene and rhodamine are directed against the two proteins whose interaction is being analysed allowing the location of both proteins and hence potential colocalization to be examined (Lagunoff and Roiman, 1995). These latter two methods are more useful for confirming suspected interactions.

7.4 Future work

To further analysis the recombinant viruses generated in this thesis, *in vivo* characterization in terms of LD_{50} and establishment/reactivation from latency should be carried out. This would allow investigation into the possible roles of both ICP34.5 and ORF P in HSV-1 latency and virulence.

To further investigate the role of ORF O and P, new recombinant viruses expressing either ORF O and ORF P separately should be constructed and characterized both *in vitro* and *in vivo*. Monoclonal antibodies raised against both ORF O and ORF P proteins should be used to detect expression of these two proteins from the recombinants (see below).

To determine which region of ORF P is involved in the interactions identified, 3 GST fusion proteins which express the aminoterminus, carboxyterminus and middle region of ORF P should be constructed and used in GST pulldwns. To identify further interacting proteins, a yeast-two-hybrid screen should be carried out with both intact and truncated versions of ORF P.

ORF O and ORF P are overlapping genes. The GST ORF P fusion protein used in this thesis, did not allow investigation into possible interactions of ORF O with both cellular and viral proteins. Thus either a GST-ORF O fusion protein which expresses only ORF O should be used in the GST pulldown assay or alternatively ORF O should be used in a yeast-2-hybrid screen. It would be good idea to compare both the ORF O and ORF P interacting proteins.

The antisera used in this study, 128 and 129, were rabbit polyclonal sera raised against a GST-ORF P fusion protein. Use of GST fusion proteins tends to generate rabbit polyclonal sera with high background. If monoclonal antibodies were raised against ORF P these higher specific antibodies might produce less background on Western blots allowing detection of low levels of ORF P. Similarly monoclonal antibodies against an ORF O fusion protein could be raised.

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